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Cucumber Mildew Resistance

Identification of Cucumber Genes Involved in Susceptibility and Resistance to Powdery and Downy Mildew

Jeroen A. Berg

Thesis

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TABLE OF CONTENTS

Chapter 1

General Introduction

Ever since the domestication of the first plant species at the dawn of the Neolithic, humans have selected the most nutritious, well-tasting and appealing looking plants for propagation, thereby improving crop germplasm to the point where cultivated crops are almost unrecognisable from their wild progenitors. After the discovery (and subsequent re-discovery) of Mendel's laws of heredity [1], twentieth century plant breeders were able to breed new, higher yielding varieties in a systematic and informed way, which sped up the plant breeding process. Eventually crop improvement, together with the introduction of synthetic fertilizers and pesticides, enabled the Green Revolution during the 1950s and 1960s which greatly increased the yields of (cereal) crops and thus increased food security, especially in developing regions [2].

Over two-hundred years ago, Malthus warned that population growth was an exponential phenomenon, whereas food production could at best grow linearly, implying that food would become increasingly scarce, resulting in massive starvation [3]. Whereas the increase in food production over the last 200 years has far exceeded Malthusian predictions, partially thanks to the above-mentioned advances in plant breeding, it is true that the world population has sharply increased from ca. 1 billion in Malthus' days to over 7 billion at present, and is expected to grow to more than 11 billion in the coming century [4]. This sets new challenges for agricultural research, calling for a "Green Revolution 2.0" [2]. Furthermore, agricultural intensification can have adverse environmental effects regarding biodiversity, water use, soil degradation and chemical run-off, which are serious issues that need to be addressed. Plant breeding efforts could help to develop new crop genotypes which are for example less input demanding (water, fertilizers) or more disease resistant, thereby limiting the need for pesticides, thus reducing the associated negative effects on the environment.

Whereas the previous focus on staple foods such as rice, wheat and maize was indeed very efficient in increasing the availability of calories per capita, and thus in decreasing hunger, micronutrient availability is lagging behind, partially due to a decrease in dietary diversity. Even though the annual amount of fruits and vegetables produced worldwide has steadily increased during the last half century, only a very small minority of the world's population has access to the recommended 400 g of fresh fruits and vegetables per day [5], and consequently insufficient fruit and vegetable consumption belongs to the top 10 of risk factors contributing to mortality, estimated to cause annually 2.7 million deaths [6]. This underlines the importance of making fruits and vegetables easily available to the world population as a whole.

One of the main challenges in crop production is the constant threat by infections from various pests, such as insects and nematodes, and pathogens such as fungi, oomycetes,

bacteria and viruses. Plants have co-evolved with numerous plant pathogenic organisms, and as such have developed complex layers of defence to minimize the damage by pathogen species, thereby creating a natural balance between plants and pathogenic species. However, in an agricultural setting, a single plant species, and often even a single or a few highly related genotypes of that species, are grown at high densities in vast tracks of land, thereby creating an artificial "paradise" for compatible plant pathogens. As such, plant pathogens can cause devastating epidemics. Notorious epidemics include for example the infamous *Phytophthora infestans* epidemic on potatoes of the 1840s which caused the Great Famine in Ireland, costing over a million lives [7], and the Panama disease on banana, caused by *Fusarium oxysporum*, which nearly wiped out commercial banana production during the 1950s [8].

The research described in this thesis aimed at increasing our understanding of the interactions between the vegetable crop cucumber (*Cucumis sativus* L.) and two of its most notorious pathogens, the downy mildew (DM) causing oomycete *Pseudoperonospora cubensis* (Berk. & M.A. Curtis) Rostovzev and the powdery mildew (PM) causing fungal species *Podosphaera xanthii* (Castagne) U. Braun & Shishkoff and *Golovinomyces orontii* (Castagne) V.P. Heluta. From a practical point of view, identifying (alleles of) genes which can contribute to resistance to these pathogens can aid plant breeding efforts to create more resistant cucumber cultivars. From a more fundamental point of view, investigating these genes and their potential working mechanisms can teach us more about plant-parasite interactions in general, knowledge which might eventually also be translated to other pathosystems.

CUCUMBER

Cucumbers are the third most consumed group of vegetables, after onions and tomatoes, with an annual production of over 83 million metric tonnes. Over 75% of all cucumbers produced worldwide are grown in China [9]. Cucumber is grown in glass greenhouses or plastic tunnels as well as in open fields. Several market types of cucumber are recognised, including cucumbers that are more suited for fresh consumption and cucumbers intended for pickling, as well as various region-specific types. Whereas cucumbers are generally known to have an elongated shape and are harvested unripe, when they have a green colour, cucumber fruits occur in all sizes, shapes and colours, ranging from white to yellow or orange, from almost round to very long and thin, and can be smooth-skinned or have warts and/or spines (Figure 1). Cucumber plants are vines, which can either creep on the ground without support or be led to climb upon a trellis or wire using its long, spiralling tendrils. The plant has an angular stem, simple palmate leaves, and has large, brightly yellow, unisexual flowers. Usually cucumber plants are monoecious (i.e.

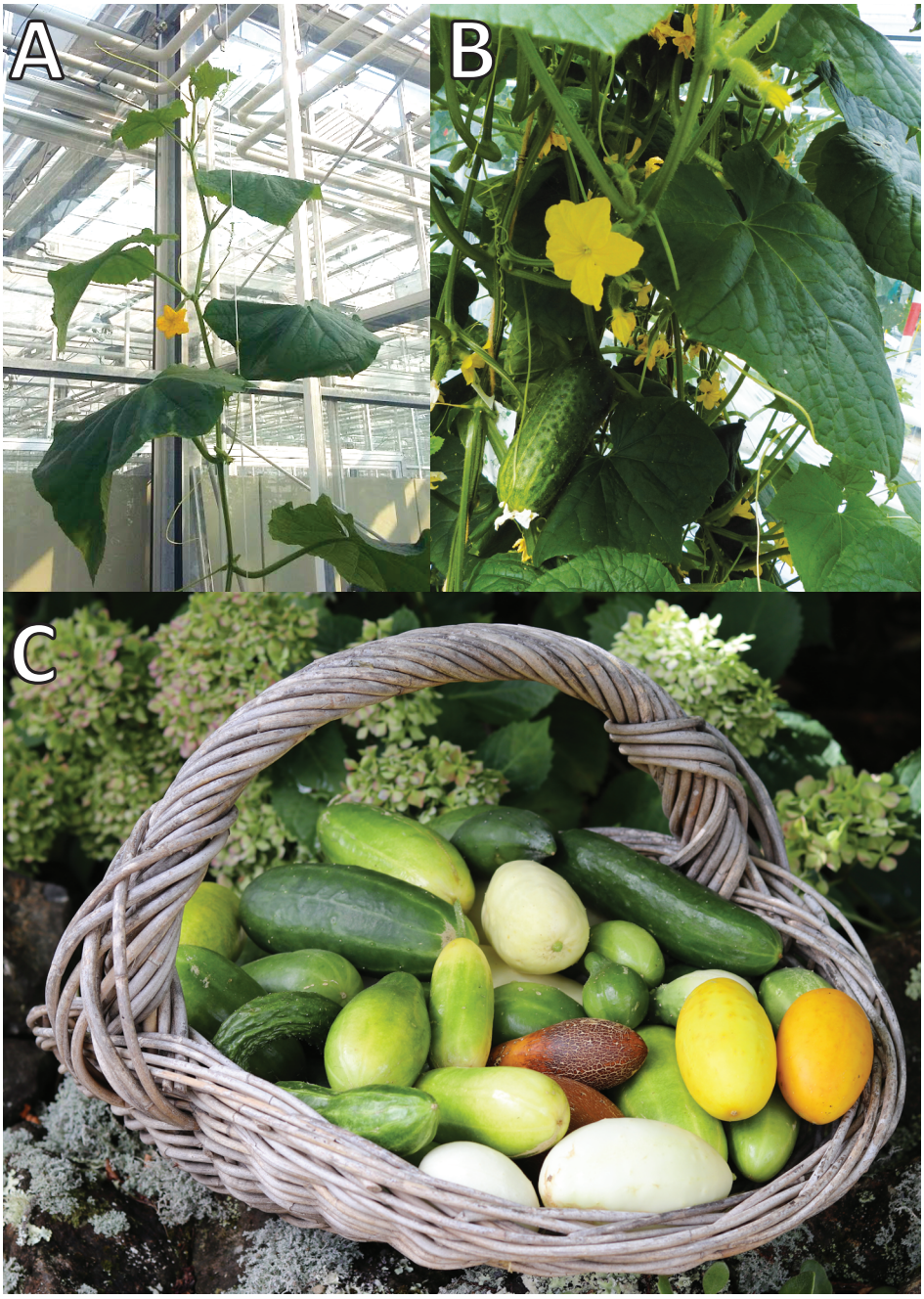

Figure 1 – (A) European greenhouse-type cucumber, growing on a wire. (B) Pickling type cucumber. (C) A collection of heirloom cucumber varieties and landraces illustrate that cucumber fruits occurs in many colours and sizes. Photo courtesy of Lynda Hallinan, NZ Gardener.

having male and female flowers growing on the same plant), although gynoecious (i.e. having only female flowers) cultivars are also commonly developed, which improves fruit yield per plant. Some cultivars are parthenocarpic, whereas others usually require pollination by insects such as honeybees.

The wild progenitor of domesticated cucumber (*C. sativus* var. *sativus*) is *C. sativus* var. *hardwickii*, which is thought to originate from the Himalayas and currently grows in the wild in India and neighbouring countries as well as in Southeast Asia [10]. Cucumber is thought to have been domesticated by Indian people at least 3000 years ago [11]. Apart from cultivated cucumber and wild cucumber, one other botanical variety is commonly recognized, i.e. *C. sativus* var. *xishuangbannanesis*, an orange-fleshed semi-wild variety which is eaten in the southern Chinese prefecture of Xishuangbanna [12]. Cultivated cucumbers were brought eastward and introduced in China ca. 2000 years ago, and were frequently cultivated there for at least 1600 years [13]. Whereas it was previously thought that cucumbers were known in the western world already during the time of the ancient Greek and Roman empires, it appears from lexicographic studies that westward expansion of cucumber cultivation occurred between 1500 and 700 years ago, whereas more ancient references to cucumber production appear to refer to mistranslations of snake melon, *C. melo* var. *flexuosus*, which has an elongated shape and culinary usage similar to that of cucumber [14]. A recent genomic variation map obtained by resequencing a core collection of 115 cucumber genotypes revealed that cultivated cucumber cultigens could be divided in clearly separated clusters containing Eurasian and East-Asian cucumbers, respectively, whereas Indian cucumbers (including both wild cucumbers and local landraces) were far more diverse than both clusters of cultivated cucumbers [15].

The *Cucumis* genus contains over 50 species, most of which are endemic to Africa, southern Asia and Australia. Apart from cucumber, the genus also includes the domesticated species melon (*C. melo* L.) [10]. The *Cucumis* genus belongs to the Cucurbitaceae family, together with 125 other genera, including several genera containing cultivated crop species, e.g. *Citrullus* (watermelon), *Cucurbita* (pumpkins and squashes) and *Luffa* (sponge gourd). Whereas *C. sativus* has a diploid genome with a chromosome number of 2n = 14, all other *Cucumis* species, including the domesticated melon (*C. melo*), have chromosome numbers of 2n = 24. This apparent reduction in chromosome numbers in cucumber poses a reproductive barrier, limiting the possibilities of introgressing traits from wild *Cucumis* species to cultivated cucumber. However, it was shown to be possible to create amphidiploid hybrids between *C. sativus* and its closest relative, *C. hystrix*, through embryo rescue and subsequent chromosome doubling [16].

The genome of cucumber is estimated to have a haploid size of 367 Mb [17]. As the first vegetable species, the genome of cucumber genotype "Chinese Long inbred line 9930" was sequenced in 2009, resulting in a reference genome with a length of 243.5 Mb, containing 26,682 predicted genes [18]. Whereas the reference genome assembly was >30% shorter than the predicted haploid genome size, K-mer analysis of sequencing reads used to construct the reference genome assembly indicated a genome size of ca. 350 Mb, indicating that a large proportion of the genome was indeed not covered by the genome assembly. Reads which could not be assembled into scaffolds largely represented repetitive satellite sequences, which according to FISH (fluorescence *in situ* hybridization) analysis were located in centromeric and telomeric regions of the genome [18]. It was shown that there were no recent whole-genome duplications and relatively few tandem duplications in the cucumber genome, explaining the relatively small genome compared to many other crops. Later, deep RNAseq of several cucumber tissues of the same genotype and additional large-insert whole genome sequencing reads enabled an improved assembly and annotation of the same genotype, which was slightly smaller than the first version (i.e. 197 Mb and 23,248 predicted genes) mainly due to the removal of contaminating bacterial segments and redundant repetitive sequences [19]. Furthermore, *de novo* genome assemblies of several other cucumber genotypes were released, i.e. the North American pickling type inbred "Gy14", the European inbred line "B10" and the wild cucumber (*C. sativus* var. *hardwickii*) accession PI 183967 [15, 20, 21]. In addition, a core collection of 115 cucumber genotypes, representing diverse geographical origins and market types, was re-sequenced [15]. The availability of these genomic resources provide an excellent starting point for those who want to study genes of cucumber.

According to a survey among cucurbit growers, shippers and producers, the highest priority in cucumber breeding should be increasing disease resistance. Cucumber can suffer from several diseases, the most threatening of which are currently Downy Mildew and *Phytophthora* fruit rot, but also of great importance are Powdery Mildew, *Fusarium* wilt, Gummy stem blight and a variety of viral diseases [22]. In this thesis, the main focus is on Downy Mildew (DM) and Powdery Mildew (PM), diseases which are caused by two evolutionary unrelated groups of pathogens, which nevertheless share a rather similar obligate biotrophic lifestyle.

CUCURBIT DOWNY MILDEW

Cucurbit downy mildew is caused by *Pseudoperonospora cubensis* (Berk. & M.A. Curtis) Rostovzev, an oomycete belonging to the order Peronosporales, family Peronospora-

ceae. The pathogen was initially discovered in herbarium specimens originating from Cuba in 1868, explaining the name of the species [23]. The first description of *P. cubensis* observed on living plants was in 1903 by Rostovzev in the Botanical Gardens of Moscow. The family Peronosporaceae includes 17 different genera, the most widespread of which are *Bremia*, *Hyaloperonospora*, *Peronospora, Pseudoperonospora* and *Plasmopara*, representatives of which cause DM on a wide variety of cultivated hosts. Furthermore, the order Peronosporales includes the genus *Phytophthora*, species in which cause devastating diseases in many host species, and which differ from DM causing Peronosporaceae by not being obligately biotrophic. Molecular phylogenies suggest that DM causing species evolved from a *Phytophthora*-like ancestor, and as such the genus *Phytophthora* is paraphyletic, unless all DM species are considered to be part of the *Phytophthora* genus [24]. The main differences between genera within Peronosporaceae, apart from their host range, are in the shapes and branching patterns of the sporophores or sporangiophores, as well as in their ability to form motile zoospores, which is lost in several genera [24]. The closest relative of *P. cubensis* is *P. humuli*, the causal agent of DM on hops (*Humulus* spp.), which is morphologically almost indistinguishable from *P. cubensis*. It was recently shown that both species can infect each other's native hosts with some success in detached leaf experiments under controlled conditions [25]. All species causing DM are obligate biotrophs, meaning that they require living plant hosts to survive, and are impossible to culture on synthetic media.

P. cubensis can infect all aerial parts of the cucumber plant, but is primarily a foliar disease. The earliest symptoms of cucurbit DM are usually chlorotic lesions on the adaxial surface of infected leaves (Figure 2A). Such lesions are restricted by major leaf veins in cucumber, creating typical irregular angular lesions, whereas lesions on other cucurbit hosts can be more circular. After several days, lesions usually become necrotic, starting from the centre of the lesion. Within days following the initial infection, growing lesions might coalesce, eventually leading to senescence of the entire leaf(Figure 2B). A drastic reduction in canopy limits the photosynthetic capabilities of the infected host, reducing fruit formation, whereas increased sun exposure of fruits due to defoliation may lead to sun scalding of fruits and secondary infections by other pathogens. Symptom development is highly dependent on the environment; lower temperatures and higher humidity can slow symptom development even though the pathogen can still develop within the leaf. Under appropriate environmental conditions, sporulation can be observed on the abaxial sides of lesions (Figure 2C), which has a brownish grey "downy" appearance [26].

The primary infective unit of *P. cubensis* is the asexual sporangium, which grows on branched sporangiophores, protruding through the stomata of infected leaves. Spo-

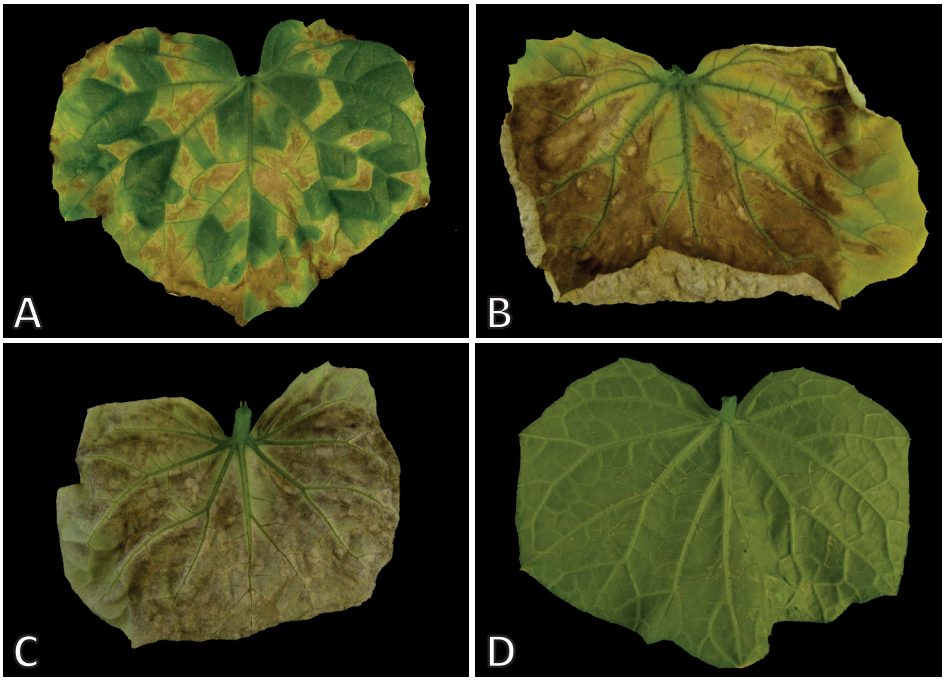

Figure 2 – (A-C) Symptoms of downy mildew on leaves of susceptible genotype HS279 (A) Chlorotic lesions can be observed, which have an angular shape due to restriction by major leaf veins. (B) necrotic lesions coalesce, eventually leading to leaf senescence. (C) brownish grey sporulation can be observed on the abaxial leaf surface. (D) The abaxial side of a resistant cucumber accession (PI 197088) remains free of sporangia.

rangia are usually grey to purple coloured, lemon-shaped with a papilla at one end, and measure 20 to 40 by 14 to 25 µm in diameter. Sporangia easily dislodge from sporangiophores, and are transported by wind or splashes of water (e.g. raindrops). When sporangia fall on a leaf of a compatible host plant, they can germinate under preferential circumstances (i.e. in presence of water and optimally between 10-20 °C), after which they produce ca. 5-15 biflagellate zoospores by cytoplasmic cleavage. Zoospores actively swim in the direction of stomata, where they encyst and subsequently form a germ tube, which develops an appressorium to penetrate through the stomatal aperture. Once inside the stomatal cavity, hyphae grow intercellular to colonize the mesophyll layer, and penetrate mesophyll cells, wherein they form specialized intracellular feeding structures known as haustoria. Under optimal environmental conditions, sporangiophores can emerge from the host within 5-7 days, usually on the abaxial side of the leaves, completing the asexual life cycle of *P. cubensis* [26, 27].

Additional to asexual reproduction, *P. cubensis* is able to propagate sexually, thereby forming thick-walled resting structures known as oospores, which can overwinter in

decayed plant debris in order to infect new plants in the next growing season. Oospores are round, golden yellow, ca. 36 µm in diameter, and can survive for over 10 months. However, observations of oospores under field conditions are very rare. *P. cubensis* is usually heterothallic, i.e. it requires a combination of two strains with opposing mating types colonizing the same leave in order to reproduce sexually. Mating type appears to be significantly correlated to host preference, with A1 mating type strains primarily being found on cucumber and melon whereas A2 mating type strains are primarily found on pumpkins and watermelon. As such it is thought that occasional sexual reproduction increases the evolutionary potential of *P. cubensis* as it allows recombination leading to new, potentially more virulent and/or fungicide resistant strains of the pathogen, but plays only a minor role regarding the overwintering ability of the pathogen [28]. Instead, the primary source of inoculum during spring appears to be long-range dispersal from subtropical regions in which host plants can grow year-round. Evidence suggests that under favourable wind conditions, asexual sporangia can travel up to 1000 km from their inoculum source [29]. Additionally, the pathogen could overwinter on perennial wild cucurbit species such as *Bryonia dioica* which grows in Northern Europe [30], and/or in greenhouses were cucurbits are cultivated year-round.

Although *P. cubensis* in general has a broad host range covering at least 60 different species within the Cucurbitaceae family, specific isolates of the pathogen often have a narrower host range. In 1987, a study on host specificity of eight *P. cubensis* isolates discriminated five pathotypes, characterized by specific subsets of compatible hosts. Interestingly, all five pathotypes were able to infect cucumber and melon variety *C. melo* var. *reticulatus*. Whereas pathotype 1 was only able to infect these two hosts, pathotypes 2-5 had increasingly larger host ranges, being able to infect *C. melo* var. *conomon* (pathotype 2-5), *C. melo* var. *acidulus* (pathotype 3-5), watermelon (pathotype 4-5) and pumpkin (pathotype 5) [31]. Later, other differential sets, including more cucurbit species and genotypes, extended the amount of recognized pathotypes to 67, although the majority of the isolates belonged to 11 pathotypes whereas the other pathotypes were rather rare [32]. Nine isolates of the pathogen, corresponding to pathotypes 1 and 3-6 were sequenced, indicating that there are two separate phylogenetic lineages, one corresponding to pathotypes 1 and 3 and mating type A1, and the other to pathotypes 4-6 and mating type A2 [33].

CUCURBIT POWDERY MILDEW

Cucurbit powdery mildew can be caused by two different ascomycete fungal species. One of these species is *Podosphaera xanthii* (Castagne) U. Braun & Shishkoff, which is

also often (erroneously) referred to as the closely related species *P. fusca* (Fr.) U. Braun & Shishkoff, and which was formerly known by various other names, most importantly *Sphaeroteca fuliginea* and *Erisyphe fusca* [34, 35]. The other species causing PM in cucurbits is *Golovinomyces orontii* (Castagne) V.P. Heluta, formerly known as *G. cichoracearum* and *Erisyphe cichoracearum*. Both species belong to the ascomycete order Erysiphales, family Erysiphaceae, species in which can cause powdery mildew on nearly 10.000 species of flowering plants [36]. However, within this family *G. orontii* and *P. xanthii* belong to the tribes Golovinomyceteae and Cystotheceae, respectively, which diverged ca. 75 million years ago [37]. Based on vegetative growing myceliae and asexual reproductive structures of both pathogens (i.e. anamorphs), the species are rather hard to distinguish, the only outstanding difference in appearance being the presence of fibrosin bodies in conidiospores of *P. xanthii*, which can be visualized using light microscopy, and which are absent in *G. orontii*. Sexual fruiting bodies (chasmothecia) of both species (i.e. teleomorphs) are considerably different, but as sexual reproduction is rather rare in both species, these differences do usually not help much in the correct identification of the two fungi [36, 38]. For these reasons, prevalence of either species over the other in a given area was in the past sometimes hard to determine. However, with the advance of molecular techniques, it has become easier to identify species by screening species-specific polymorphisms in the ITS and 18s rDNA sequences. Although the prevalence of either pathogen is rather variable and region-dependent, the general consensus is that *P. xanthii* is the major causal agent of PM in cucurbits, especially in warmer climates and in greenhouses, whereas *G. orontii* occurs mainly in open field production under colder climates [39, 40]. *G. orontii* has a broad host range, including hosts from at least eight plant families. Phylogenetic evidence suggests that *Golovinomyces* species initially evolved on hosts of the Asteraceae family, after which a subset growing on hosts in the genus *Lactucae* expanded their host range to other plant families, including Cucurbit species [41]. Interestingly, *Podosphaera* species, which have an even broader host range, causing PM on more than 1000 angiosperm plant species from at least 40 different plant families, also co-evolved with Asteraceae hosts before acquiring the ability to infect other plant species. *P. xanthii* isolates isolated from cucurbit hosts were found to be cross-infective with isolates of the same rDNA haplotype from six other hosts representing various non-related families, implying that this specific subtype of *P. xanthii* has a very broad host range [42].

Similarly to DM, PM is primarily a foliar disease, although other aerial tissues (especially stems, petioles, and hypocotyls) are also frequently infected. In contrast to DM causing pathogens however, which grow inside of the plant host, PM fungi are usually ectoparasites (with the exception of four genera in tribe Phyllactinieae), growing on the epidermis of leaves. The primary symptom of PM infections is therefore the develop-

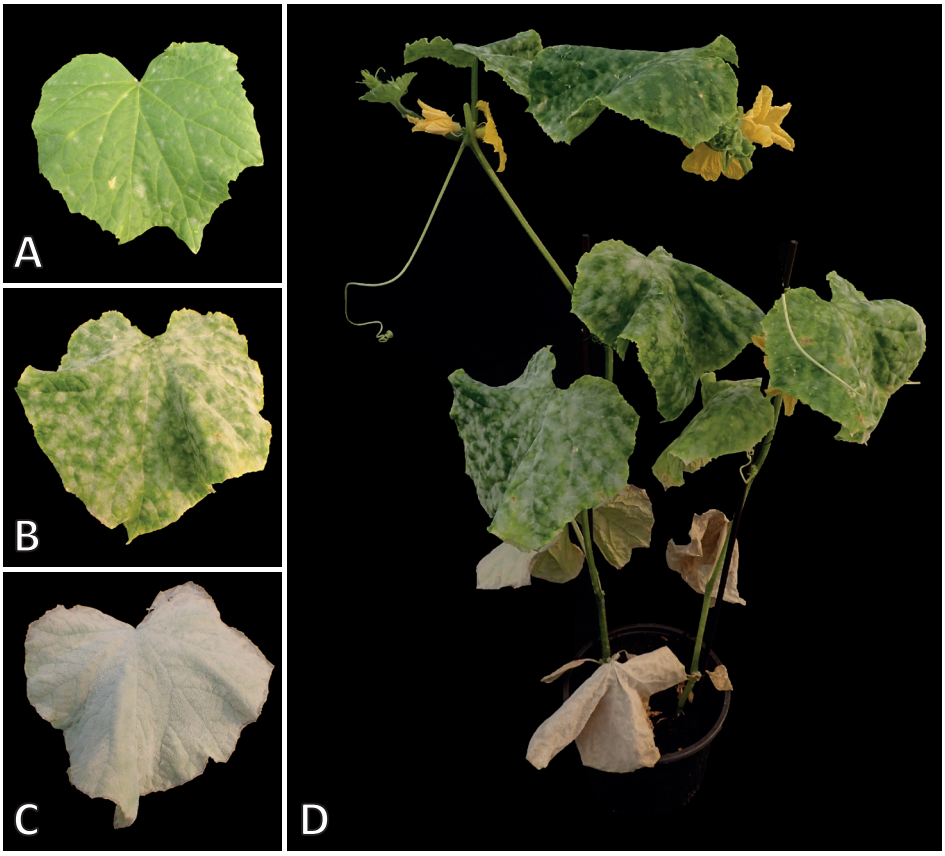

Figure 3 – Symptoms of powdery mildew on leaves of susceptible genotype 'Hoffmans Johanna'. (A) Initial fungal colonies. (B) When the disease progresses, the infected leaf might become chlorotic. (C) Heavily infected leaves eventually become completely covered in a white layer of spores and mycelium. (D) Two powdery mildew infected cucumber plants showing leaves with various degrees of symptoms.

ment of white, powdery fungal colonies covering the plant surface (Figure 3). While the pathogen feeds on the host, mycelial growth on the leaf surface prevents photosynthesis, often causing heavily infected leaves to turn chlorotic under certain environmental circumstances, which might eventually lead to death of the infected leaf. Similarly to DM, PM infected plants have lower yields due to reduced photosynthetic efficiency, and a decrease in foliage may lead to malformed and sun-burned fruits [38].

The asexual life cycle of PM fungi starts with the landing of a conidiospore on a susceptible host leaf. In contrast to most other fungi and to oomycetes causing DM, PM fungi do not require water in order to germinate. A conidiospore secretes esterases and cutinases, which can occur within a minute after landing on the leaf surface, in order to

break the leaf cuticle and fasten the spore to the host. Within the first hour a primary germ tube is formed, which attaches itself to the leaf through a cuticular peg. Within ten hours the primary germ tube elongates and induces the formation of an appressorial germ tube, which breaks the cell wall of an epidermal cell through a combination of turgor pressure and secretion of cell wall degrading enzymes. After successfully invading the plant cell, a haustorium is formed, surrounded by an extrahaustorial matrix in order to protect it from host defence responses. After successful infection, the hyphae elongate and branch, forming a colony which establishes multiple secondary haustoria. After several days an expanding colony will form conidiophores, which grow perpendicular to the leaf surface. In contrast to the sporangiophores of *P. cubensis*, which causes DM, conidiophores of PM causing fungi are unbranched. Both cucurbit PM species have catenate conidiophores, meaning that multiple conidiospores ripen on the same conidiophore in a chain, with the oldest, most developed conidiospore growing at the end and new conidiospores developing at the basal part of the conidiophore. Through wind or mechanical stimulation of the leaf, ripe conidia are dislodged, and may be carried by the wind in order to infect new hosts, thus completing the life cycle [36, 38].

Both cucurbit PM species also have sexual stages, although field observations indicate that these are rather rare [38]. When hyphae of two strains with opposing mating types meet, they can form reproductive structures known as antheridia (male) and ascogonia (female). After fertilization, the resulting binucleate cell and surrounding mononucleate hyphae from both parents grow to form a chasmothecium, the sexual fruiting body of PM fungi. Within the chasmothecium, nuclear fusion followed by meiosis leads to the development of a single ascus containing eight ascospores (*P. xanthii*) or multiple asci, containing two ascospores per ascus (*G. orontii*) [43]. Chasmothecia can withstand extreme temperatures for long periods, and as such can serve as overwintering or oversummering structures, releasing ascospores upon optimal environmental conditions in order to start a novel disease cycle [36].

Whereas both PM species have broad host ranges, extending far beyond cucurbit hosts, a differential set of six cucurbit genotypes (representatives of three genera and five different species) can be used to discriminate various pathotypes of both fungi, each defined by a combination of possible hosts [44]. However, all pathotypes observed so far are virulent on (susceptible) cucumber, and differ only for their pathogenicity on other cucurbit hosts, which is reminiscent of the situation for DM. Furthermore a differential set of eleven melon genotypes is used to define physiological races of both species, as race-specific PM resistance is observed in melon, in contrast to other cucurbit species [44, 45]. Even though the discrimination of different pathotypes and races appears to be of lesser importance for scientists primarily interested in cucumber mildew resistance,

given that all pathotypes and races can infect cucumber, they can be useful tools to study the pathogen population structure. Furthermore it is an interesting open question why cucumber generally appears to be more widely susceptible to pathogens causing both PM and DM compared to other cucurbit species.

PATHOGEN RESISTANCE IN PLANTS: GENETIC CONCEPTS AND MOLECULAR MECHANISMS

When studying the interactions between a phytopathogenic organism and its plant host, some genotypes of the host are usually more resistant to the pathogen than others. This resistance can be complete, when a certain genotype cannot be infected by the pathogen at all (incompatible interaction, full resistance), but can also be partial, when the genotype is still infected by the pathogen although to a lower level compared to fully susceptible genotypes. Obviously, "fully susceptible" is a relative term, as novel host genotypes which are even more susceptible might later be discovered. The inheritance of resistance is of high importance to plant breeders. When a segregating population is developed, by crossing a fully resistant genotype with a fully susceptible genotype (followed by a round of self-fertilization of the resulting progeny, assuming the parental lines are homozygous), several possible outcomes can be observed:

- In some cases, a proportion of the progeny will be fully resistant, whereas the remainder of the progeny is fully susceptible. In such cases, the resistance is governed by (one or more) so-called resistance genes (*R* genes), which are inherited according to Mendelian laws. Usually the resistance conferred by these genes is dominantly inherited. Sometimes however, resistance is found to be recessively inherited. In such cases, loss-of-function alleles of so-called susceptibility (*S*) genes are responsible, as susceptibility is the dominantly inherited trait. In simple scenarios a single dominant R gene is inherited, leading to a 3:1 distribution of resistant and susceptible plants, respectively, in an F2 population, whereas a single *S*-gene leads to a segregation of 1:3. However, a resistant plant might also have multiple *R* or *S* genes, leading to other ratios.
- In other cases, resistance and susceptibility are not inherited as discrete classes, but there is rather a continuum of disease phenotypes, ranging from completely resistant to completely susceptible with various levels of partial resistance in between. In such cases, resistance is governed by multiple genes, each with a partial effect (i.e. horizontal resistance genes, partial resistance genes). Whereas each individual partial resistance gene is obviously inherited according to Mendelian laws, the occurrence of multiple combinations of partial resistance genes – sometimes with epistatic interactions between genes – in the population leads to a (more or less)

continuous distribution of partial resistance phenotypes. Furthermore, apart from genetic variation there might be environmental variation adding to the continuous distribution of disease phenotypes. In scenarios involving large numbers of partial resistance genes with relatively small effects, disease phenotypes are expected to be approximately normally distributed, given a sufficiently large population.

Qualitative resistance and the Gene-for-Gene hypothesis

Whereas major, dominantly inherited *R* genes are easy to incorporate in a breeding program, and offer full resistance, the downside is that usually such resistances are easily overcome by novel strains of the pathogen, sometimes already shortly after the introduction of the new *R* gene in commercial material [46]. In the 1940s, Flor studied the inheritance of pathogenicity in *Melampsora lini*, a basidiomycete fungus which causes leaf rust on flax (*Linum usitatissimum*) and which has a life cycle with a sexual stage, enabling genetic studies. He found that when crossing physiological races of the fungus which differ in pathogenicity on a genotype of flax with a single R gene, the pathogenicity of the F2 offspring of the fungus segregated as a monogenic recessive trait. When the flax genotype had two, three or four *R* genes, F2 cultures of the pathogen segregated for as many recessive pathogenicity genes. To explain this phenomenon, Flor proposed the gene-for-gene hypothesis, which states that for every (dominant) *R* gene in a host plant, there exists a corresponding (dominant) avirulence (*Avr*) gene in the pathogen, the product of which is recognised by the product of the *R* gene. When the pathogen has the recessive (loss-of-function) *avr* allele, it is not recognized by a plant with the corresponding *R* gene, leading to susceptibility. In total, Flor described 26 *R/Avr* pairs in the flax-rust pathosystem. Furthermore Flor and others noted that often several *R* genes were either allelic to one another or very tightly linked, as it was often difficult (e.g. for the flax *M* locus of resistance genes) or impossible (e.g. for the flax *L* locus of resistance genes) to find recombinants in populations derived from crossing two parental lines with *R* genes against different physiological races of a pathogen [47]. Gene-for-gene relationships between *R* genes and *Avr* genes were subsequently found to be the rule rather than an exception in numerous other pathosystems, including powdery mildews [48].

Significant breakthroughs between the 1970s and early 1990s in the field of molecular biology, such as the invention of Sanger sequencing [49], *Agrobacterium tumefaciens* mediated genetic transformation [50], transposon tagging [51] and the polymerase chain reaction (PCR) [52] opened up the possibility to clone the first *R* and *Avr* genes during the 1990s. After the first *R* genes were cloned, it was found that *R* genes from various plant species against completely unrelated pathogens often encoded rather conserved proteins [53]. Four out of the first six cloned *R* genes encoded proteins with

leucine rich repeat (LRR) domains, which were already known to be often involved in protein-protein interactions. Three of these R proteins (RPS2, N and L6, respectively conferring resistance to the bacterial pathogen *Pseudomonas syringae* in *Arabidopsis thaliana* , to tobacco mosaic virus in tobacco and to the fungus *M. lini* in flax) had a cytoplasmic localization, and contained conserved nucleotide binding site (NBS) domains, similar to those found in Toll and Interleukin-1 receptors which are involved in animal innate immune systems [54–56]. The other protein with an LRR domain, Cf-9 for *Cladosporium fulvum* resistance in tomato, localized to the plasma membrane, with the LRR domain being extracellular [57]. A fifth cloned R gene, *PTO* for *P. syringae* resistance in tomato, encoded a second plasma membrane-anchored protein, with an intracellular protein kinase domain, and an extracellular domain without LRR repeats, but with homology to other plasma membrane-anchored protein kinases [58].

According to a recent review, 25 years after the cloning of the first *R* gene, 314 different genes involved in resistance have been cloned, eighty percent of which belong either to the *Nucleotide-binding site, Leucine Rich Repeat* (*NBS-LRR*) family similar to *RPS2*, *N* and *L6*, or to the *Receptor-like Kinase/Protein* (*RLK/RLP*) family, similar to *Cf9* and *PTO* [59]. Both gene families are very abundant in plant genomes, and occur frequently in clusters, enabling tandem duplication, potentially leading to novel *R* genes. The NBS-LRR family harbours 61% of all cloned resistance genes to date, and can either directly recognize pathogen encoded effector proteins, or (more often) indirectly perceive alterations of host proteins, caused by pathogen encoded effector proteins. *NBS-LRR* genes form an abundant gene family in most plant genomes, usually occurring with more than hundred homologs per plant genome. Whereas only a minority of *NBS-LRR* genes has experimentally been shown to be an *R* gene, roles of other *NBS-LRR* genes are unknown, and as such they might be *R* genes of unknown specificity and/or be involved in basal defence. Furthermore those *NBS-LRRs* might not have a specific role as such, but rather represent a "reservoir" for the evolution of *R* genes with novel specificities. NBS-LRR proteins generally have at least three domains: a variable N-terminal domain, usually consisting either of a Toll/Interleukin-1 receptor (TIR) domain or a coiled-coil (CC) domain, an LRR domain which confers the substrate specificity, and an NBS domain, which is thought to act as a "molecular switch", repressing the activation of downstream signalling components when the ligand of the NBS-LRR protein is not bound. As NBS-LRR proteins generally have a cytoplasmic localization, their cognate AVR proteins should be translocated into the host cell in order to be perceived [59, 60].

RLK genes on the other hand, encode proteins which extend to the outside of the host cell plasma membrane, and can therefore perceive *Avr* gene products which are located in the apoplast. RLK proteins have diverse extracellular domains facilitating

perception of cognate ligands, transmembrane helices to anchor the proteins to the plasma membrane, and conserved intracellular kinase domains to confer a signal unto signalling cascades when perceiving the cognate ligand. Whereas the largest group of RLK proteins (ca. 30%) have extracellular LRR domains similar to those in NBS-LRR proteins, over 40 other, non-homologous extracellular domains have been identified in RLK proteins [61]. The *RLK* gene family is even more abundant than the *NBS-LRR* gene family, with several hundreds of copies per plant genome, making up ca. 2% of the total amount of protein encoding genes in the genomes of *Arabidopsis thaliana* and rice. *RLK* genes are not only involved in pathogen perception, but can have various other roles, for example in the perception of hormones regulating growth and development, in self-incompatibility during sexual reproduction and in signalling of abiotic stresses [62]. Some genes encode proteins with extracellular domains and transmembrane helices, but without a kinase domain (RLPs), such as Cf-9. Such RLPs are often shown to depend on the regulatory LRR-RLK SOBIR1 in order to transduce a signal [63].

Our current understanding of the plant innate immune system is that several RLKs, termed pattern recognition receptors (PRRs) perceive broadly conserved pathogen associated molecular patterns (PAMPs) attributed to classes of pathogens, such as chitin derived from fungal cell walls, flagellin and elongation factor Tu (EF-Tu) from bacteria, or heptaglucan from the cell walls of oomycetes [64]. These PRRs confer broad basal resistance against non-adapted pathogens, which is termed PAMP-triggered immunity (PTI). However, adapted pathogens encode a plethora of secreted proteins in order to block or repress plant defence responses (effector proteins), leading to a compatible interaction and thus to effector-triggered susceptibility (ETS). On their turn, some genotypes of the host plant have evolved to encode RLK and NBS-LRR proteins which perceive some of these effectors, triggering a stronger defence response termed effector triggered immunity (ETI), usually associated with a hypersensitive response (HR) [65]. The RLK and NBS-LRR proteins perceiving effectors are the products of the *R* genes identified decades earlier by Flor and others, whereas the effectors they recognize are the products of the corresponding *Avr* genes.

Recessive resistance and Susceptibility genes

Whereas the majority of genes involved in resistance were shown to be dominantly inherited, following the gene-for-gene hypothesis, exceptions to this general rule, in the form of recessive resistances, were also found. Especially resistances against viral diseases are frequently recessively inherited [66, 67]. However, recessive resistances against other pathogens, including powdery mildews, have also been described [68–70]. In contrast with dominant resistance genes, recessive resistances appear to be more difficult to overcome by strains of the pathogen, making those resistances more durable.

During the 1990s, several forward genetic screens identified *Arabidopsis* mutants with increased disease resistance. Usually, mutations leading to increased resistance had pleiotropic effects, such as dwarf growth and the presence of lesions in absence of the pathogen, due to constitutive defence responses associated with accumulation of the defence hormone salicylic acid (SA) and transcriptional activation of *Pathogenesis Related* (*PR*) genes. As usual for mutant alleles, these mutant phenotypes (including disease resistance) were inherited recessively. Usually, these recessive resistant mutants had broad resistance against multiple strains of pathogens, and even against multiple unrelated pathogen species [71–74]. However, several mutants with enhanced (recessively inherited) powdery mildew resistance were found without constitutive defence responses, which were called *pmr* mutants (powdery mildew resistant). Although these mutants did not constitutively express high levels of defence related genes, the amplitude of defence responses upon inoculation was enhanced compared to wild type plants in several of these mutants [75–77]. The identification of these genes gave rise to the concept of "Susceptibility genes", or *S*-genes, as the functional, dominant allele of these genes confers susceptibility to pathogens [78]. In retrospect, recessive resistances identified in earlier decades were likely also caused by loss-of-function alleles of *S*-genes.

A prime example of the durability of recessive resistances is the barley *mlo* mutant, which is resistant to the PM-causing fungus *Blumeria graminis* f.sp. *hordei*. A recessive mutant allele of this gene, which confers resistance, was developed over 75 years ago by X-radiation experiments. Barley varieties with *mlo* resistance have since been grown in the field for several decades without breaching of resistance by virulent new mildew races in the field to date, providing evidence for the durability of *S*-gene based resistance [79]. After the barley MLO gene was cloned [80], it was found that MLO genes are conserved throughout the plant kingdom and occur in plants as a multi-copy gene family [81, 82]. Recently, Kusch et al. [83] provided evidence for the occurrence of MLO-like genes in representatives of all groups of land plants, including mosses and gymnosperms, in related unicellular algae, and even in distantly related eukaryotes such as Amoebozoa and Chromalveolata. The latter group intriguingly includes plant pathogens such as *Phytophtora infestans* and *Hyaloperonospora arabidopsidis*.

In many plant species, including *Arabidopsis*, tomato, pea, pepper, tobacco, wheat and potentially also grapevine and peach, *MLO* genes have been found to be involved in PM susceptibility [84–90]. It has been demonstrated by phylogenetic analysis that the *MLO* gene family can be divided in seven clades [83]. All identified *MLO S*-genes cluster in two clades, namely clade IV for *S*-genes in monocot species and clade V for dicot species. The other five clades harbour MLO-like genes that have not been found to be

S-genes [82, 83]. Whereas the roles of the majority of *MLO* genes outside of clades IV and V are unknown, several *Arabidopsis* mutants in *MLO* genes not belonging to clade V were studied: mutant alleles of clade I gene *AtMLO4* and *AtMLO11* have a disturbed root thigmomorphogenesis, characterised by an unusual root curling phenotype, as such these genes are likely involved in root growth [91]. Furthermore, mutants in clade III gene *AtMLO7*, also known as *nortia*, have defects in fertility, caused by overgrowth of the pollen tube by the synergids of the female gametophyte [92]. Recently, the clade VI gene *AtMLO3*, which is closely related to the clade V genes involved in PM susceptibility, was studied in detail. It was found that this gene apparently was involved in defence-related outputs such as hormone balance, and loss-of-function mutants shared pleiotropic phenotypes with the *S*-gene *AtMLO2*, such as random callose depositions and early leaf senescence. However, no effects at all were found in susceptibility against PM causing fungi nor other tested pathogens in *Atmlo3* mutants, showing that although clade V and clade VI *MLO* genes apparently have some overlap in triggered pathways, clade VI genes are not considered to be *S*-genes [93].

In contrast to *R* genes, which are rather conserved as discussed above, usually belonging to either the *NBS-LRR* or the *RLK* family, differing only in their ability to recognise specific ligands, *S* genes are wildly diverse, belonging to various gene families, each contributing to susceptibility in their own specific way. In a review, van Schie and Takken distinguished three major phases during which *S*-genes facilitate susceptibility: genes which facilitate host recognition and penetration by the pathogen, genes which play a role as negative regulator of immune signalling, and genes which fulfil metabolic needs of the pathogen [94]. Whereas *S* genes all contribute to susceptibility, the fact that they are not lost during evolution implies that they play important roles for the host plant, and as such, mutant alleles are often associated with pleiotropic effects, which may impede their use in plant breeding.

Whereas resistance due to dominant *R* genes is easily overcome by loss-of-function mutations in their cognate *Avr* genes in the pathogen, overcoming *s*-gene based resistance is generally thought to require gain-of-function mutations in the pathogen, which is much rarer. As such, resistance based on disabled *S*-genes is predicted to be much more durable than *R*-gene based resistance.

Quantitative resistance

As discussed above, resistance is sometimes inherited quantitatively rather than qualitatively. The term "quantitative disease resistance" is not used in a consistent way in literature, and might in some places refer to a partially resistant phenotype of a given

plant, whereas it can in other places refer to the genetic architecture in a given population, where multiple loci have a quantitative effect.

Compared to qualitative disease resistance, much less is known about the causal genes underlying quantitative resistance loci (QRL). This is in part caused by the difficulties regarding fine-mapping of quantitative traits. Prior to the development of DNA-based genetic markers, genetic maps were generally not dense enough to map complex quantitative traits, and hence it was difficult to select for quantitative resistance. With the advent of molecular markers, starting with RFLP (restriction fragment length polymorphism) markers in the 1980s [95], it became feasible to map complex traits such as quantitative disease resistance, enabling the identification of quantitative trait loci (QTL), defined as chromosomal regions spanning two or more molecular markers which are significantly correlated with the evaluated trait [96].

However, whereas many resistance QTL were mapped, identification of underlying causal genes often remained problematic, as fine-mapping of the QTL to narrow intervals containing few predicted genes generally is impeded by variation due to other segregating loci. Therefore, the common approach is to develop near isogenic lines (NILs) segregating only for a single QTL in a uniform genetic background. In such populations, a single locus causing a partial resistance phenotype segregates according to Mendelian ratios, and can therefore be positionally cloned similar to qualitative *R* genes [96]. Obviously, this approach works for loci with a large enough effect to distinguish discrete phenotypes, but remains troublesome for small-effect QTL.

Whereas actual cloning of causal genes for quantitative disease resistance is rather rare, especially compared to the overwhelming number of cloned qualitative *R* genes, evidence so far suggests that quantitative disease resistance genes are rather diverse: on several occasions, quantitative resistance genes were shown to encode *NBS-LRR* genes, similar to the majority of the qualitative *R* genes. Apparently, some "weak" *NBS-LRR* genes have partial effects rather than conferring full resistance. In other cases, QTL for resistance are recessively inherited, and as such represent *s* genes with a partial effects, for example due to partial redundancy with homologous genes. In yet other cases, quantitative resistance genes were found to be involved in basal defence (encoding pattern recognition receptors recognizing conserved PAMPs), in signalling pathways (e.g. transcription factors, MAP kinases) or in defence responses downstream of pathogen perception, such as the production of phytoalexins (secondary metabolites which are toxic to the pathogens) and breakdown of pathogen-secreted toxic compounds. Finally, in yet other cases quantitative disease resistance can be a pleiotropic effect of genes

involved in the development of morphological traits, such as stomatal density and/or openness [96–99].

Generally, quantitative resistance is considered to be more durable than race-specific *R* gene based resistance, because pathogens need multiple mutations to overcome each of the quantitative resistance genes, some of which might require gain of function mutations. Mutations leading to overcoming a single partial resistance gene will have only a limited advantage to the pathogen, and will therefore probably be selected for less strongly in the population [97].

CUCUMBER MILDEW RESISTANCE BREEDING

PM resistance breeding

The earliest references to cucumber resistance breeding stem from the 1940s and 1950s. Several researchers, especially from the United States, introgressed Asian sources of resistance in modern cultivars with good horticultural traits. The first modern resistant lines were created in the Puerto Rico Agricultural Research station by introgressing resistances from Chinese germplasm, giving rise to "Puerto Rico" or PR lines, which were resistant to both powdery mildew (PM) and downy mildew (DM). Inheritance of PM resistance in PR37 was shown to be recessive, and supposedly due to multiple genes with partial effects [100], whereas DM resistance in the same line was "probably polygenic", without clear conclusions on the mode of inheritance [101]. Other studies identified PM resistant accessions from India (PI 197087, cv. Bangalore), Burma (PI 200815 and PI 200818) and Japan (cv. Natsufushinari), which were usually also described to be inherited as polygenic, recessive traits [69, 102, 103].

In 1968, Kooistra at the Institute for Horticultural Plant Breeding in Wageningen analysed cucumber genotypes which were reported to be resistant during the 1940s and 1950s, but found that all of these cucumbers were only partially resistant [69]. Of these, the Japanese line Natsufushinari stood out as the most promising genotype, although it was not completely PM resistant. Crosses between Natsufushinari and both Burmese lines (PI 200815 and PI 200818) gave rise to completely susceptible F1 plants, indicating that resistances in both lines are recessive, and caused by different genes, whereas a cross between both Burmese lines gave a resistant F1, indicating that the resistance in both accessions is similar. In F2 populations from Natsufushinari x Burmese lines segregation ratios implied that full resistance was governed by three recessive gene, of which two came from Natsufushinari and one from the Burmese lines. Furthermore, it was noted that leaves of plants with PM resistance developed chlorotic/necrotic spots

when grown in a greenhouse, also in absence of the pathogen. There appeared to be a strong correlation between powdery mildew resistance and chlorosis/necrosis, but the identification of several F2 plants with good resistance and absence of chlorosis/necrosis suggested that this trait is linked to resistance rather than a pleiotropic effect of the causal resistance gene. A line denoted as NPI was released combining resistance genes from Natsufushinari with the resistance gene from the Burmese accessions. This NPI line was subsequently frequently used in Dutch cucumber breeding [69].

Shanmugasundaram [104] was the first to discriminate between "hypocotyl resistance", also called "intermediate resistance", and "leaf" or complete resistance. The intermediate resistance phenotype was defined by a lower amount of sporulating colonies on leaves, and hypocotyls being completely free of colonies. 20 accessions were studied, of which six with various levels of resistance were used for genetic studies. Crosses between accessions indicated the existence of a single recessive gene, "*s*", controlling hypocotyl resistance. Complete resistance depended on homozygous presence of the same recessive gene but also needed a second, dominant gene "*R*", as well as absence of a dominant inhibitor gene "*I*" [104].

In 1992 Zijlstra and Groot performed disease tests on 177 cucumber accessions, finding 108 partially resistant accessions. By crossing the 53 most resistant accessions with a completely susceptible line, it was determined that in each of these lines resistances were caused by recessive genes, as F1 plants were always highly susceptible. By crossing the same 53 lines with the previously developed line "NPI", it was tested whether these accessions had previously known genes or novel recessive genes. It was found that six accessions had completely different resistance genes, whereas ten other lines probably had some genes in common with NPI, combined with new recessive genes [105].

In 2005, American researchers screened all 977 cucumber accessions in the United States National Plant Germplasm System for PM resistance, indicating that 94 accessions were either highly or intermediately resistant. Of these 94 accessions, all but eight were originally collected in Southern or Eastern Asia, predominantly from China, India and Japan. The remaining eight accessions, collected from various places in Europe as well as Hawaii and Kenya, represented breeding lines from institutes and companies rather than original old varieties from those countries, and likely originated due through introgression of Asian resistances as well [106].

The above mentioned reports studied the inheritance of PM resistance based on segregation ratios, but did usually not try to map causal resistance genes, although in

some cases genetic linkage with other traits such as DM resistance and "dull green fruit colour" was described [107]. Due to the advances in molecular markers, as well as the fact that PM resistance in cucumber is usually a quantitative trait, recent reports usually take a QTL mapping approach to describe the inheritance of PM resistance.

The first study using a QTL approach was in 2006, using a population of recombinant inbred lines (RILs) derived from a cross between PI 197088 (highly resistant, from India) and cv. Santou (intermediate resistance, from Japan). As the intermediate resistance from cv. Santou is temperature sensitive (i.e. the genotype is partially resistant at high temperatures, but not at lower temperatures), two experiments were performed on the same set of RILs, at 20°C and 26°C. A single major QTL was detected at both temperatures, whereas one and two additional minor QTLs were detected at 20°C and 26°C, respectively [108].

Later, several other researchers performed QTL mapping, identifying various numbers of QTL, although QTL on chromosome 5 were often reported to have the largest effects. As the physical locations of flanking markers are usually not reported, it is often not feasible to directly compare the intervals discovered by different studies. Of special interest is the analysis by He et al. [109], who performed QTL mapping separately for hypocotyl resistance, cotyledon and true leaf resistance in a population derived from resistant inbred line WI 2757. They identified six QTL, of which a QTL on chromosome 5 explained the majority of the variation in hypocotyl resistance and additionally played a highly significant role in cotyledon and leaf resistance. The authors concluded that this is most likely the recessive gene *s* previously suggested to cause hypocotyl resistance while being necessary for complete resistance, and noted that several other groups also mapped major QTL at the same interval [104, 109].

Whereas the far majority of all identified QTL for PM resistance in cucumber are inherited recessively, a recent report by Xu et al. [110] described fine-mapping of a dominant resistance gene, conferring nearly complete PM resistance. The gene was fine-mapped to a locus on chromosome 1, containing eight predicted genes, two of which were strongly upregulated by PM inoculation, and which were not expressed in the susceptible parent. Both genes encoded receptor-like kinases with cysteine-rich extracellular domains. The resistant parent was a previously unmentioned genotype from Northern China (Jin5-508) [110].

In conclusion, all sources of PM resistance in cucumber identified during the last century originate from Asian countries, and are in most cases due to combinations of (usually recessive) genes with partial effects. In many cases, frequently used sources of

resistance were shown to be allelic to one another. QTL mapping studies have shown that a recessive gene on chromosome 5 plays the most important role in PM resistance, conferring "hypocotyl" resistance, whereas various other QTL were described adding to overall PM resistance. The fact that PM resistance is usually recessively inherited in cucumber, suggests that these resistances are often caused by loss-of-function mutations in susceptibility genes.

DM resistance breeding

As described above, the earliest publications on resistance in cucumber referred to the "PR" lines developed at the Puerto Rico Agricultural Research station by introgressing resistance from Chinese accessions. These PR lines were shown to be resistant to both PM and DM, although it is not known whether this broad resistance is due to the same genes, or whether these lines have multiple resistance genes for both diseases [101, 111]. The first commercial DM resistant cultivar, "Palmetto", was released in 1948, and was derived from PR 40. However, within two years after its release, all "Palmetto" fields were found to be infected with DM, indicating that this resistance was broken [112].

Hereafter, a novel promising source of DM resistance was found in the Indian accession PI 197087, characterised by the development of small brown hypersensitive lesions upon inoculation with extremely sparse sporulation [113]. Interestingly, the same Indian accession was also used in PM resistance breeding [103]. Whereas the original paper by Barnes and Epps did not mention the inheritance of DM resistance in PI 197087, later reports on cucumber lines derived from PI 197087 suggested that resistance was caused by a single recessive gene, called "*p*" or "*dm-1*", genetically linked to a recessive gene for PM resistance [107, 114]. Limited numbers of other sources of DM resistance were studied, presumably due to the adequate level of resistance conferred by the *dm-1* gene. The *dm-1* locus was recently fine-mapped, and a candidate gene was found in the fine-mapped interval, the *Staygreen* (*Sgr*) gene, involved in chlorophyll breakdown. Resistant accessions with *dm-1* all had a missense mutation in this gene. However, a clear mechanism by which *Staygreen* loss-of-function mutations contribute to DM resistance was not proposed [115]. Since the year 2004, new strains of the pathogen emerged which are virulent on plants with *dm-1* [32, 33, 116].

Following the loss of *dm-1* as a source of DM resistance, 1300 plants in the United States National Plant Germplasm System were evaluated for DM resistance in large multi-year, multi-location experiments, including test sites in the United States, Europe and India. Out of these experiments, two Indian accessions (PI 197088 and PI 605996) as well as one accession originating from Pakistan (PI330628) were found to be consistently the most resistant to post-2004 DM [117]. Interestingly, one of these Indian accessions,

PI 197088, was collected simultaneously and on the same location as PI 197087, the source of *dm-1*, indicating that these genotypes are probably related. Subsequently, these three resistant accessions were crossed with one another, and it was found that in the F2 generation several plants were susceptible, indicating that the resistance in each of the three lines is (at least partially) due to different genes, opening up the possibility to combine resistances [118].

Several mapping studies aimed at unravelling the genetic architecture of DM resistance in these resistant accessions. Especially PI 197088 was the subject of multiple QTL mapping experiments by various research groups, each arriving at different conclusions regarding the number of QTL and the contribution of each of these QTL to overall resistance. However, one or more QTL on chromosome 5 as well as a QTL on chromosome 4 were detected as major QTL by all groups, usually accompanied by several minor QTL on other chromosomes, which might be environment- and/or inoculum source dependent [119–122]. One study investigated inheritance of DM resistance derived from PI 330628, describing four QTL, of which again QTL on chromosomes 4 and 5 were major QTL [123], but it remains to be seen whether these QTL are due to the same causal genes as the major QTL from PI 197088. To our knowledge, DM resistance in the third highly resistant cucumber accession, PI 605996, is not yet mapped.

In conclusion, DM in cucumber was controlled for many decades by a loss-of-function allele in the susceptibility gene *dm-1*, but this resistance was overcome. Several promising accessions with high DM resistance exist, and mapping studies indicate that especially loci on chromosomes 4 and 5 contribute to DM resistance in two of these accessions.

SCOPE AND OUTLINE OF THIS THESIS

The aims of this thesis were to identify genes involved in cucumber-mildew interactions, in order to better understand these pathosystems, thus providing new leads for the breeding of mildew resistant cucumbers. As resistances against both PM and DM were previously shown to be usually recessive, special attention is given to the concept of susceptibility genes (*S* genes), loss-of-function alleles of which can contribute to effective and durable resistance.

In **Chapter 2** we studied a cucumber homolog of the barley *MLO* gene, named *CsaMLO8*. *MLO* is the first and most well-known example of an *S* gene, loss-of-function mutations of which provide PM resistance in many different plant species. The physical location

of *CsaMLO8* in the cucumber genome on the interval of the well-known "hypocotyl resistance" QTL, as well as its homology to several *MLO* genes proven to be *S*-genes in other plant species led us to suspect that a loss-of-function allele of this gene could be causal for hypocotyl resistance. We identified a retrotransposable element in the coding sequence of *CsaMLO8* in a genotype with hypocotyl resistance, leading to a loss-of-function allele. We proved that the WT *CsaMLO8* gene is a functional *MLO* homolog by heterologous overexpression of the gene in an *mlo* mutant tomato genotype, restoring full susceptibility to tomato PM caused by *Oidium neolycopersici*, whereas overexpression of the mutant *Csamlo8* allele did not restore susceptibility, proving that this loss-of-function mutation leads to PM resistance.

In **Chapter 3** we continued our work on cucumber *MLO* genes by studying *CsaMLO1* and *CsaMLO11*, both closely related homologs of *CsaMLO8*. We showed by heterologous overexpression in tomato that each of these three cucumber *MLO* genes can restore PM susceptibility to *O. neolycopersici* in the *mlo* tomato too, indicating that all three genes are functional *S* genes. We searched for mutant alleles of either *CsaMLO1* or *CsaMLO11* in resequenced cucumber accessions, but did not find any loss-of-function mutations. By studying the expression of all three cucumber *MLO* genes, we found that *CsaMLO8* is expressed to a much higher extent compared to the two other genes in tissues relevant for PM, likely explaining why *CsaMLO8* mutations confer partial PM resistance whereas mutant alleles of the other *MLO* genes were apparently not selected for.

In **Chapter 4**, the focus of this thesis shifts towards DM. We studied introgression lines derived from Indian accession PI 197088, which is currently the most frequently used source of DM resistance, in order to identify causal genes. We found that a major QTL on chromosome 4 consisted of three subQTL, each of which could be distinguished by markedly different resistance phenotypes. A transcriptomic approach led to the identification of a novel *RLK* gene, *CsLRK10L2*, as the most likely candidate for one of these subQTL, being highly upregulated upon inoculation, and being lost due to a 551 bp deletion in susceptible genotypes. Heterologous overexpression of this gene in *Nicotiana benthamiana* triggered a fast, strong, necrotic response, indicating that this gene can activate defence responses, culminating in cell death. The evolutionary history of this gene as well as roles of homologous genes in *Arabidopsis thaliana* are discussed.

In **Chapter 5**, the work from the previous chapter is continued by identification of a candidate gene for another subQTL contributing to DM resistance of accession PI 197088. A loss-of-function mutation in the amino acid transporter *CsAAP2A* was found, caused by the integration of a transposable element, leading to abolishment of *CsAAP2A* expression in resistant genotypes. As *CsAAP2A* represents a novel susceptibility gene,

involved in amino acid transport, the effect of the mutation in this gene on amino acid dynamics was investigated by gas chromatography. We found that inoculation with *P. cubensis*, the causal agent of DM, led to a drastic increase in amino acid levels in infected leaves. However, in leaves of partially resistant plants being homozygous for the natural *csaap2a* mutation, this increase in amino acid concentration was significantly lower than in the susceptible control, indicating that the mentioned mutation prevents the flow of nutrients towards the pathogen, thereby decreasing the pathogen's fitness.

Finally, in **Chapter 6** I integrate and discuss the results of the previous chapter. Implications of these results on cucumber-mildew interactions and perspectives for future research as well as cucumber breeding are given.

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Chapter 2

A transposable element insertion in the susceptibility gene *CsaMLO8* results in hypocotyl resistance to powdery mildew in cucumber

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ABSTRACT

Background: Powdery mildew (PM) is an important disease of cucumber (*Cucumis sativus* L.). *CsaMLO8* was previously identified as a candidate susceptibility gene for PM in cucumber, for two reasons: 1) This gene clusters phylogenetically in clade V, which has previously been shown to harbour all known *MLO*-like susceptibility genes for PM identified in dicot species; 2) This gene co-localizes with a QTL on chromosome 5 for hypocotyl-specific resistance to PM.

Results: We cloned *CsaMLO8* alleles from susceptible and resistant cucumber genotypes, the latter carrying the QTL for hypocotyl resistance. We found that insertion of a non-autonomous Class LTR retrotransposable element in the resistant genotype leads to aberrant splicing of *CsaMLO8* mRNA. Heterologous expression of the wild-type allele of *CsaMLO8* in a tomato *mlo*-mutant restored PM susceptibility. However, heterologous expression of the *CsaMLO8* allele cloned from the resistant cucumber genotype failed to restore PM susceptibility. Furthermore we showed that inoculation of susceptible cucumber with the PM pathogen *Podosphaera xanthii* induced transcriptional upregulation of *CsaMLO8* in hypocotyl tissue, but not in cotyledon or leaf tissue. This coincides with the observation that the QTL at the *CsaMLO8*-locus causes full resistance in hypocotyl tissue, but only partial resistance in cotyledons and true leafs. We studied the occurrence of the loss-of-function allele of *CsaMLO8* in cucumber germplasm by an *in silico* approach using resequencing data of a collection of 115 cucumber accessions, and found that this allele was present in 31 out of 115 accessions.

Conclusions: *CsaMLO8* was characterised as a functional susceptibility gene to PM, particularly in the hypocotyl where it was transcriptionally upregulated upon inoculation with the PM pathogen *P. xanthii.* A loss-of-function mutation in *CsaMLO8* due to the insertion of a transposable element was found to be the cause of hypocotyl resistance to PM. This particular allele of *CsaMLO8* was found to occur in 27% of the resequenced cucumber accessions.

Keywords: Powdery mildew, *MLO*, Susceptibility gene, Cucumber (*Cucumis sativus* L.), Hypocotyl resistance, Non-autonomous transposable element

BACKGROUND

Cucumber (*Cucumis sativus* L.) is an economically important crop, with an annual global production of over 65 megatons [1]. Powdery mildew (PM) is one of the most widespread diseases in cucurbits, and a limiting factor for cucumber production. Two species of fungi have been reported to cause PM in cucumber, i.e. *Podosphaera xanthii* (synonymous with *P. fusca*, previously named *Sphaerotheca fuliginea*) and *Golovinomyces cichoracearum* (previously named *Erysiphe cichoracearum*). Of these, *P. xanthii* is considered to be the main causal agent of PM in cucurbits [2, 3].

Breeding of resistant cucumber varieties has been undertaken for several decennia (e.g. [4–6]), but underlying resistance genes have to date not been functionally characterised. As the genome of cucumber ('Chinese long' inbred line 9930) was published in 2009 [7], and several other cucumber accessions have been resequenced [8, 9], the time is now ripe to identify causal genes for cucumber resistance to mildew diseases.

Traditionally, breeding of disease resistant crops is performed by introgression of resistance (*R*) genes, often from wild relatives of the crop. R proteins, most commonly of the nucleotide-binding, leucine-rich-repeat (NB-LRR) type, are able to recognise either corresponding avirulence (*Avr*) gene products of the pathogen, or degradation products of host factors associated with pathogen attack [10]. This triggers a defence response in the host cell, often associated with a hypersensitive response (HR), leading to cell death [10]. As *R* genes recognise very specific products, introgression and subsequent employment of a new *R* gene puts selective pressure on the pathogen to evolve in such a way that it is no longer recognised by the host plant. Therefore, *R*-gene based resistance is often breached by new, virulent, races of the pathogen quite soon, especially for versatile pathogens, such as powdery mildew fungi [10].

An alternative for *R*-gene mediated resistance is the identification of impaired susceptibility (*S*) genes [11]. Most pathogens require cooperation of their host plant to be able to successfully establish a compatible interaction [12]. This is especially true for biotrophic pathogens such as mildew species, as they greatly rely on a long-lasting interaction with (living) host cells to facilitate their propagation [12]. Therefore, the expression of several host genes is essential for the pathogen. Such genes can be regarded as *S* genes, and can function for instance in facilitating host recognition and penetration, negative regulation of host defences or fulfilling metabolic and structural needs of the pathogen [12]. Loss-of-function mutations in a *S* gene is thought to lead to durable, broad spectrum, recessively inherited resistance [13, 14].

The barley *mlo* gene is one of the best-known examples of an impaired *S* gene. After it first was found in the 1940s in a mutagenized barley population [15], deployment of loss-of-function *mlo* alleles in barley has resulted in PM resistant barley varieties. These have been grown in the field for several decades already without breaching of resistance by virulent new mildew races to date, providing evidence for the durability of *S*-gene based resistance [16]. After the barley *MLO* gene was cloned [17], it was found that *MLO* genes are conserved throughout the plant kingdom and occur in higher plants as a multi-copy gene family [18, 19]. In several plant species, *MLO*-like genes have been found to be involved in PM susceptibility, such as *Arabidopsis*, tomato, pea, pepper, tobacco, bread wheat and potentially also grapevine and peach [20–27]. It has been found that in phylogenetic trees of the *MLO* gene family all *MLO*-like S-genes for PM detected in monocotyledonous species cluster in clade IV, whereas all *MLO*-like *S*-genes identified in dicotyledonous species cluster in clade V. The other clades (I, II, III and VI) harbour *MLO*-like genes that have not been proven to be *S*-genes [19].

The genome of cucumber harbours 13 putative *MLO*-like genes [28]. Of these, three (i.e. *CsaMLO1*, *CsaMLO8* and *CsaMLO11*,with respective Cucurbit Genomics Database IDs [Csa1M085890.1], [Csa5M623470.1] and [Csa6M292430.1]) cluster in clade V of the *MLO* gene family, and can therefore be considered candidate *S*-genes for powdery mildew resistance [28]. *CsaMLO8* is of particular interest, as its position on the genome (Chr5: 24,827,408..24,831,456) co-localizes with *pm5.2*, a recently identified major QTL explaining 74.5% of the phenotypic variation for 'hypocotyl' resistance in F3 families derived from the resistant cucumber inbred line WI 2757 [29]. 'Hypocotyl' or intermediate resistance of cucumber to PM was previously shown to be a recessively inherited monogenic trait in crossings between several cucumber lines, and was characterised by completely resistant hypocotyl, stem and petiole tissue and partially resistant leaves and cotyledons. Hypocotyl resistance is suggested to play an important role in overall PM resistance of cucumber, as it appears that complete resistance in leaves is not possible without the recessive hypocotyl resistance gene [5]. In breeding practice loss of the hypocotyl resistance allele leads to PM susceptible seedlings. The allele is present in almost all modern pickling cucumber varieties, and most of the resistant long cucumber varieties (Freddy Hermans, personal communications), showing the agricultural significance of hypocotyl resistance in cucumber.

Here, we report the cloning of *CsaMLO8* from both susceptible and (hypocotyl) resistant cucumber genotypes. We show that at the transcript level the allele obtained from the resistant genotype has deletions of 72 or 174 bp due to alternative splicing, caused by the insertion of a LTR retrotransposable element in this gene at the genomic level. Complementation of the tomato *mlo*-mutant with the wild-type and Δ174 alleles of

CsaMLO8 showed that wild-type *CsaMLO8* is a functional susceptibility gene (*S*-gene), whereas the Δ174 allele has lost its function as *S*-gene, thus leading to PM resistance. Furthermore, qRT-PCR showed that *CsaMLO8* is transcriptionally upregulated upon inoculation with *P. xanthii* in hypocotyl tissue, but not in leaves or cotyledon, explaining why loss-of-function of *CsaMLO8* provides particularly resistance in the hypocotyl.

RESULTS

Cloning and sequencing of the *CsaMLO8* coding sequence from susceptible and resistant genotypes

We performed RT-PCR using RNA derived from either a susceptible wild-type cucumber cultivar or a resistant breeding line known to be homozygous for the *hypocotyl resistance* QTL as a template. Whereas the product we obtained from the susceptible genotype was of the expected size (i.e. 1726 bp), we obtained two different products from the resistant genotype, both smaller than expected (Figure 1A). Sequence analysis revealed that the *CsaMLO8* mRNA variant obtained from the susceptible genotype was identical to the predicted coding sequence. The two mRNA products obtained from the resistant genotype however had (non-frameshift) deletions of respectively 72 and 174 bp. The 174 bp deletion variant corresponds to a loss of the complete 11th exon of the *CsaMLO8* gene, whereas the 72 bp deletion variant corresponds to the loss of a fragment of the 11th exon with canonical splice sites (5'-GT and AG-3') (Figure 1B). Furthermore, the coding sequence of the resistant genotype has five (synonymous) SNPs compared to the reference genome (Additional file 1).

To determine the impact of the 72 and 174 bp deletions found in the mRNA on the predicted CsaMLO8 protein sequence, the predicted CsaMLO8 protein was aligned to a dataset of MLO proteins encoded by clade V S-genes from several other species i.e. *Arabidopsis*, barrel clover, pea, lotus, tomato, pepper and tobacco (Additional file 2). It appeared that the region encoded by the deleted area in the 72 and 174 bp deletion variants is highly conserved among different MLO proteins (Figure 1C). Furthermore, the transmembrane structure of the CsaMLO8 protein (wild-type allele) was predicted using HMMTOP 2.1 software [30]. The predicted transmembrane structure of the wild-type protein was largely consistent with the barley MLO structure determined by Devoto et al. [18, 19]. The 72 and 174 bp deletions correspond to removal of a region of 24 respectively 58 amino acid residues in the (predicted) third cytoplasmic loop of CsaMLO8 (Figure 1D).

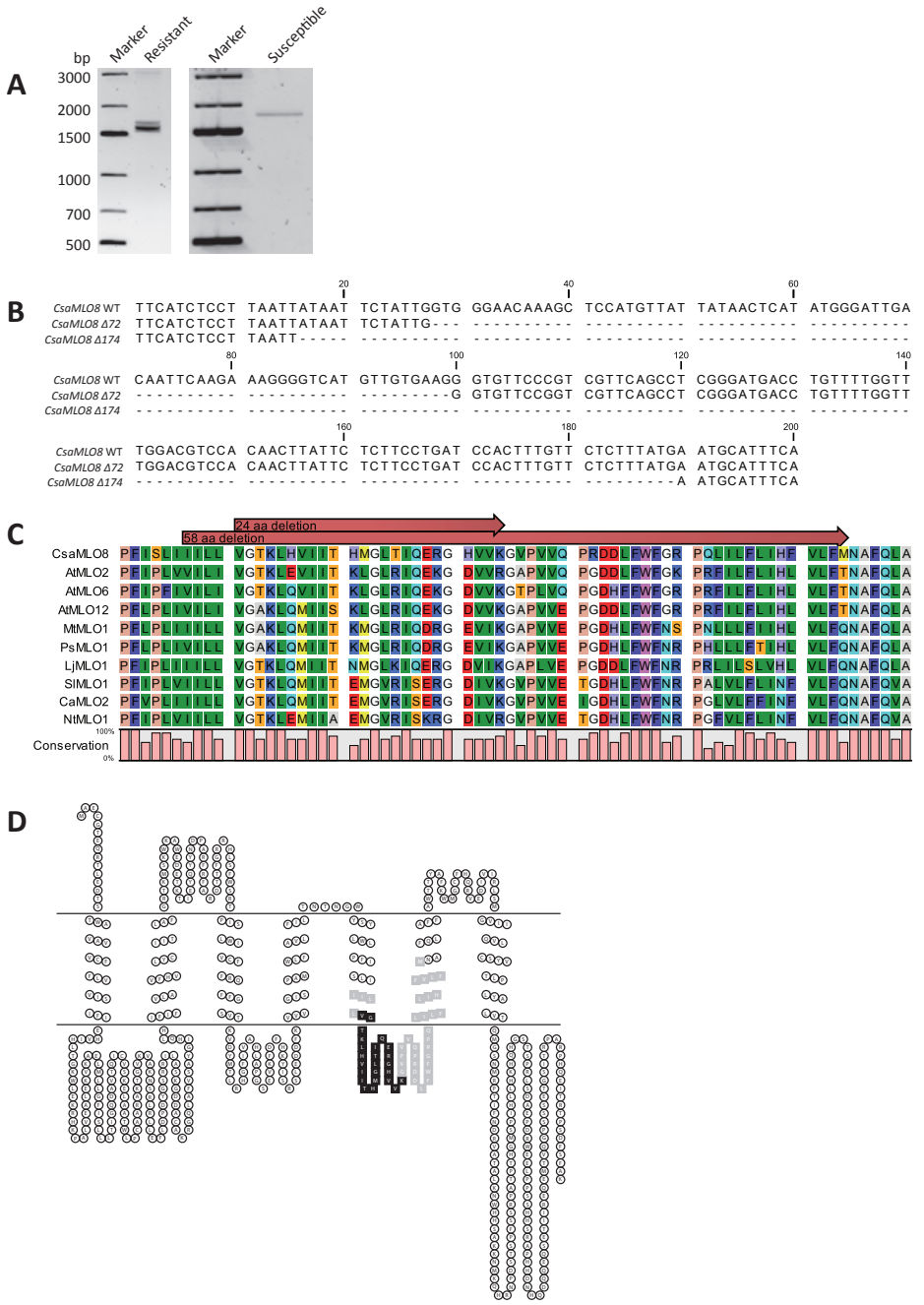

Figure 1 – Characterization of *CsaMLO8* alleles from resistant and susceptible cucumber genotypes

Figure 1 – Characterization of *CsaMLO8* alleles from resistant and susceptible cucumber genotypes
A) cDNA of resistant (left panel) and susceptible (right panel) cucumber genotypes was used as template for PCR with *CsaMLO8* specific primers. Amplified products were analysed on 1.25% agarose gels. Whereas the product amplified from cDNA of the susceptible genotype gives a single band of the expected size, cDNA of the resistant genotype results in two separate bands, both of a smaller size than expected.
B) Full length *CsaMLO8* amplified from cDNA from susceptible and resistant cucumber genotypes was sequenced. A partial alignment is shown between the (wild-type) sequence as obtained from the susceptible genotype and the sequences from two deletion variants (Δ72 and Δ174) obtained from the resistant genotype. Numbers are relative to the start of the alignment.
C) Partial alignment of the CsaMLO8 protein and other proteins encoded by clade V *MLO* S-genes of several species. Amino acid residues are coloured according to the RasMol colour scheme. The 24 and 58 amino acid residues deleted in the proteins encoded by the Δ72 and the Δ174 variants of *CsaMLO8* are indicated by red arrows. A bar graph underneath the alignment indicates the conservedness of each amino acid position.
D) Graphic representation of the transmembrane structure of the predicted CsaMLO8 protein, determined using HMMTOP 2.1 [30]. The plasma membrane is indicated by two horizontal lines. Amino acid residues highlighted in black are predicted to be deleted in the protein encoded by the Δ72 variant of the *CsaMLO8* gene, residues highlighted in black and grey are predicted to be deleted in the protein encoded by the Δ174 variant of the *CsaMLO8* gene.

The relative transcript abundances of the two *CsaMLO8* splice variants characterised by the 72 and 174 bp deletions were determined by qRT-PCR using splice junction spanning primers on different tissues (i.e. hypocotyl, cotyledon and true leaf) of PM resistant cucumber, either inoculated with PM or non-inoculated. It appeared that the 174 bp deletion splice variant was the most abundant isoform, whereas the 72 bp deletion splice variant was less abundant in each tissue regardless whether tissues were inoculated or not (Additional file 3).

Complementation of *SlMLO1* loss-of-function tomato mutant with *CsaMLO8* WT and *CsaMLO8Δ174*

The sequence analysis of the transcripts of *CsaMLO8* from susceptible and resistant genotypes led to the hypothesis that *CsaMLO8* is a functional *S*-gene for PM, whereas the 174 bp deletion allele (*CsaMLO8Δ174*) has lost its function as *S*-gene. To test these hypotheses, both alleles were overexpressed in a previously described tomato *mlo*-mutant, which carries a mutation in the tomato *SlMLO1* gene and is resistant to tomato powdery mildew, *Oidium neolycopersici* [21].

Cuttings of ten independent transgenic individuals per construct (*35S::CsaMLO8* WT and *35S::CsaMLO8Δ174*) were challenged with the tomato PM pathogen *O. neolycopersici*. Powdery mildew susceptibility was evaluated qualitatively, by looking for PM symptoms on the leaves (Figure 2A, Additional file 4). Six out of ten individual

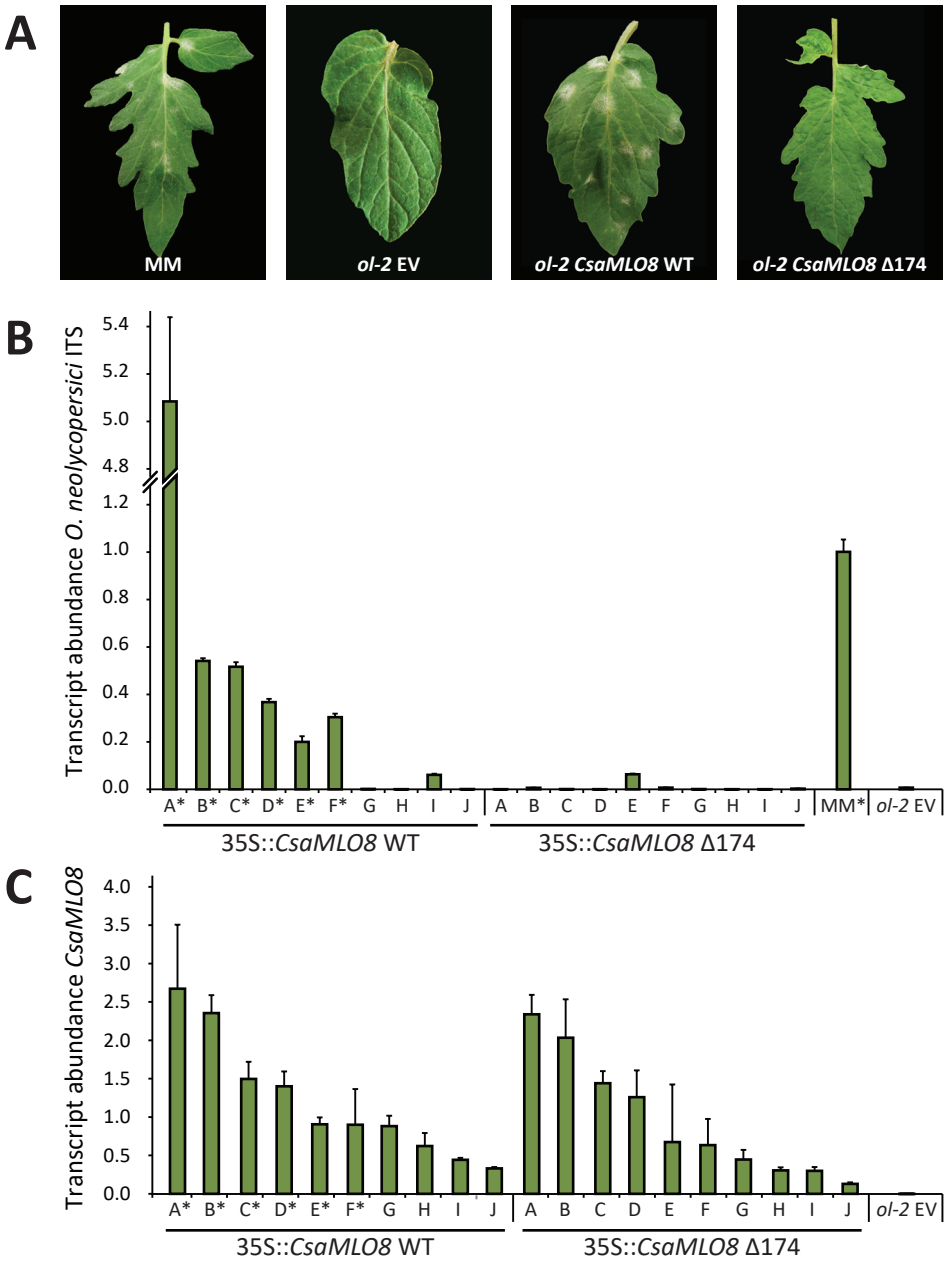

Figure 2 – Complementation of ol-2 tomato with *CsaMLO8* WT restores PM susceptibility, whereas complementation with *CsaMLO8*Δ174 does not

The PM resistant ol-2 tomato mutant with a deletion in *SlMLO1* [21] was transformed with either a 35S::*CsaMLO8* WT construct, a 35S::*CsaMLO8*Δ174 construct, or an empty vector (EV) control. Cuttings from these transformants were inoculated with a *Oidium neolycopersici* spore suspension. As additional control we used the wild-type, susceptible cv. Moneymaker (MM).

Figure 2 – Complementation of ol-2 tomato with *CsaMLO8* WT restores PM susceptibility, whereas complementation with *CsaMLO8*Δ174 does not
A) The phenotype of susceptible control MM, resistant EV transformed ol-2, and transgenic individuals overexpressing either *CsaMLO8* WT or *CsaMLO8*Δ174 in ol-2 background. Photographs were taken 16 days post inoculation.
B) Relative quantification by qPCR of the ratio between *O. neolycopersici* and plant gDNA in susceptible MM, resistant EV transformed ol-2, and transgenic individuals overexpressing either *CsaMLO8* WT or *CsaMLO8*Δ174 in ol-2 background. Fold changes were normalised relative to the susceptible control MM. based on macroscopic evaluation. Bars represent the average fold change over 3 technical replicates. Error bars indicate standard deviation. Asterisks indicate plants scored as susceptible to powdery mildew.
C) Relative quantification by qRT-PCR of the ratio between *CsaMLO8* expression and expression of tomato housekeeping gene *SlEF-*α in EV transformed ol-2 and transgenic individuals overexpressing either *CsaMLO8* WT or *CsaMLO8*Δ174 in ol-2 background. Bars represent the average fold change over 3 technical replicates. Error bars indicate standard deviation.

transformants expressing *CsaMLO8* WT were scored as susceptible to PM, whereas none of the transformants expressing *CsaMLO8Δ174* were scored as susceptible to PM. PM susceptibility was confirmed quantitatively, by performing qPCR on DNA isolated from inoculated leaves, using *O. neolycopersici* specific primers. This showed that the biomass of *O. neolycopersici* in plants scored as susceptible to PM was at least 0.20, relative to the biomass in the susceptible control MM, whereas the biomass in plants scored as resistant was less than 0.20 (Figure 2B). Furthermore, transcript abundances of the transgenes in each of the transgenic individuals were determined by qRT-PCR using *CsaMLO8* specific primers (Figure 2C). This confirmed that transcript levels of *CsaMLO8* WT and *CsaMLO8Δ174* were comparable. The six *CsaMLO8* WT transformants scored as susceptible to PM had a higher *CsaMLO8* expression than the four *CsaMLO8* WT transformants scored as resistant to PM.

Sequencing and characterization of a transposable element in *CsaMLO8*

To investigate the cause of the deletions in the *CsaMLO8* coding sequence, we performed PCR using DNA from both the susceptible and resistant cucumber genotypes as a template, with primers designed to amplify the region that contained the deletions in *CsaMLO8*. The product amplified from the susceptible genotype had the expected size (i.e. 346 bp), whereas the product amplified from the resistant genotype was larger (ca. 1500 bp, Figure 3A). Sequence analysis of the amplified product revealed a 1449 bp insertion in the genomic DNA sequence of the resistant genotype compared to the susceptible genotype. This insertion in the DNA of the resistant genotype coincided with the region that contained the deletion in the *CsaMLO8* mRNA of this genotype. Characterization of this genomic insertion by a dot-plot (Figure 3B) revealed the presence of long terminal repeats (LTRs) with a length of ca. 200 bp. An alignment between the first and last 200 bp of the insertion confirmed the presence of 184 bp long LTRs

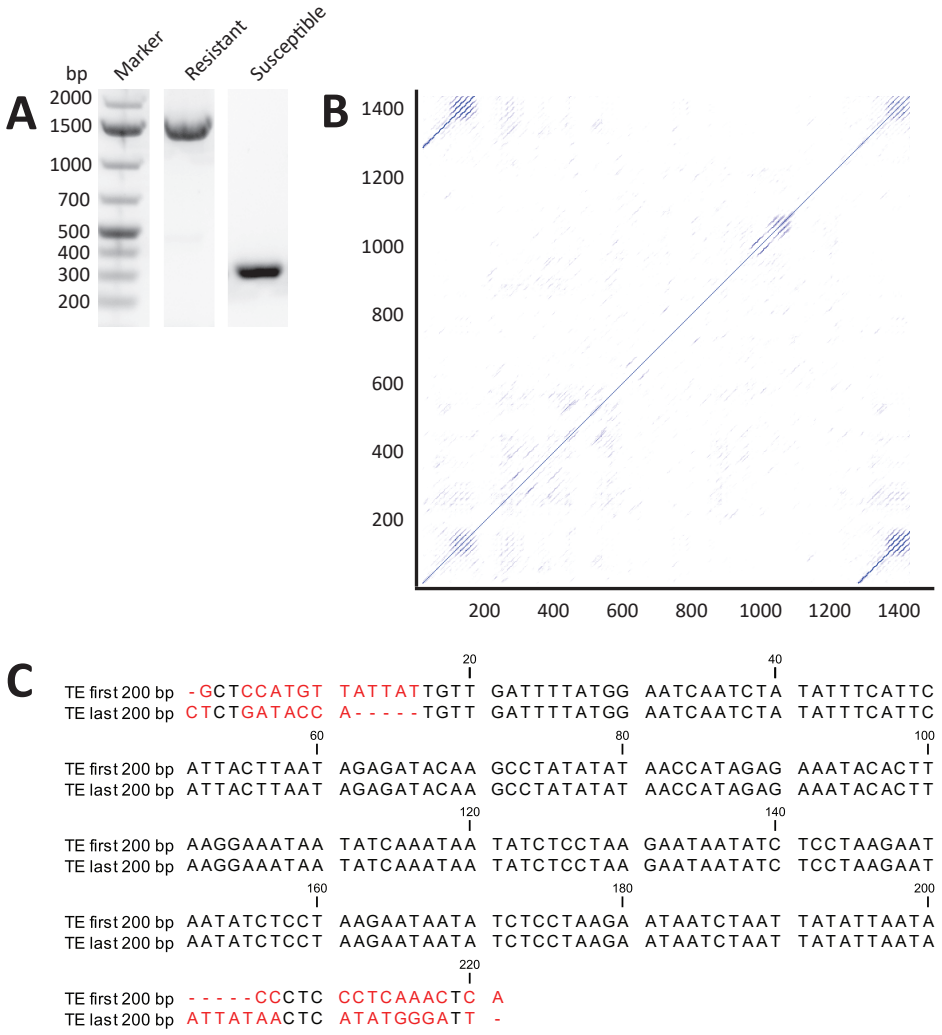

Figure 3 – Amplification and sequencing of *CsaMLO8* from genomic DNA isolated from the resistant genotype reveals the insertion of an 1449 bp long Transposable Element (TE)

A) The genomic region of *CsaMLO8* in which deletions in the coding sequence were observed in the resistant genotype was amplified from DNA isolated from both the susceptible and resistant genotypes. Amplified products were analysed on 1.25% agarose gel. Whereas the product amplified from the susceptible genotype was of the expected size, the product amplified from the resistant genotype was larger than expected.

B) The product amplified from the resistant genotype as described in **(A)** was sequenced, which revealed an insertion with a length of 1449 bp. A dot-plot was made of the insertion to see whether the sequence contains repetitive elements.

Figure 3 – Amplification and sequencing of *CsaMLO8* from genomic DNA isolated from the resistant genotype reveals the insertion of an 1449 bp long Transposable Element (TE)
C) The first and last 200 bp of the insertion, plus 15 bp of *CsaMLO8* before and after the insertion were aligned to one another, to verify the presence of long terminal repeats (LTRs). Non-aligned parts of the sequence are highlighted in red. It can be seen that the first 184 bp of the insertion are completely identical to the last 184 bp of the insertion. There is a duplication of 5 bp from *CsaMLO8* before and after the insertion (Target site duplication, 5'-ATTAT-3').
D) Schematic representation of the insertion. The locations of LTRs and the 3' TSD are indicated.

beginning with a 5'-TG-3' and ending with a 5'-TA-3' (Figure 3C). The LTRs share 100% sequence identity with one another. After the 3' LTR, there is a duplication of the 5 bp of *CsaMLO8* before the insertion (Target Site Duplication, TSD, 5'-ATTAT-3'). No open reading frames (ORFs) could be detected in the insertion. Taken together, these findings led us to the conclusion that the insert is most likely a non-autonomous transposable element (TE) of Class I, Order LTR, according to the transposable element classification scheme proposed by Wicker et al. [31].

Similar TEs in the cucumber genome

In an attempt to identify homologous, potentially autonomous, transposable elements in the cucumber genome, we performed a BLASTn search on the cucumber reference genome (Chinese long inbred line '9930', v2) with the LTR sequence of the TE found in *CsaMLO8* as query. We identified 169 putative homologous LTRs. A previously designed tool [32] was used to screen the genome for regions bordered by two putative homologous LTR sequences. Two putative homologous LTR sequences within a window of 20 kb were considered to be the borders of a putative homologous TE. The 20 kb window was decided upon based on the observation that LTR retrotransposons are generally between 3-15 kb of size [33], the only exception to our knowledge being the very large *Ogre* retrotransposons found in legumes [34], which have ca. 5 kb LTRs and are therefore ca. 22 kb in size. A total of 44 putative TEs was identified, randomly distributed over all seven chromosomes of the cucumber reference genome (Figure 4, Additional file 5). For 20 putative TEs, the complete sequence in between the LTRs was extracted from the genome, and compared to the sequence of the TE found in *CsaMLO8* (Additional file 6). It was found that most of the putative TEs have a length comparable to the *CsaMLO8*-TE, being between 1 and 2 kb. One putative TE was considerably larger than average, with 7,142 bp, whereas one putative TE was considerably smaller than average, i.e. 367 bp. In only one out of the 20 putative TEs (TE37), an open reading frame (ORF) could be detected. This ORF, with a length of 411 bp, does not lead to a predicted protein with any similarity to known proteins according to a BLASTp search against all non-redundant protein databases, and is therefore considered a false positive ORF. We conclude that we could not detect an autonomous TE that contained the genes that could have been responsible for the insertion of the non-autonomous TE in *CsaMLO8.*

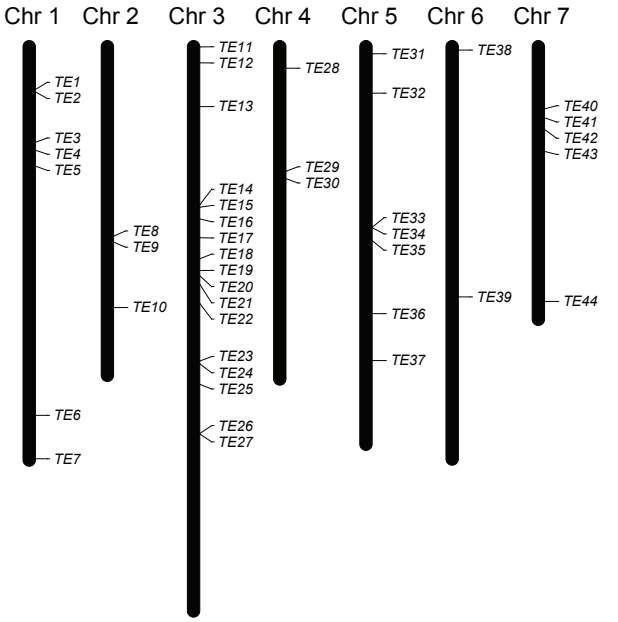

Figure 4 – There are 44 putative homologous TEs in the cucumber reference genome
A BLASTn search was performed on the cucumber reference genomes with the LTR sequence of the TE found to be inserted in *CsaMLO8*. Pairs of putative LTRs within 20 kb of one another were considered borders of putative TEs. 44 putative TEs were identified, chromosomal locations of which are indicated

Occurrence of the TE-allele of *CsaMLO8* in cucumber germplasm

We were interested to see how frequently the TE-allele of *CsaMLO8* we have characterised in our resistant cucumber genotype occurs in the cucumber germplasm. As Qi et al. (2013) resequenced a core collection of 115 very divergent cucumber accessions [8], we decided to perform an *in silico* search for the presence of the mutant *CsaMLO8* allele containing the TE) and/or the wild type (WT) allele among those genotypes. For 21 resequenced accessions (18%) we could only detect reads indicating presence of the TE-allele. For 82 resequenced accessions (71%) we could only find reads indicating presence of the WT-allele. For 10 accessions (9%) we found reads indicating presence of both alleles. For the remaining two accessions (2%), presence of neither of the alleles could be identified (Table 1, Additional file 7). The TE-allele of *CsaMLO8* was present in three out of the four geographic groups of accessions (i.e. East Asian, Eurasian and Indian but not Xishuangbanna) as defined by Qi et al. [8]. One of the 31 accessions in which the TE-allele of *CsaMLO8* was detected (i.e. PI 215589) belongs to the wild form of cucumber, *Cucumis sativus* var. *hardwickii,* whereas the other 30 accessions belong to the cultivated form of cucumber, *C. sativus* var. *sativus.*

Table 1 - 31 out of 115 resequenced cucumber accessions have the TE-allele of CsaMLO8.
Total reads of 115 recently resequenced cucumber accessions [8] were assayed in silico for the presence of reads indicating the presence of either the allele of CsaMLO8 characterised by the insertion of a TE, or the wild-type allele. The amount of reads indicating presence of either the TE-allele or the WT-allele of CsaMLO8 is given. Database number, accession names and geographic groups of accessions were obtained from [8].

Accession number NCBI SRA	TE-allele reads	WT-allele reads	Putative genotype[A]	PI or CGN number	Name Accession	Group[B]
SRR543205	9	0	Hom.	PI 215589	13598	I
SRR543216	17	0	Hom.	V05A0674	Bei Jing Xiao Ci	A
SRR543221	1	9	Het.	V05A1333	Liao Tong Mi Ci	A
SRR543223	19	0	Hom.	V05A0920	He Cha Huang Gua	A
SRR543224	19	0	Hom.	V05A1115	Qian Qi Li Huang Gua	A
SRR543225	1	7	Het.	V05A0985	Ye San Bai	A
SRR543226	23	0	Hom.	V05A0428	Liao Yang Ye San	A
SRR543228	1	0	Hom.	-	228	A
SRR543230	18	0	Hom.	V05A0522	Huang Gua	A
SRR543231	5	8	Het.	V05A0552	Qing Dao Qiu Ye Er San	A
SRR543240	1	13	Het.	CGN19828	-	A
SRR543242	22	0	Hom.	V05A0034	Da Ci Huang Gua	A
SRR543243	12	1	Het.	V05A1427	Qiu Huang Gua	A
SRR543244	1	7	Het.	V05A0291	Leng Lu Huang Gua	A
SRR543246	1	0	Hom.	-	Bai Ye San	A
SRR543251	4	0	Hom.	-	2004348	A
SRR543252	11	0	Hom.	CGN20266	Hok	E
SRR543253	6	0	Hom.	-	151G	E
SRR543257	5	0	Hom.	CGN20512	752	E
SRR543258	9	0	Hom.	CGN20515	Gy 3 (S4)	E
SRR543264	2	0	Hom.	-	65G	E
SRR543265	11	0	Hom.	-	G8	E
SRR543267	14	0	Hom.	V05A0726	Jin Yan Er Hao	A
SRR543269	10	3	Het.	CGN19579	1972 B-2	E
SRR543271	15	0	Hom.	CGN19844	2163	E
SRR543272	2	5	Het.	PI 234517/CGN20898	SC 50	E
SRR543274	11	0	Hom.	CGN21627	Spartan Garden MSU-C7-63	E
SRR543275	4	0	Hom.	-	Marketmore76	E
SRR543276	4	0	Hom.	-	GY14	E
SRR543281	9	3	Het.	PI 482412	TGR 580	I
SRR543293	6	8	Het.	PI 605943	USM 307	I

[A]: Hom. = Homozygous, Het. = Heterozygous
[B]: I = Indian, A = East Asian, E = Eurasian

Inoculation with *P. xanthii* induced transcription of *CsaMLO8* in hypocotyl tissue, but not in leaf tissue of susceptible cucumber

MLO genes involved in PM susceptibility are upregulated in several plant species several hours after inoculation (e.g [26, 35, 36]). To see whether the same holds true for *CsaMLO8,* we performed qRT-PCR experiments to quantify *CsaMLO8* transcript abundances in hypocotyl, cotyledon and leaf tissues of PM susceptible and resistant cucumber plants, prior to and at 4, 6, 8 and 24 hours after PM inoculation (Figure 5). For PM susceptible plants, we found that in hypocotyl tissue *CsaMLO8* transcript abundance was significantly higher at 4 hpi (p = 0.037) and 6 hpi (p = 0.004) compared to the transcript abundance prior to inoculation (0 hpi). The significant difference had disappeared 8 hpi (p = 0.212) and 24 hpi (p = 0.281). Contrastingly, *CsaMLO8* transcript abundances in cotyledons and true leaves were not significantly altered at any of the evaluated time points after PM inoculation (p > 0.05) (Figure 5A).

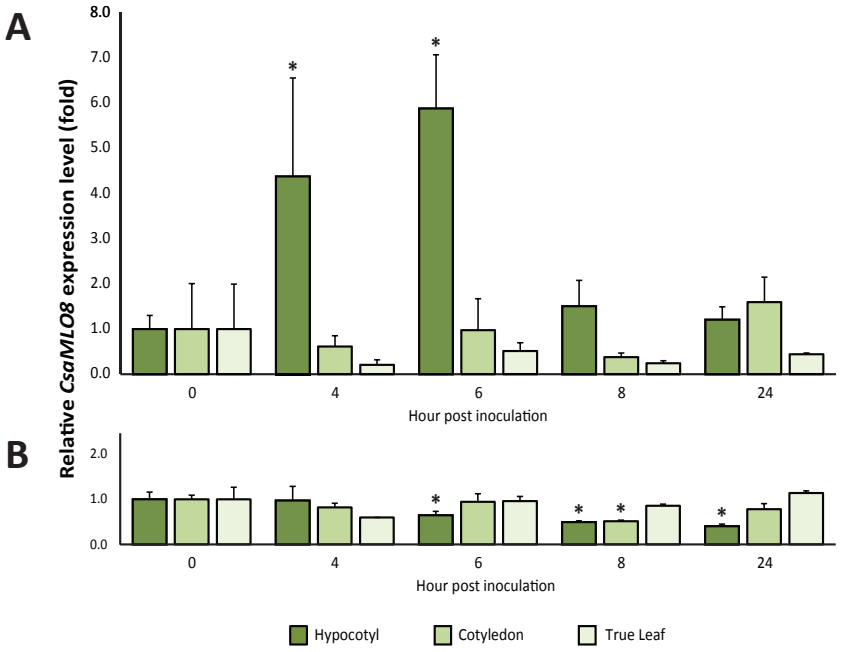

Figure 5 – *CsaMLO8* transcription is induced after inoculation with *Podosphaera xanthii* in hypocotyl tissue, but not in cotyledon or true leaf tissue
Susceptible **(A)** and resistant **(B)** cucumber seedlings were inoculated with a *P. xanthii* spore suspension. Prior to and 4, 6, 8 and 24 hours post inoculation, hypocotyl, cotyledon and true leaf tissue were harvested and immediately frozen in liquid nitrogen. Relative quantification of *CsaMLO8* expression was performed by qRT-PCR. Fold changes were normalised relative to *CsaMLO8* expression prior to inoculation. Bars represent the average fold change over three independent biological replicates. Error bars indicate standard errors of the mean. Asterisks indicate significant differences to the expression prior to inoculation (Student's T test, p<0.05)

For PM resistant plants, we found that *CsaMLO8* transcript abundance was not significantly higher in any tissue at any time point after inoculation compared to the transcript abundance prior to inoculation ($P > 0.05$). In hypocotyl tissue, transcript abundance was significantly lower at 6 hpi ($P = 0.046$), 8 hpi ($p = 0.006$) and 24 hpi ($p = 0.009$) compared to the transcript abundance prior to inoculation (0 hpi). In cotyledon tissue, transcript abundance was significantly lower at 8 hpi ($P = 0.002$) compared to the transcript abundance prior to inoculation (Figure 5B).

DISCUSSION

CsaMLO8 is a functional susceptibility gene for PM in cucumber

Several studies characterised some, but not all, clade V *MLO* genes as being required for PM susceptibility in different dicotyledonous plant species [20–23, 25–27]. Here we have shown that heterologous expression of the cucumber gene *CsaMLO8* in *Slmlo1* mutant tomato background restored PM susceptibility, providing evidence for the role of *CsaMLO8* as a susceptibility gene for PM in cucumber (Figure 2). As the role of clade V *MLO* genes in susceptibility to PM seems to be evolutionary conserved between divergent dicotyledonous plant families, e.g. Brassicaceae [20], Solanaceae [21, 23, 25], Fabaceae [22], Vitaceae [26], Rosaceae [27, 36] and now also Cucurbitaceae, it is probable that in other economically important species belonging to the family Cucurbitaceae, such as melon (*Cucumis melo*) and pumpkin (*Cucurbita pepo*) clade V *MLO* genes will also play a role in PM susceptibility. Indeed, in a patent application a functional complementation of *Arabidopsis Atmlo2*, *Atmlo2,6* and *Atmlo2,6,12* mutants by a melon *MLO*-like gene was claimed to partially restore PM susceptibility, based on the percentage of diseased leaf area in 4 to 9 primary transformants [37]. Alignment of this melon *MLO* gene with the three Clade V genes of cucumber revealed that the gene from melon is most similar to *CsaMLO8,* and less alike to the two other Clade V genes (i.e. *CsaMLO1* and *CsaMLO11*) [28]. This is consistent with our finding that *CsaMLO8* is an *S*-gene for PM. In tomato we observe that complementation of *SlMLO1* loss-of-function mutants with *CsaMLO8* restores PM susceptibility, with individual transformants with higher *CsaMLO8* expression generally being more susceptible to PM than transformants with lower *CsaMLO8* expression (Figure 2). It seems possible that in the case of complementation of *Arabidopsis* mutants by the melon *MLO* gene there is also a quantitative effect due to different levels of melon *MLO* expression in individual transformants, leading to the conclusion that the melon *MLO* gene only partially restores susceptibility whereas it is actually due to the fact that transgene expression was not high enough to fully complement the loss of *AtMLO* function.

Transposon insertion in *CsaMLO8* leads to aberrant splicing and therefore to loss of the *S*-gene function

By cloning *CsaMLO8* from cDNA of a PM resistant cucumber genotype that is homozygous for the *hypocotyl resistance* QTL, we found evidence for aberrant splicing of *CsaMLO8* in this genotype, leading to products with deletions of respectively 72 and 174 bp in exon 11, compared to the WT gene. We showed that these deletions are predicted to lead to loss of 24 respectively 58 amino acid residues in the third cytoplasmic loop of the CsaMLO8 protein, in a highly conserved region between clade V MLO proteins from different species (Figure 1). As it was previously shown that cytoplasmic loop-loop interplay is required for MLO function [38], we anticipated that such rather big deletions in one of the cytoplasmic loops, if the protein should properly fold at all, would lead to loss-of-function of the protein. Indeed, we showed here that expression of the Δ174 variant of *CsaMLO8* in *Slmlo* mutant tomato background failed to restore PM susceptibility (Figure 2). This makes cucumber, after barley [17], tomato [21] and pea [22], the fourth plant species in which a natural mutation in an *MLO* gene has been found to lead to resistance. Although we did not try to complement *Slmlo* mutant tomato with the 72 bp deletion variant of *CsaMLO8*, and thus cannot rule out the possibility that it is (partially) functional as an S gene, we expect that the result will be similar to the 174 bp deletion variant, given the conservedness of the deleted region.

To determine the reason for the aberrant splicing of *CsaMLO8* in the resistant cucumber genotype, we set out to amplify and sequence the genomic region of *CsaMLO8* in which the deletions were detected. In this way, we discovered a 1,449 bp insertion in exon 11 of the gene compared to the reference genome. Sequence analysis of the insertion revealed the presence of 100% identical LTRs and TSDs, but no open reading frames or any similarity to known proteins or genes (Figure 3), leading to the conclusion that the insertion is probably a Class I, Order LTR (retro)transposable element (TE), following the TE classification scheme proposed by Wicker et al. [31]. The fact that the LTRs are completely identical to one another is an indication that the TE is relatively recently inserted. The integration of a transposable element in a *MLO* gene, leading to aberrant splicing of transcripts and in that way to loss of gene function, is reminiscent of the findings in the pea *PsMLO1* gene, where in one of the alleles (found in PM resistant pea cultivar JI 2302) the integration of an *Ogre* LTR retrotransposon lead to aberrant splicing [22].

We analysed putative TEs with similar LTRs (Figure 4), and found no functional ORFs in these TEs, confirming that we are dealing with a family of non-autonomous TEs. Additionally, a large amount of LTR singlets (i.e. LTR sequences without a partner) were detected, as only 88 out of the 169 detected LTRs could be assigned to a putative TE

(Additional file 5). LTR singlets presumably originate from the unequal recombination between two LTRs of a single element [39], or from assembling errors of the reference genome. It is known that plant genomes are to a great extent shaped by the integration of large amounts of transposable elements, with LTR retrotransposons being the most abundant among them (e.g. [40, 41]). The cucumber genome was shown to be no exception to this, with 24% of the genome consisting of transposable elements and LTR retrotransposons comprising 10.4% of the genome [7]. To our knowledge, the TE we found to be inserted in *CsaMLO8* is the first TE with a reported effect on a cucumber gene. It seems likely that more TEs with an effect on genes in cucumber will be found in the future.

CsaMLO8 is upregulated upon *P. xanthii* inoculation in hypocotyl tissue only

Resistance to PM in cucumber has previously been reported to be tissue specific, with an important, recessively inherited gene providing full PM resistance in hypocotyl tissue and partial resistance in leafs [5]. Recently, PM resistance of cucumber was mapped in multiple tissues separately. The strongest QTL for hypocotyl resistance, *pm5.2* was mapped on chromosome 5, in a region containing *CsaMLO8* [29]. In this study, we showed that *CsaMLO8* was, in susceptible cucumber, transcriptionally upregulated in hypocotyl tissue at 4 and 6 hours post inoculation, but not in cotyledon or leaf samples (Figure 5A). Apparently, the ability of the pathogen to upregulate *CsaMLO8* expression is specific for hypocotyl tissue. Therefore, we postulate that it is very well possible that PM resistance caused by a loss of function allele of *CsaMLO8* would also be specific for hypocotyl tissue.

Interestingly, *CsaMLO8* was not found to be transcriptionally upregulated in hypocotyl tissue (or any other tissue) in the resistant cucumber line (Figure 5B). This is in sharp contrast with the findings in barley [35] where transcription of the *MLO* gene seemed to be even stronger induced upon PM inoculation in *mlo* loss-of-function mutants compared to wild type plants. In tomato it was found that transcription of the *SlMLO1* gene was slightly upregulated upon PM inoculation in *slmlo1* loss of function mutants, but to a far lesser extent than in wild type plants [21]. Although it remains a question why the pathogen is unable to upregulate *CsaMLO8* expression in our resistant cucumber line several explanations might be offered, e.g. lesser transcript stability of the mutant *CsaMLO8* transcripts, differences in the promotor region of the mutant allele of *CsaMLO8* or differences in other genes required for *CsaMLO8* expression compared to the susceptible cultivar.

Previously, RNA-seq experiments on cucumber leaf tissue revealed that of the thirteen *CsaMLO* genes only *CsaMLO1*, another clade V *MLO* gene, was transcriptionally up-

regulated after inoculation with *P. xanthii* [28]. This is consistent with our finding that *CsaMLO8* is not upregulated in leaf samples after PM inoculation (Figure 5). It is possible that *CsaMLO1* and *CsaMLO8* are functionally redundant, but are specifically expressed in separate tissues (i.e. *CsaMLO1* specific in leaf tissue and *CsaMLO8* in hypocotyl tissue). To our knowledge there are no other examples of tissue specialization in *MLO*-like *S* genes of other species. In *Arabidopsis*, which also has three clade V *MLO* genes, *Atmlo2* mutants were found to be partially resistant, double mutants *Atmlo2*/*Atmlo6* or *Atmlo2*/*Atmlo12* were more resistant than *Atmlo2* single mutants, and triple mutants *Atmlo2*/*Atmlo6*/*Atmlo12* were completely resistant [20]. It is not yet known by what mechanism *MLO* genes are transcriptionally upregulated upon PM infection, although it would seem intuitive to hypothesise that it is an active process caused by an effector of the fungus. Given the tissue specificity of *MLO* upregulation in cucumber, this might be an interesting model to investigate the mechanism of *MLO* upregulation by PM fungi.

The transposon insertion allele of *CsaMLO8* occurs frequently in cucumber germplasm

Interestingly, during the preparation of this manuscript, another group reported the fine-mapping of a QTL for PM resistance on the long arm of chromosome 5, which they called *pm5.1*, to a region of 170 kb containing 25 predicted genes. The main candidate gene in this region was found to be a *MLO* like gene, which appears to be the same as *CsaMLO8* in our study. By cloning and sequencing of this gene from genomic DNA of their resistant parent, line S1003, as well as two additional unrelated resistant lines, S02 and S06, they found that they contained a 1449 bp insert in the 11th exon of the gene [42]. Sequence analysis indicates that the location and sequence of the insertion found in their study are completely identical to the LTR retrotransposon described in this study. These researchers did not report on cloning the coding sequence of *CsaMLO8* in their material, nor on complementation experiments.

Additionally, a patent was filed describing an allele of *CsKIP2*, a gene claimed to provide PM resistance, shown to harbour a 72 bp deletion in the coding sequence [43]. Although it is not shown in the patent, the occurrence of this allele is claimed to be caused by the integration of a transposon-like element in the 11th exon of the gene. Sequence analysis revealed that *CsKIP2* is in fact the same gene as *CsaMLO8*, and the 72 bp deletion allele they describe is the same as the 72 bp deletion we found in our material. Interestingly the patent does not describe the 174 bp deletion which we found, but an *in silico* prediction showed that the 174 bp deletion variant would not be amplified by the primers they chose to amplify the partial *CsaMLO8* sequence. In the patent no functional proof is given that this allele of *CsaMLO8* indeed leads to resistance.

As several groups independently found the same allele of *CsaMLO8* in different, to our knowledge unrelated, resistant cucumber genotypes, we were interested to know how often this allele occurs in the global cucumber germplasm. Therefore, we performed an *in silico* screen on a collection of 115 recently resequenced cucumber accessions [8] for the presence and/or absence of the transposable element (TE) allele of *CsaMLO8*. We found evidence for the presence of the TE-allele, either homozygously or heterozygously, in at least 31 out of the 115 accessions (Table 1), indicating that this particular allele of *CsaMLO8* occurs quite often. For some accessions only a small number of reads indicating presence/absence of the TE allele was found, potentially due to a low read coverage at this locus. It is therefore possible that in some accessions now identified as homozygous for either the TE-allele or the WT allele of *CsaMLO8*, reads indicative of the other allele were missed due to low read coverage, so there might be some heterozygous accessions misidentified as being homozygous for one of the alleles.

As we found that the TE allele of *CsaMLO8* leads to PM resistance, it might have been selected for by cucumber breeders, by selecting for the most resistant plants. Interestingly one of the accessions found to have the TE-allele of *CsaMLO8* was PI 215589, a wild accession of *C. sativus var. hardwickii* collected in India in 1954. This indicates that the TE-allele of *CsaMLO8* does occur in the wild, and might have been introgressed in cultivated cucumber from PI 215589 or a related *hardwickii* accession.

CONCLUSIONS

In this study we provide evidence for a role of *CsaMLO8* as a *S* gene for powdery mildew (PM) susceptibility. We show that complementation by *CsaMLO8* overexpression in *Slmlo1* mutant tomato background restores PM susceptibility. We also show that a mutant allele of *CsaMLO8* cloned from resistant cucumber fails to restore PM susceptibility. As *CsaMLO8* is located in the region where a QTL for hypocotyl specific resistance was detected, we determined *CsaMLO8* expression in different tissues of PM inoculated plants, and found that *CsaMLO8* was only transcriptionally upregulated in hypocotyl tissue. On this basis we conclude that the mutant allele of *CsaMLO8* is causal to the observed hypocotyl resistance towards PM in cucumber.

METHODS

Plant materials and fungal strain

Two cucumber genotypes were used in this study: the PM susceptible cv. Sheila and an advanced breeding line, related to the resistant cv. Anaxo, homozygous for a recessively inherited QTL on chromosome 5 conferring hypocotyl resistance (*pm-h*).

Two tomato genotypes were used: PM susceptible cv. Moneymaker (MM), and a PM resistant breeding line *ol-2*, homozygous for a 19 bp deletion mutation in the coding sequence of *SlMLO1* [21].

Unless otherwise indicated, plants were grown under standard conditions in a closed greenhouse.

An isolate of *P. xanthii* (causing PM in cucumber) was obtained from infected cucumber plants in the greenhouse of a seeds company from The Netherlands and maintained on cv. Sheila in a greenhouse compartment at Wageningen University, The Netherlands. The species of the isolate was confirmed by sequencing of the ITS sequence from fungal DNA by primer pair 5'- CGTCAGAGAAGCCCCAACTC-3' (ITS *P. xanthii* Forward) and 5'-AGCCAAGAGATCCGTTGTTG-3' (ITS *P. xanthii* Reverse) (data not shown).

The Wageningen isolate of *Oidium neolycopersici* (tomato PM) was maintained on cv. MM as described [44].

Cloning and sequencing of *CsaMLO8* CDS

Young leaves of cucumber cv. Sheila and the resistant breeding line were harvested and immediately frozen in liquid nitrogen. Total RNA was isolated by using the RNeasy Kit (Qiagen, Germany). Possible DNA contamination of RNA samples was removed by treatment with DNase I, Amp Grade (Invitrogen life technologies, U.S.A.). cDNA was synthesised using 2 µg of RNA samples with an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, U.S.A.).

For amplification of *CsaMLO8* coding sequences, cDNA was amplified with primers 5'- caccCTGCCTCTCCACATGCATAA-3' (Full length *CsaMLO8* Forward) and 5'-GCGCCCTG-TACATGAAGAAC-3' (Full length *CsaMLO8* Reverse). As template 50 ng cDNA was used in 50 µl reactions using 1 u *PfuUltra* II Fusion HS DNA polymerase (Agilent Technologies, U.S.A.), 1x reaction buffer, 1mM dNTP and 200 nM of each primer. Cycling conditions were: 1 min. initial denaturation at 95°C, followed by 40 cycles of 20 sec. denaturation at 95°C, 20 sec. annealing at 60°C and 2 min. extension at 72°C. Reactions were finished

by 3 min. incubation at 72°C. PCR products were separated by gel electrophoresis in ethidium bromide stained agarose gels. Bands were cut out and purified using QIAquick Gel Extraction Kit (Qiagen, Germany). Purified products were cloned into Gateway-compatible vector pENTR D-TOPO (Invitrogen life technologies, U.S.A.) and transformed to chemically competent *Escherichia coli* strain One Shot TOP10. Presence of the right fragment was assessed by colony PCR using primers and conditions as above. Plasmids were recovered using the Qiaprep spin miniprep kit (Qiagen, Germany). Sequencing reactions were performed in triplicates using pUC/M13 forward and reverse sequencing primers (GATC Biotech, Germany).

Complementation of tomato *ol-2* mutant with *CsaMLO8* WT and *CsaMLO8Δ174*

Entry plasmids pENTR:*CsaMLO8* WT and pENTR:*CsaMLO8Δ174*, obtained as described above, were transferred by Gateway LR cloning into binary vector pK7WG2, which harbours the constitutively active 35S Cauliflower Mosaic Virus promotor and the *nptII* marker gene for kanamycin resistance [45]. Recombinant plasmids were transformed to chemically competent *E. coli* strain dh5α. Positive recombinant bacterial colonies were screened by colony PCR using *CsaMLO8* specific primers as described above, and sequenced. Recombinant plasmids were recovered using the Qiaprep spin miniprep kit (Qiagen, Germany). pK7WG2:*CsaMLO8* WT and pK7WG2:*CsaMLO8Δ174* binary vectors were transformed to electrocompetent cells of *Agrobacterium tumefaciens* strain AGL1-virG by electroporation [46].

Cotyledon explants of *ol-2* mutant tomato seedlings were transformed as previously described [25]. Obtained tomato transformants were assessed for presence of *CsaMLO8*, the *nptII* marker gene and the 35S CaMV promotor sequence by PCR with primers 5'-caccCTGCCTCTCCACATGCATAA-3' (Full length *CsaMLO8* forward) and 5'-GCGCCCTGTA-CATGAAGAAC-3' (Full length *CsaMLO8* reverse), 5'-GAAGGGACTGGCTGCTATTG-3' (*nptII f*orward) and 5'-AATATCACGGGTAGCCAACG-3' (*nptII r*everse), and 5'-TACAAAGGCG-GCAACAAACG-3' (35S forward) and 5'-AGCAAGCCTTGAATCGTCCA-3' (35S reverse), with conditions as described above.

For each of the two transformations with a different construct, ten independent transgenic plants were selected, and were assessed for *CsaMLO8* expression by qRT-PCR using primer pair sequences specific for *CsaMLO8* 5'-GCGACGGCATTGAAGAACTG-3' (Forward) and 5'-AGGAGACATGCCGTGAGTTG-3' (Reverse). As housekeeping gene for normalization of *CsaMLO8* expression in tomato, *SlEF-*α was used, with primer pair 5'-ATTGGAAACGGATATGCCCCT-3' (*SlEF-*α forward) and 5'-TCCTTACCTGAACGCCTGTCA-3' (*SlEF-*α reverse). qRT-PCR was performed using the CFX96 Real-Time PCR machine (Bio-

Rad Laboratories, U.S.A.). Each 10 µl reaction contained 300 nM of each primer, 1 µl (50ng) cDNA template and 1 x iQ SYBR Green Supermix (Bio-Rad Laboratories, U.S.A.). Cycling conditions were an initial denaturation step of 95°C for 3 min., followed by 40 cycles of 10 sec. denaturation at 95°C and 30 sec. annealing and extension at 60°C, finished by a melt cycle of 0.5°C increment per 10 sec. from 65°C to 95°C.

Evaluation of PM resistance of *ol-2* tomato, overexpressing *CsaMLO8* WT or *CsaMLO8Δ174*

Cuttings originating from ten individual transgenic plants per construct (two cuttings per plant) were inoculated with *O. neolycopersici.* Cuttings of an empty vector (EV) transformed *ol-2* plant and the susceptible cultivar Moneymaker (MM) were used as controls. A spore suspension was prepared by washing heavily infected leaves of cv. MM with water, and adjusting the spore concentration to 8×10^4 conidiospores/ml . The spore suspension was evenly sprayed on the cuttings. Sixteen days after inoculation the disease severity was assessed by eye, and scored as either susceptible (sporulating powdery mildew colonies visible on leaves) or resistant (no powdery mildew symptoms at all). Additionally, leaf samples were taken for quantification of *O. neolycopersici* biomass. Infected leaves (the 2nd or 3rd leaf) were sampled for each cutting. Total plant and fungal DNA was extracted using the DNeasy Plant Kit (Qiagen, Germany). Isolated DNA was used for qPCR with primer pair 5'-CGCCAAAGACCTAACCAAAA-3' (*Oidium* ITS forward) and 5'-AGCCAAGAGATCCGTTGTTG-3' (*Oidium* ITS reverse), specific for the internal transcribed spacer (ITS) of *O. neolycopersici* ribosomal DNA, to quantify *O. neolycopersici* biomass, and with *SlEF-* primers as described above for normalization. qPCR was performed using the CFX96 Real-Time PCR machine (Bio-Rad Laboratories, U.S.A.). Each 10 µl reaction contained 300 nM of each primer, 2 µl (20ng) cDNA template and 1 x iQ SYBR Green Supermix (Bio-Rad Laboratories, U.S.A.). Cycling conditions were identical to those described above for quantification of *CsaMLO8* expression in transformed tomato.

Amplification, sequencing and characterization of *CsaMLO8*-insertion

DNA was isolated from young leaves of cucumber cv. Sheila and the resistant breeding line, which were immediately frozen in liquid nitrogen after harvesting, using the DNeasy Plant Kit (Qiagen, Germany). DNA was amplified with primers 5'-AGCATTTTGCCATCCATACTTCA-3' (*CsaMLO8* insertion region Forward) and 5'-CTGCAAGCACAGGATGAATGTC-3' (*CsaMLO8* insertion region Reverse). As template 30 ng DNA was used in 25 µl reactions using 1.25 u DreamTaq DNA polymerase (Thermo Scientific, U.S.A.), 1x DreamTaq buffer, 0.8 mM dNTP and 200 nM of each primer. Cycling conditions were: 3 min. initial denaturation at 95°C, followed by 35 cycles of 30 sec. denaturation at 95°C, 30 sec. annealing at 57°C and 2 min. extension at 72°C. Reactions were finished

by 5 min. incubation at 72°C. PCR products were visualised by staining with GelRed and electrophoresis on agarose gels. PCR products were purified using Qiaquick PCR purification kit (Qiagen, Germany). Sequencing reactions were performed in duplo, using primers 5'-AGCATTTTGCCATCCATACTTCA-3' (*CsaMLO8* insertion region Forward), 5'-ACGAAGAGCGAAACGAAGAA-3' (*CsaMLO8* insertion sequencing Forward), 5'- GCTCCT-GCCCAATTCAGACC-3' (*CsaMLO8* insertion sequencing Reverse) and 5'-CTGCAAGCACAG-GATGAATGTC-3' (*CsaMLO8* insertion region Reverse) (GATC Biotech, Germany). Obtained sequences were aligned using CLC Genomics Workbench 7.5 software. The consensus sequence for the amplified region was extracted from the alignment. This consensus sequence was aligned to the genomic reference sequence of *CsaMLO8* to determine the exact location and sequence of the insertion.

A dot plot was constructed for the sequence of the insertion, using CLC Genomics Workbench 7.5 standard settings. The first and last 200 bp of the insertion sequence were extracted and aligned to each other to identify the length and sequence of the LTRs. The sequence of the insertion was scanned for open reading frames using CLC Genomics Workbench 7.5 standard settings, which gave no results.

In silico mining of the cucumber reference genome for homologous TEs

The previously determined LTR sequence of the *CsaMLO8*-TE was used as query to perform a BLASTn search in the genome of the cucumber reference genome (Chinese long inbred line '9930', v2 [7]) to identify putative homologous LTRs. The resulting output was stored as a tabular file. A python script described by Wolters et al. (2014) was used to search for LTR matches within 20 kb from each other [32]. Sequences with a length smaller than 20 kb flanked by two LTRs were considered as putative homologous TEs, and were extracted from the genome using the BEDtools suite [47]. The list of putative TEs was manually curated to remove sequences with two LTRs in opposite directions (two instances) and sequences with large (>100 bp) gaps (25 instances). In three instances, putative TEs were found to be nested (i.e. three LTRs were found to be within 20 kb of each other), in which cases the smaller putative TEs were discarded in favour of the bigger, nested model. Putative TEs were aligned to one another and to the *CsaMLO8*-TE using CLC Genomics Workbench 7.5 software, to determine sequence identity compared to the *CsaMLO8*-TE. Putative TEs were screened for open reading frames using CLC Genomics Workbench 7.5 standard settings. Putative TEs were used as query to perform tBLASTx searches to the REPbase database [48].

In silico screening of resequenced lines for presence of *CsaMLO8*-TE allele

Reads of the resequencing project of 115 cucumber accessions by Qi et al. [8] were downloaded from the NCBI short read archive, accession SRA056480. By a simple Bash script, total reads were screened for the presence of 30 bp sequences comprised of:

1.) The last 15 bp of *CsaMLO8* before the TE insertion and the first 15 bp of the TE insertion, in forward (5'-GCTCCATGTTATTATTGTTGATTTTATGGA-3') or reverse (5'-TCCATAAAATCAACAATAATAACATGGAGC-3') orientation;
2.) The last 15 bp of the TE insertion and the first 15 bp of *CsaMLO8* after the TE insertion, in forward (5'-TATATTAATAATTATAACTCATATGGGATT-3') or reverse (5'-AATCCCATATGAGTTATAATTATTAATATA-3') orientation;
3.) The 30 bp of *CsaMLO8* surrounding the TE insertion site, without TE sequence, in forward (5'-GCTCCATGTTATTATAACTCATATGGGATT-3') or reverse (5'-AATCCCATATGAGTTATAATAACATGGAGC-3') orientation.

The number of detected reads per accession with each of the six bait sequences was stored as a tabular file. The total number of reads indicating presence of the TE allele and the total number of reads indicating presence of the WT allele were summated, the genotype of the accessions was determined to be either homozygous TE-allele, homozygous WT-allele or heterozygous.

CsaMLO8 expression analysis PM-inoculated cucumber

PM susceptible and resistant cucumbers were grown in a climate chamber at 20°C (day) and 16°C(night), with a 16h/8h day/night cycle, and a relative humidity of 90%. 18 days post seeding, plants were inoculated with a *P. xanthii* spore suspension by spray method, using inoculum that was obtained by washing heavily infected cucumber leaves with water. The inoculum was adjusted to a final concentration of 1.0×10^4 conidia/ml. The spore suspension was evenly sprayed on leaves, cotyledons and hypocotyl of the seedlings. Prior to inoculation and at 4, 6, 8 and 24 hours post inoculation (hpi), from three individual plants per time point hypocotyl, cotyledon and (first) true leaf samples were harvested separately, and were immediately frozen in liquid nitrogen.

Total RNA was isolated using the MagMAX-96 Total RNA Isolation kit (Ambion, U.S.A.). cDNA was synthesised using 1 µg of RNA samples with an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, U.S.A.). Before use in qRT-PCR, cDNA samples were diluted 10-fold.

Quantitative real-time PCR was performed using a CFX96 Real-Time PCR machine (Bio-Rad Laboratories, U.S.A.). Primer pair sequences specific for *CsaMLO8* 5'-GCGACGGCATTGAAGAACTG-3' (Forward) and 5'-AGGAGACATGCCGTGAGTTG-3' (Reverse) were

used to quantify *CsaMLO8* expression. Primer pairs specific for cucumber housekeeping genes *TIP41*, *CACS* and *EF-α*, as described by Warzybok et al. [49], were used for normalization of *CsaMLO8* expression. Each 10 µl reaction contained 300 nM of each primer, 1 µl (50ng) cDNA template and 1 x iQ SYBR Green Supermix (Bio-Rad Laboratories, U.S.A.). Cycling conditions were an initial denaturation step of 95°C for 3 min. followed by 40 cycles of 10 sec. denaturation at 95°C and 30 sec. annealing and extension at 60°C, finishing with a melt cycle of 0.5°C increment per 10 sec. from 65°C to 95°C.

Two technical replicates for each sample were tested. *CsaMLO8* expression of each sample was determined by the $\Delta\Delta C_t$ method [50], normalised by the geometric mean of the three housekeeping genes. Averages and standard errors of *CsaMLO8* transcript abundance were calculated over three biological replicates per tissue/time point combination, and statistical significance of differences in $\Delta\Delta C_t$ value between time points 4, 6, 8 and 24 hpi and 0 hpi were determined, using Student's T-tests.

Relative quantification of *CsaMLO8* transcript isoforms in resistant cucumber

cDNA samples of non-inoculated and inoculated (6 hpi) resistant cucumber tissues, obtained as described above, were used to quantify relative transcript abundance of the Δ174 and Δ72 splice isoforms. Quantitative real-time PCR was performed using a CFX96 Real-Time PCR machine (Bio-Rad Laboratories, U.S.A.). Four primer pairs were designed to specifically amplify one of the two *CsaMLO8* splice isoforms: 5'-CTCCTTAATTAATGCATTTCAGC-3' (Forward) with 5'-CTTGTATGATAACCCCCATTGAG-3' (Reverse) or 5'-TTCATTGTTGCACATCTTGC-3' (Forward)with 5'-AAGCTGAAATGCATTAATTAAGG-3'(Reverse) for specific quantification of *CsaMLO8*Δ174 and 5'-ATTCTATTGGGTGTTCCCGTC-3' (Forward) with 5'-CTTGTATGATAACCCCCATTGAG-3' (Reverse) or 5'-TTCATTGTTGCACATCTTGC-3' (Forward)with 5'-GAACGACGGGAACACCCAAT-3'(Reverse) for specific quantification of *CsaMLO8*Δ72. Primer pairs specific for cucumber housekeeping genes *TIP41*, *CACS* and *EF-α*, as described by Warzybok et al. [49], were used for normalization of *CsaMLO8* expression. Each 10 µl reaction contained 300 nM of each primer, 1 µl (50ng) cDNA template and 1 x iQ SYBR Green Supermix (Bio-Rad Laboratories, U.S.A.). Cycling conditions were an initial denaturation step of 95°C for 3 min. followed by 40 cycles of 10 sec. denaturation at 95°C and 30 sec. annealing and extension at 60°C, finishing with a melt cycle of 0.5°C increment per 10 sec. from 65°C to 95°C.

Two technical replicates for each sample were tested. *CsaMLO8* expression of each sample was determined by the $\Delta\Delta C_t$ method [50], normalised by the geometric mean of the three housekeeping genes. Averages and standard errors of *CsaMLO8* splice isoform abundance were calculated over three biological replicates per tissue, per tissue the

average of the relative abundances calculated with the two different primer pairs per splice isoform was calculated.

AUTHORS' CONTRIBUTIONS

JAB, MA, YB and HJS designed the experiments. JAB, MA and MSM performed the experiments. FWKH and WHV provided resources. JAB drafted the manuscript. MA, WHV, RGFV, YB and HJS critically revised the manuscript. All authors read and approved the manuscript.

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ADDITIONAL DATA

All figures and additional datafiles described in this chapter were deposited online, and can be accesed through doi:10.4121/uuid:9b31358d-b8da-449a-8ae8-0fea922bc186

Additional file 1

Format: PDF

Full length alignment of *CsaMLO8* WT, *CsaMLO8*Δ72 and *CsaMLO8*Δ174 coding sequences.

Additional file 2

Format: PDF

Multiple sequence alignment of MLO proteins encoded by clade V *MLO* S-genes from different species.

Additional file 3

Format: PDF

Relative quantification of *CsaMLO8*Δ174 and *CsaMLO8*Δ72 transcript abundances by qRT-PCR on cDNA samples obtained from non-inoculated **(A)** or inoculated **(B)** cucumber tissue samples. Fold changes were normalised relative to *CsaMLO8*Δ174 expression. Bars represent the average fold change over three independent biological replicates. Error bars indicate standard errors of the mean.

Additional file 4

Format: PDF

Photographs of 20 independent ol-2 tomato plants transformed with either *CsaMLO8* WT or *CsaMLO8*Δ174.

Additional file 5

Format: XLSX

Complete overview of putative LTRs and putative TEs homologous to the TE identified in *CsaMLO8*.

Additional file 6

Format: PDF

Multiple sequence alignment of the TE identified in *CsaMLO8* and putative homologous TEs.

Additional file 7

Format: XLSX

Full table of 115 resequenced accessions. The amount of reads identified is given at the overlap between *CsaMLO8* and the start of the insertion in forward (TE start-F) and reverse (TE start-R) direction, at the overlap between the end of the insertion and *CsaMLO8* in forward (TE end-F) and reverse (TE end-R) direction, and at the site of the insertion with only *CsaMLO8* sequence in forward (WT-F) and reverse (WT-R) direction.

Chapter 3

Functional Characterization of Cucumber (*Cucumis sativus* L.) Clade V *MLO* Genes

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ABSTRACT

Background: Powdery mildew (PM) causing fungi are well-known pathogens, infecting over 10.000 plant species, including the economically important crop cucumber (*Cucumis sativus* L.). Loss-of-function mutations in clade V *MLO* genes have previously been shown to lead to recessively inherited broad-spectrum resistance to PM in several species. In cucumber, one clade V *MLO* homolog (*CsaMLO8*) was previously identified as being a susceptibility factor to PM. Two other closely related homologs (*CsaMLO1* and *CsaMLO11*) were found, but their function was not yet unravelled.

Methods: *CsaMLO1* and *CsaMLO11* were cloned from cucumber and overexpressed in a tomato *mlo* mutant. The transcript abundances of all three *CsaMLO* genes in different cucumber tissues were quantified using qRT-PCR and RNA-seq, with and without inoculation with the cucumber PM fungus *Podosphaera xanthii*. Allelic variation of *CsaMLO1* and *CsaMLO11* was screened *in silico* in sequenced cucumber germplasm.

Results: Heterologous overexpression of all three *CsaMLO* genes in the tomato *mlo* mutant restored susceptibility to PM caused by *Oidium neolycopersici*, albeit to a different extent: whereas overexpression of *CsaMLO1* or *CsaMLO8* completely restored susceptibility, overexpression of *CsaMLO11* was only partially able to restore PM susceptibility. Furthermore, it was observed by qRT-PCR and RNA-seq that *CsaMLO8* was significantly higher expressed in non-inoculated cucumber compared to the other two *MLO* genes. However, inoculation with *P. xanthii* led to upregulation of *CsaMLO1*, but not to upregulation of *CsaMLO8* or *CsaMLO11*.

Conclusions: Both *CsaMLO1* and *CsaMLO11* are functional susceptibility genes, although we conclude that based on the transcript abundance *CsaMLO8* is probably the major clade V *MLO* gene in cucumber regarding providing susceptibility to PM. Potential loss-of-function mutations in *CsaMLO1* and *CsaMLO11* have not been identified. The generation and analysis of such mutants are interesting subjects for further investigation.

Keywords: Cucumber (*Cucumis sativus* L.), Powdery Mildew, *MLO*, Susceptibility genes, Gene expression

BACKGROUND

Powdery mildew (PM), caused by ascomycete fungi of the order *Erysiphales*, is one of the most well-known plant diseases [1]. PM fungi are able to cause disease on leaves, stems, flowers and fruits of nearly 10.000 different angiosperm plant species, including various economically important plants, such as cucumber (*Cucumis sativus* L.). PM in cucumber can be caused by two different species, *Golovinomyces orontii* and *Podosphaera xanthii*. In greenhouses and open field cultivation in warm regions, *P. xanthii* appears to be the most occurring agent of PM in cucurbits, whereas *G. orontii* is the major PM species on cucurbits grown in the open field in colder regions [2].

According to the gene-for-gene concept, plants have dominantly inherited resistance genes (*R*-genes), encoding R proteins which recognize the products of corresponding avirulence genes (*Avr*-genes) of pathogens, triggering defence responses [3, 4]. Even though this can give a strong, complete resistance, the pathogen can mutate, lose or silence the recognized *Avr* gene to break the resistance, leading to a new virulent race of the pathogen, often within a few years after the commercial introduction of a new *R* gene. The cloning of *R* genes in several plant species has led to the finding that they typically encode receptor proteins of various classes with leucine-rich repeat (LRR) domains [3]. An exception to *R*-gene mediated resistance was discovered in X-ray irradiated summer barley populations in the 1940's [5]. A recessively inherited monogenic resistance was observed, which was active against all known isolates of barley PM (caused by *Blumeria graminis* f. sp. *hordei*). Later, other alleles at the same genetic locus were obtained in various barley genotypes, including a naturally occurring mutant allele that was found in resistant Ethiopian barley landraces [6]. The durability of this so-called *mlo* (*Mildew Locus O*) resistance is exemplified by the fact that cultivars with this type of resistance have been extensively cultivated since the 1980s without new races of the pathogen breaking the resistance [7]. By positional cloning, the causal gene for *mlo* based resistance was isolated [8]. It was found to encode a plasma membrane-anchored protein with seven transmembrane helices, reminiscent of animal G-protein-coupled receptors [9].

After the barley *MLO* gene was cloned, it was found that mutations in homologs of this gene in other plant species can also lead to recessively inherited resistance to different PM causing fungi. In the model species *Arabidopsis thaliana* T-DNA insertion mutations in three *MLO* homologs contribute to PM resistance, although a mutation in one of the three genes (*AtMLO2*) has a larger effect compared to mutations in the other two genes (*AtMLO6* and *AtMLO12*). The effect of loss of function of *AtMLO6* or *AtMLO12* is only additive in *Atmlo2* background, but not detectable when *AtMLO2* is intact [10]. In several

crop species, i.e. tomato [11], pea [12], cucumber [13] and tobacco [14], recessively inherited PM resistance with similar characteristics compared to barley *mlo* resistance was indeed found to be caused by naturally occurring mutations in *MLO* homologs. In other species, e.g. pepper, wheat, apple and grapevine, knockdown of *MLO* homologs by virus induced gene silencing (VIGS) or RNA interference (RNAi) led to PM resistance [15–18]. This indicates that *mlo*-based resistance is very common in plants, rather than a particular oddity occurring in barley. It has been shown that *mlo*-based resistance depends on the formation of cell wall depositions (papillae) by the plant cell directly beneath PM penetration attempts [19]. The molecular basis of *mlo*-based resistance is however yet poorly understood, although it has been shown that it depends on the function of several molecular components, such as the BAX-inhibitor protein (BI-1) which plays a role in control of programmed cell death [20]; on an intact actin cytoskeleton [21] and on the t-SNARE proteins PEN1 (*Arabidopsis*) and ROR2 (barley) involved in targeted exocytosis [22].

Since the year 2000, when the first plant genome sequence was published, i.e. that of the model plant *Arabidopsis thaliana* [23], an increasing number of plant genomes has been sequenced. In all available sequenced plant genomes *MLO* homologs occur as medium sized gene families with seven to thirty-nine *MLO* genes per plant species [24]. In phylogenetic analyses of the *MLO* gene family, it has been found that *MLO* genes can be divided into seven phylogenetic clades, although not all plant species harbour representatives of all clades [25]. The mosses, representing the most basal lineages of land plants, have *MLO* homologs only one of the clades, i.e. clade I. In other lineages of plants, especially in angiosperm species, the *MLO* gene family has diversified. However, several plant species apparently lost genes in some of the *MLO* clades during evolution, such as the monocotyledonous family of the Poaceae which has no *MLO* homologs in clades V and VI, or several dicotyledonous species such as *Arabidopsis thaliana* and tomato which have lost clade IV *MLO* homologs, even though a basal angiosperm species, *Amborella trichopoda,* has homologs of clade I to VI [25].

Not all the *MLO* genes found so far have been characterised as being required for susceptibility towards PM fungi. For most *MLO* genes mutant phenotypes have not been described yet, although there are examples of clade I *mlo* mutants with a defect in root formation [26] and of clade III *mlo* mutants with defects in pollen tube perception [27] and pollen hydration [28]. So far, all *MLO* genes involved in PM susceptibility (i.e. susceptibility genes, S-genes) have been found to group either in clade IV (for monocotyledonous species) or clade V (for dicotyledonous species). It has been shown that heterologous overexpression of the barley clade IV *MLO* gene can functionally complement loss of function mutations in clade V *MLO* genes in tomato [29], exemplifying that

although there are significant differences in amino acid sequence between clade IV and V MLO proteins, they are functionally conserved.

In cucumber, the genome sequence of which was published in 2009 [30], thirteen *MLO* homologs have previously been identified. Of these thirteen homologs, three genes were found to group phylogenetically in clade V. These three genes, named *CsaMLO1*, *CsaMLO8* and *CsaMLO11*, should therefore be considered as potential PM S-genes in cucumber [31]. In other cucurbit crops, i.e. melon (*Cucumis melo*), watermelon (*Citrullus lanatus*) and pumpkin (*Cucurbita pepo*), similar numbers of clade V *MLO* genes have been identified, although pumpkin has four rather than three clade V *MLO* genes [32]. Phylogenetic analysis reveals that the last common ancestor of these cucurbit crops already had at least three clade V *MLO* genes.

Of the three cucumber clade V *MLO* genes, *CsaMLO8* has previously been proven to be a susceptibility gene for PM caused by *P. xanthii*. From cucumber genotypes with recessively inherited PM resistance a natural *Csamlo8* mutant allele was cloned [13, 33]. While heterologous overexpression of the wild-type *CsaMLO8* was able to functionally complement *mlo* loss-of-function mutants in both tomato [13] and *Arabidopsis* [33], the mutant allele failed to restore susceptibility. The mutation in *Csamlo8* has been caused by the integration of a retrotransposable element in the coding sequence of the gene [13, 34]. This mutant allele was found to occur frequently in cultivated cucumber germplasm [13], and additionally two other *Csamlo8* loss-of-function mutations have been found in resistant genotypes due to either a frameshift indel leading to an early stop codon, or a SNP in an intron-exon junction causing aberrant splicing of the pre-mRNA [33].

In a review of co-localization of cucumber *MLO* genes with QTLs for PM resistance, Schouten et al. (2014) mentioned that two previously described QTLs for PM resistance co-localized with the other two cucumber clade V *MLO* genes, *CsaMLO1* and *CsaMLO11* [31]. Fukino et al. (2013) performed QTL analysis in a RIL population derived from a cross between the PM resistant genotype CS-PMR1 (an inbred line derived from the PM resistant wild cucumber accession PI 197088) and the moderately susceptible genotype Santou, a native Japanese cultivar [35]. Of the nine detected QTLs for PM resistance, one QTL (*pm1.1*) co-localized with *CsaMLO1*, whereas another QTL (*pm6.1*) co-localized with *CsaMLO11*. The resistance associated with *pm1.1* was contributed by the allele from CS-PMR1, while the Santou allele contributed to resistance at the *pm6.1* locus.

Here we report the functional characterization of two cucumber clade V *MLO* genes, *CsaMLO1* and *CsaMLO11*. We show that heterologous overexpression of either of the

genes in a tomato *mlo* mutant led to restoration of susceptibility to PM. Furthermore, we investigated the transcription profile of the three cucumber clade V *MLO* genes in various tissues, both prior to and after inoculation with the PM causing fungus *P. xanthii*. Also, we screened a set of 115 resequenced cucumber accessions *in silico* for potential loss of function mutations in either of the clade V *MLO* genes, and resequenced two additional cucumber genotypes with reported PM resistance QTLs in regions containing either *CsaMLO1* or *CsaMLO11*.

RESULTS

Functional analysis of cucumber clade V *MLO* genes by complementation of a tomato *mlo* mutant

We amplified the clade V *MLO* genes *CsaMLO1* [Csa1M085890] and *CsaMLO11* [Csa6M292430] from cDNA of a cucumber inbred line. PCR products were of the expected sizes (1.749 bp and 1.782 bp, respectively) and sequences were identical to the reference genome of the PM susceptible genotype 'Chinese Long 9930' [30]. To test whether these genes are susceptibility genes, *CsaMLO1* and *CsaMLO11* were overexpressed in a tomato *mlo* mutant, which is resistant to PM due to a mutation in the *SlMLO1* gene [11]. It was expected that if *CsaMLO1* and/or *CsaMLO11* are susceptibility genes, overexpression in the tomato *mlo* mutant would lead to restoration of susceptibility to PM. After transformation, cuttings from eight (*CsaMLO1*) or seven (*CsaMLO11*) individual transformants were obtained and inoculated with *Oidium neolycopersici*, the causal agent of PM in tomato. Sporulation was observed on five of the eight *CsaMLO1* transformants and on two of the seven *CsaMLO11* transformants (Table 1).

In order to confirm the ability of *CsaMLO1* and *CsaMLO11* to restore PM susceptibility, primary transformants were self-pollinated to obtain T2 families. T2 families were obtained from two individual transformants per gene. Additionally, two T2 families were obtained from previously described *CsaMLO8* [Csa5M623470] overexpressing transformants [13]. From each T2 family, 22 to 30 plants were sown. Plants were screened by PCR for the presence of the overexpression construct. It was found that the T2 populations obtained from the *CsaMLO1* and *CsaMLO11* transformants segregated for the presence of the transgene in ratios close to 3:1, suggesting one transgene insertion site per individual primary transformant. The T2 populations obtained from (both) the *CsaMLO8* transformants showed skewed segregation patterns with either one out of 22 (T2-A) or one out of 30 (T2-B) individuals not having the transgene, suggesting multiple inserts per individual transformant.

Table 1 – Cuttings of five out of eight *CsaMLO1* transformants and two out of seven *CsaMLO11* transformants were found to be susceptible to *Oidium neolycopersici*.

Gene	Transformant	Transgene expression	PM symptoms
CsaMLO1	35S::*CsaMLO1*-A	2.3	+
	35S::*CsaMLO1*-B	1.3	+
	35S::*CsaMLO1*-C	1.2	+
	35S::*CsaMLO1*-D	0.8	-
	35S::*CsaMLO1*-E	0.7	-
	35S::*CsaMLO1*-F	0.4	-
	35S::*CsaMLO1*-G	0.4	+
	35S::*CsaMLO1*-H	0.1	+
CsaMLO11	35S::*CsaMLO11*-A	0.3	-
	35S::*CsaMLO11*-B	0.3	-
	35S::*CsaMLO11*-C	0.2	+
	35S::*CsaMLO11*-D	0.2	-
	35S::*CsaMLO11*-E	0.1	-
	35S::*CsaMLO11*-F	0.1	-
	35S::*CsaMLO11*-G	0.1	+

The tomato Slmlo1 mutant, with a frameshift deletion in the SlMLO1 gene [11], was transformed with either a 35S::CsaMLO1 construct or a 35S::CsaMLO11 construct. CsaMLO1 or CsaMLO11 expression in each of the primary transformants (one sample per individual transformant) was quantified relatively to housekeeping gene SlEF-α using qRT-PCR. Two cuttings per individual transformant were inoculated with Oidium neolycopersici, the causal agent of powdery mildew (PM) in tomato. Disease phenotypes were scored based on whether or not PM symptoms were visible on the leaves at 10 days post inoculation.

T2 families were inoculated with *O. neolycopersici* and PM symptoms were scored based on a 0-3 scale, with 0 being completely free of PM symptoms and 3 being fully infected. The non-transformed *slmlo1* mutant did not show any PM symptoms (i.e. all plants scored a disease index of 0), whereas in the susceptible control (Moneymaker) 75% of the plants scored the maximum disease index of 3, and 25% of the plants scored a disease index of 2 (Figure 1). An overall analysis showed significant differences between the groups (Kruskal-Wallis test, $P < 0.05$). Stepdown post hoc analysis revealed that overexpression of *CsaMLO8* or *CsaMLO1* restored susceptibility completely, leading to a susceptibility level not significantly different from Moneymaker ($P > 0.05$). However, overexpression of *CsaMLO11* restored susceptibility only partially, giving disease indices between 0 and 1, which is significantly higher than the resistant control *slmlo1* ($P < 0.05$) but significantly lower than the susceptible control Moneymaker ($P < 0.05$).

Per family, five plants (positive for the presence of the overexpression construct) were randomly chosen to measure the expression of the transgene using qRT-PCR (Additional

file 1). It was found that the transgene expression in both *CsaMLO8* T2 families was significantly higher than in the *CsaMLO1* T2-A family and both of the *CsaMLO11* T2 families (ANOVA with Bonferroni post hoc test, P < 0.05), whereas there was no significant difference in transgene expression between *CsaMLO1* and *CsaMLO11* T2 families (ANOVA with Bonferroni post hoc test,P > 0.05). The *CsaMLO1* T2-B family did not have a significantly different transgene expression compared to either of the other T2 families (ANOVA with Bonferroni post hoc tests,P > 0.05). Transgene expression was not detectable in (untransformed) susceptible Moneymaker or resistant *mlo* control plants.

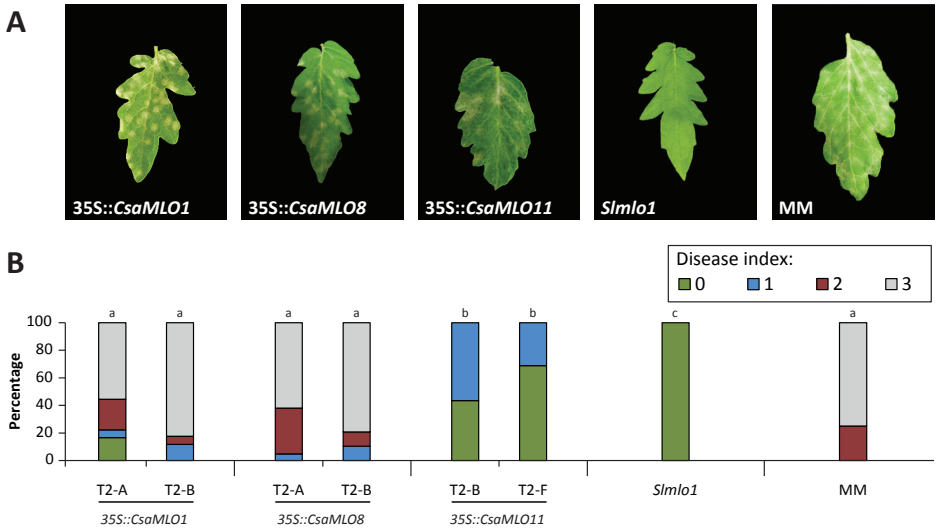

Figure 1 – Complementation of a tomato mlo mutant with either *CsaMLO1*, *CsaMLO8* or *CsaMLO11*
The tomato *mlo* mutant, with a frameshift deletion in the *SlMLO1* gene [11], was transformed with either a 35S::*CsaMLO1* construct, a 35S::*CsaMLO8* construct or a 35S::*CsaMLO11* construct (Table 1). Two individual transformants per transgene were self-pollinated to obtain T2 populations. T2 plants were screened for the presence of the overexpression construct. Plants carrying an overexpression construct were inoculated with *O. neolycopersici*.
A) Representative individuals from T2 families expressing either *CsaMLO1*, *CsaMLO8* or *CsaMLO11* in the tomato *mlo* mutant, showing powdery mildew (PM) symptoms. Non-transformed tomato *slmlo1* mutant (PM resistant) and cv. Moneymaker (MM, PM susceptible) are shown as controls.
B) Disease indices were scored visually on a scale from 0 (completely resistant) to 3 (completely susceptible), as described in [11]. T2 families consisted of 16 to 29 individuals positive for the presence of the overexpression construct. Resistant and susceptible controls consisted of 12 individuals. Bars represent percentages of plants within each disease index class. Different letters above bars indicate significant differences between populations (Kruskal-Wallis test with Stepwise-Stepdown Multiple Comparisons, P < 0.05).

Transcript abundance profiling of cucumber clade V *MLO* genes

The relative transcript abundance of the cucumber clade V *MLO* genes *CsaMLO1*, *CsaMLO8* and *CsaMLO11* was determined in three different tissues (hypocotyl, cotyledon and leaf) of the susceptible cucumber cultivar Sheila, using qRT-PCR.

It was found that in each of the three tissues the transcript abundance of *CsaMLO8* was several orders of magnitude higher than that of *CsaMLO1* and *CsaMLO11* (Figure 2A), a difference which was found to be statistically significant (ANOVA with Bonferroni post hoc tests, $p < 0.05$).

To confirm this, we re-examined a previously obtained dataset consisting of RNA-seq data obtained from a variety of cucumber tissues from the reference cultivar 'Chinese Long 9930'. This showed that in all examined aerial tissues (hypocotyl, stem, cotyledon, leaf and fruit tissue) *CsaMLO8* was higher expressed (on average ca. tenfold) compared to either *CsaMLO1* or *CsaMLO11*, although in stem tissue *CsaMLO1* also appeared to be rather highly expressed. For the aerial tissues for which data on more than one biological replicate was available (leaf and fruit tissue), the observed difference in expression was found to be statistically significantly (ANOVA with Bonferroni post hoc tests, $P < 0.05$). In root and root tip tissue however, both *CsaMLO1* and *CsaMLO11* were highly expressed, whereas *CsaMLO8* was lowly expressed (Figure 2B), although this difference was not found to be statistically significant (ANOVA, $P > 0.05$). For comparison we also examined the expression of the *A. thaliana* Clade V *MLO* genes (*AtMLO2*, *AtMLO6* and *AtMLO12*) in a publicly available RNA-seq dataset of *A. thaliana* tissues [36]. We found that *AtMLO2* expression was much higher than *AtMLO6* or *AtMLO12* expression in all four sampled tissues (root, leaf, flower and fruit), although the difference in roots was smaller than in the other tissues (Additional file 2).

Subsequently we investigated the expression profile in cucumber tissues inoculated with *P. xanthii*. Samples were taken of cucumber hypocotyl, cotyledon and leaf tissue prior to and at four, six, eight and twenty-four hours post inoculation. The relative expression of *CsaMLO1*, *CsaMLO8* and *CsaMLO11* in those samples was determined using qRT-PCR (Figure 3). We found that in leaf tissue there were significant differences in *CsaMLO1* transcript abundance between time points (ANOVA, $P < 0.05$). At four, six and eight hours post inoculation *CsaMLO1* transcript abundance was significantly higher compared to the transcript abundance prior to inoculation (Bonferroni post hoc tests, $P < 0.05$). This induction of *CsaMLO1* was not significant anymore at twenty-four hours after inoculation (Bonferroni post hoc test, $P > 0.05$). *CsaMLO11* was significantly downregulated at four hours post inoculation compared to the transcript abundance prior to inoculation (ANOVA with Bonferroni post hoc test, $P < 0.05$). In cotyledon or hypocotyl

tissue there were no significant differences of Clade V *MLO* gene transcript abundance between any of the time-points (ANOVA, P > 0.05).

As these results were in conflict with the finding in [13] that *CsaMLO8* transcription was upregulated upon PM inoculation, the experiment was repeated independently, with

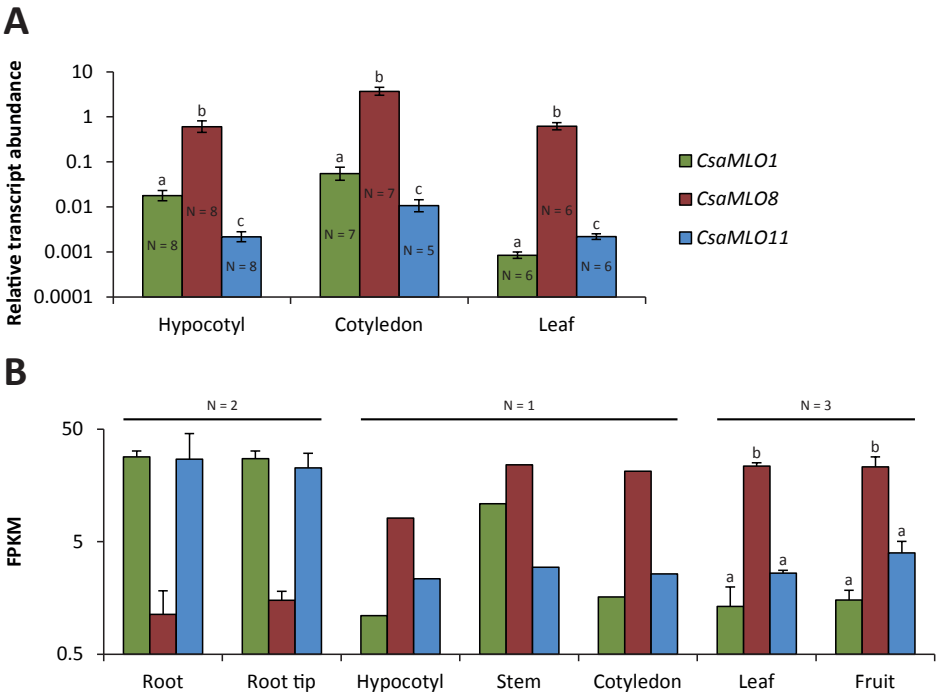

Figure 2 – Expression profile of clade V cucumber *MLO* genes in different tissues
A) The relative transcript abundances of *CsaMLO1*, *CsaMLO8* and *CsaMLO11* in three tissues of the PM-susceptible cucumber cultivar 'Sheila' (hypocotyl, cotyledon and leaf) were determined using qRT-PCR. Data were normalized, using the geometric average of the Ct values of reference genes *Ef-α* and *TIP41*. Relative transcript abundances were calculated as 2^{-dCt}. Each bar shows the average transcript abundance of five to eight biological replicates on a logarithmic scale. The number of independent biological replicates per gene/tissue combination is given in the respective bars. Error bars indicate standard error of the mean. Different letters above bars indicate significant differences between genes (ANOVA with Bonferroni post hoc tests, P < 0.05).
B) The transcript abundances in seven tissues of cucumber ('Chinese long' inbred line 9930) were determined using RNA-seq. The FPKM values (Fragments Per Kilobase of transcript per Million mapped fragments) for *CsaMLO1*, *CsaMLO8* and *CsaMLO11* in each of these tissues are shown on a logarithmic scale. The amount of independent biological replicates per tissue was either one (hypocotyl, stem and cotyledon), two (root and root tip) or three (leaf and fruit). If applicable, error bars indicate the standard error of the mean. Different letters above bars indicate significant differences between genes (ANOVA with Bonferroni (for fruit tissue) or Dunnet T3 (for leaf tissue) post hoc tests, P < 0.05).

samples of cucumber hypocotyl and leaf tissue prior to and at fours, six, eight, twelve and twenty-four hours post inoculation. The relative expression of *CsaMLO8* in those samples was determined using qRT-PCR (Additional file 3). No significant differences in *CsaMLO8* transcript abundance were observed in any of the time points compared to the transcript abundance prior to inoculation (ANOVA, P > 0.05).

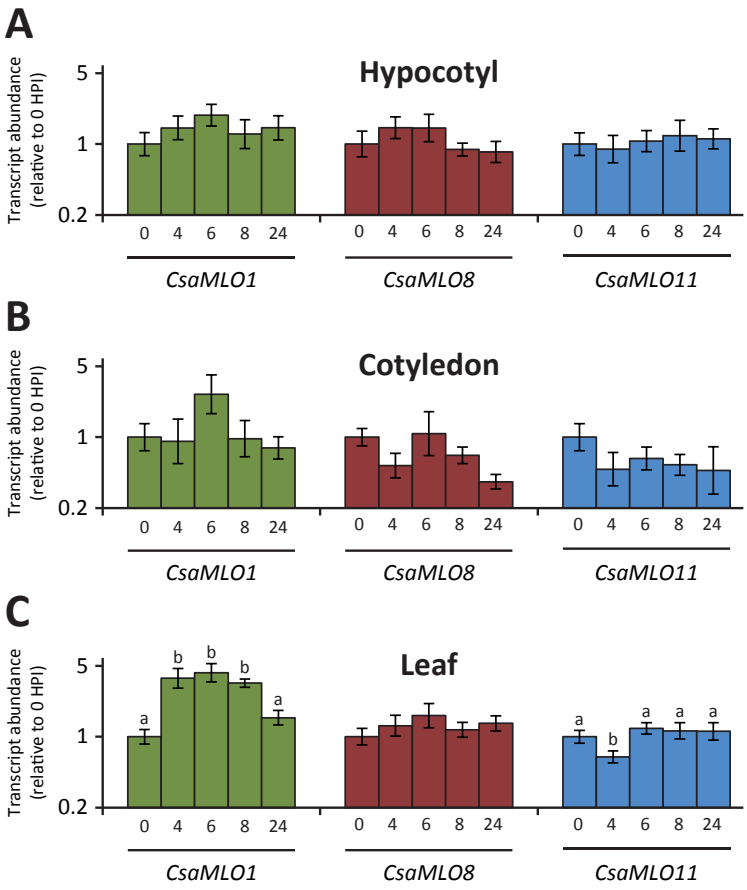

Figure 3 – Relative expression level of clade V *MLO* genes in different cucumber tissues in response to *Podosphaera xanthii* inoculation

Relative transcript abundances in three tissues (**(A)** hypocotyl, **(B)** cotyledon and **(C)** leaf) of PM-susceptible cucumber genotype 'Sheila' before and at 4, 6, 8 and 24 hours post inoculation with *P. xanthii* were determined using qRT-PCR. Data were normalized relative to the geometric average of the Ct values of reference genes *Ef*-α and *TIP41*, and subsequently normalized relative to the average dCt value at 0 hpi for each gene/tissue combination. Relative transcript abundances were calculated as 2^{-ddCt}. Each bar shows the relative expression of four to eight biological replicates on a logarithmic scale. Error bars indicate standard error of the mean. Different letters above bars indicate significant differences between time points (ANOVA with Bonferroni post hoc tests, P < 0.05).

Screening of sequenced cucumber germplasm for potential *CsaMLO1* or *CsaMLO11* mutants

We anticipated that if *CsaMLO1* and *CsaMLO11* are susceptibility genes towards PM in cucumber, loss-of-function mutations in those genes, leading to resistance, would have been selected for in cucumber germplasm. As Qi et al. published resequencing data of a collection of 115 divergent cucumber genotypes [37], we decided to screen these data for potential loss-of-function mutations in either *CsaMLO1* or *CsaMLO11*. Complete lists of detected SNPs and indels in the 115 lines were downloaded from the Cucurbit Genomics Database [38], and filtered for the chromosomal locations of *CsaMLO1* and *CsaMLO11*. Additional files 4 and 5 give an overview of all the detected SNPs and indels in *CsaMLO1* and *CsaMLO11* regions, respectively. The lists of SNPs/indels were manually curated to obtain variants in coding regions of the genes, with an effect on the predicted amino acid sequence. For *CsaMLO11* we observed no SNPs or indels with an effect on the predicted protein. For *CsaMLO1* three SNPs (and no indels) were found with an effect on the predicted amino acid sequence (Table 2). The first substitution (V170G) was in an amino acid residue conserved to be either a Valine, an Isoleucine or a Leucine in other clade V MLO proteins with a proven function as susceptibility gene (Additional file 6). The other two detected substitutions (V472I and V557I) were in non-conserved regions.

Table 2 – Non-synonymous SNPs detected in the CsaMLO1 coding sequence of 115 resequenced cucumber accessions.

Chromosome	Chromosome position	Exon number	Nucleotide substitution	Amino acid substitution
Chr1	8163552	Exon 4	T -> G	V170G
Chr1	8159763	Exon 14	G -> A	V472I

Resequencing of cucumber genotypes CS-PMR1 and Santou to find potential *CsaMLO1* or *CsaMLO11* mutants

As it was mentioned that two previously reported QTLs for PM resistance co-localize with the genomic positions of *CsaMLO1* and *CsaMLO11* [31], we hypothesized that the sources of these resistances might have mutant alleles of *CsaMLO1* and/or *CsaMLO11*, causal for the observed resistance. In order to test this hypothesis, we isolated DNA from leaves of genotypes CS-PMR1 and cv. Santou, the parental genotypes used for the QTL analysis [35], and performed whole genome sequencing (WGS). We aligned the obtained reads to the reference genome (Chinese long 9930 [30]) and identified SNPs and small indels in the genomic regions of *CsaMLO1* and *CsaMLO11* (Additional files 7-9). In the genomic region of *CsaMLO1* we identified 23 SNPs and indels in CS-PMR1 and four SNPs and indels in Santou, but none of these had an effect on the predicted encoded protein. Similarly we identified 23 SNPs and indels in the genomic region of

CsaMLO11 in CS-PMR1, neither of which had an effect on the predicted encoded protein. The *CsaMLO11* sequence of Santou was found to be identical to that of the reference genome.

In addition to calling SNPs and indels we identified two regions in genotype CS-PMR1, in intron 6 of *CsaMLO1* and intron 12 of *CsaMLO11* respectively, where we observed a local low read coverage combined with flanking read pairs with insert sizes deviating from the average or for which one of the mates in the read pair could not be mapped (Additional files 7-8). As this can be indicative for larger structural variations that are harder to characterize with short read sequencing, we amplified these regions from DNA of CS-PMR1 and sequenced the PCR products by Sanger sequencing. Compared to the reference genome, a 10 bp deletion and a 23 bp insertion in intron 6 of *CsaMLO1* (Additional file 10A), and a 231 bp deletion in intron 12 of *CsaMLO11* (Additional file 10D) were found in genotype CS-PMR1. To verify whether these large indels in intron 6 of *CsaMLO1* and intron 12 of *CsaMLO11* have any effect on the splicing of the genes, we also amplified and sequenced the corresponding regions from cDNA of CS-PMR1 and Santou by PCR, and found that there was no observable difference in PCR product size or sequence for either *CsaMLO1* (Additional file 10B-C) or *CsaMLO11* (Additional file 10E-F).

As we anticipated that the resistances of genotypes Santou and CS-PMR1 could also be caused by a difference in *CsaMLO1* or *CsaMLO11* expression rather than a difference in encoded protein sequence, we determined the relative transcript abundances of *CsaMLO1* and *CsaMLO11* in leaf tissue of both genotypes by qRT-PCR, using the susceptible genotype Sheila as a control (Additional file 11). Although the relative transcript abundance of both genes was found to be slightly higher in CS-PMR1 compared to the other two genotypes, these differences were found to be not statistically significant for either *CsaMLO1* (ANOVA, P = 0.156) or *CsaMLO11* (ANOVA, P = 0.239).

DISCUSSION

Overexpression of all three cucumber clade V MLO genes restores PM susceptibility in a tomato *mlo* mutant

Previously, it has been shown that loss of susceptibility towards PM causing fungi due to mutations in *MLO* genes can be restored by overexpression of functional *MLO* genes, both by cloning an *MLO* gene from a susceptible individual from the same plant species [10–12] as well as by heterologous expression of clade V *MLO* genes from other dicot species [13, 15, 39], and even by heterologous expression of clade IV *MLO* genes from a

monocot species [29]. This shows that even though there are considerable differences in amino acid sequence between MLO proteins, they are functionally conserved between plant species. This enabled us to study the function of cucumber clade V *MLO* genes by heterologous expression in the tomato *slmlo1* mutant background. We have shown that overexpression of either of the three clade V *CsaMLO* genes restored susceptibility to *O. neolycopersici*, the PM causing fungus in tomato, albeit to a different extent. Overexpression of either *CsaMLO1* or *CsaMLO8* resulted in full restoration of PM susceptibility towards wild-type levels. Overexpression of *CsaMLO11* on the other hand only partially complemented loss of *SlMLO1* function (Figure 1). This shows that, at least to some extent, all three genes are functionally conserved.

Overexpression of *CsaMLO11* appeared to be less efficient in restoring PM susceptibility compared to the other *CsaMLO* genes (Figure 1). Although the proteins encoded by each of the three genes are not identical, they are highly similar to one another and to clade V *MLO* genes of other species (Additional file 6). It appears difficult to attribute the lower efficiency of *CsaMLO11* compared to the other *CsaMLO* genes in restoring PM susceptibility to a particular difference in amino acid sequence.

CsaMLO8 is the major clade V *MLO* gene in aerial tissues, whereas *CsaMLO1* and *CsaMLO11* are the major clade V genes in roots

Susceptibility genes are defined as those genes that facilitate infection and support compatibility to plant pathogens [40]. Within the *MLO* gene family, homologs from clade IV (in monocotyledonous species) and V (in dicotyledonous species) have been found to be susceptibility genes [24]. However, not all clade V *MLO* genes in all dicotyledonous plant species have been found to be S-genes. For instance, in grapevine (*Vitis vinifera*) it was found that there are four clade V *MLO* genes. Silencing of one of them (*VvMLO7*) by transformation with RNAi constructs led to gain of PM resistance, whereas silencing of two other homologs only increases resistance when *VvMLO7* was already silenced. Silencing of the fourth homolog did not contribute to resistance at all [16]. This unequal genetic redundancy was previously also observed in *Arabidopsis* with one major *MLO* S-gene (*AtMLO2*) and two minor *MLO S*-genes [10]; and in tomato, with one major *MLO* S-gene (*SlMLO1*), two minor *MLO* S-genes and one clade V *MLO* homolog which does not seem to play a role in PM susceptibility [41].

We have shown, using qRT-PCR data (Figure 2A) and RNA-seq data (Figure 2B), that in aerial cucumber tissues (hypocotyl, stem, cotyledon, leaf and fruit tissue) *CsaMLO8* is several folds higher expressed than the other cucumber clade V *MLO* genes, *CsaMLO1* and *CsaMLO11*. This is reminiscent of the findings in tomato by Zheng et al. (2016), who showed that the major clade V *MLO* gene, *SlMLO1*, is much higher expressed than

the other clade V homologs in case of absence of PM [41]. Interestingly, they showed that silencing of *SlMLO1* by transformation with an RNAi construct led to gain of resistance, whereas silencing of the other *MLO* homologs did not. Indeed, a natural *slmlo1* loss-of-function mutant had previously been characterised to be resistant to PM [11], even though the other clade V *MLO* genes were presumably still intact. Additionally, we have shown that in a publicly available *Arabidopsis* RNA-seq dataset one clade V *MLO* homolog, *AtMLO2*, is much higher expressed than the other clade V homologs, *AtMLO6* and *AtMLO12* (Additional file 2). Previously it has been shown that loss-of-function mutations in *AtMLO2*, but not in *AtMLO6* or *AtMLO12* lead to (partial) resistance against PM, although double *AtMLO2/6* or *AtMLO2/12* and triple *AtMLO2/6/12* mutants showed even higher levels of resistance [10].

Recently, researchers investigated the expression pattern of *MLO* genes in a large number of tissues from *Arabidopsis* and rice, based on microarray data [42]. The data presented there for the *Arabidopsis MLO* genes are in agreement with our findings (Additional file 2), as they report that in most tissues the major *MLO* S-gene *AtMLO2* is much higher expressed than the other two clade V *MLO* genes [42]. Rice has two clade IV *MLO* genes, *OsMLO3* and *OsMLO6* [25], it was found that in most rice tissues *OsMLO3* transcription was much higher than *OsMLO6* transcription [42]. Based on this finding, the authors conclude that *OsMLO3* is likely to be the major clade IV *MLO* in rice, rather than *OsMLO6*.

Taken together, this suggests that loss-of-function mutations in the most abundantly expressed *MLO* gene have a large effect compared to loss-of-function mutations in the less abundantly expressed genes. This would imply that in cucumber, *CsaMLO8* would be the major clade V *MLO* gene concerning PM susceptibility, comparable in function to e.g. *SlMLO1* in tomato and *AtMLO2* in *Arabidopsis*. We postulate that differences in transcription efficiency between different clade V *MLO* genes in a species are the main reason for the observed unequal genetic redundancy, and that characterization of the relative transcript abundances of clade V *MLO* genes in a species may help identify the major *MLO* gene regarding susceptibility. To our knowledge, it has not been attempted in either *Arabidopsis* or tomato to express the minor clade V *MLO* genes under a strong constitutive promoter. On the basis of our results, we would expect that overexpression of e.g. *AtMLO6* or *AtMLO12* in *Atmlo2* background would be sufficient for restoration of susceptibility, if it were to be true that transcript abundance rather than differences in amino acid sequence determine which clade V *MLO* gene is the major S-gene.

Contrastingly, RNA-seq results show that *CsaMLO1* and *CsaMLO11* are highly expressed in root tissue, whereas *CsaMLO8* is not (Figure 1B). Interestingly, all three *Arabidopsis*

Clade V *MLO* genes were also found to be highly expressed in root tissue (Additional file 2). As PM causing fungi are foliar pathogens, which do not infect roots, the finding that several clade V *MLO* genes in cucumber and *Arabidopsis* are highly expressed in roots will probably not have much consequence on the interaction of plants with PM causing fungi. However, we should note that it is likely that *CsaMLO1* and *CsaMLO11* fulfil an important, yet unknown role in cucumber roots.

Loss-of-function mutations in *HvMLO* in barley and *AtMLO2*/*AtMLO6* in *Arabidopsis* lead to increased susceptibility to necrotrophic and hemibiotrophic pathogens such as leaf spot blotch disease caused by *Bipolaris sorokiniana* [43], rice blast on barley caused by *Magnaporthe grisea* [44], leaf spot disease caused by *Alternaria* spp. and late blight caused by *Phytophthora infestans* [10]. This suggests that *MLO*-genes can in some cases contribute to resistance to necrotrophic and hemibiotrophic pathogens, in contrast to their role as susceptibility gene for (biotrophic) PM causing fungi. Therefore, it might be interesting to study the effect of loss-of-function mutations in the root-expressed *CsaMLO1* and *CsaMLO11* on the interaction with necrotrophic, soil-borne cucumber pathogens such as vascular wilt caused by the necrotrophic fungus *Fusarium oxysporum* f. sp. *cucumerinum* or root rot caused by the necrotrophic oomycete *Pythium* spp. Furthermore, as it is known that barley *mlo* mutants are less efficiently colonized by mutualistic arbuscular mycorrhiza fungi [45], it could also be interesting to see the effect of *CsaMLO1* and *CsaMLO11* loss-of-function mutations on the mutualistic interaction with arbuscular mycorrhiza fungi in cucumber.

CsaMLO1 expression is induced upon PM inoculation, whereas *CsaMLO8* and *CsaMLO11* are not

For several plant species it has been shown that the expression of *MLO* susceptibility genes is induced upon inoculation with PM causing fungi (e.g. [46–48]), potentially due to the fungus actively upregulating the expression of those genes to induce susceptibility. It was previously reported based on RNA-seq data of *P. xanthii* inoculated cucumber leaves that *CsaMLO1*, but not *CsaMLO8* or *CsaMLO11* was upregulated in leaves in response to the inoculation, showing a ca. 3.5 fold upregulation in expression eight hours post inoculation [31]. Figure 3C confirms this finding, since we have found significant (ca. four-fold) upregulation of *CsaMLO1* expression in leaves at four, six and eight hours after inoculation with *P. xanthii*. As upregulation of *MLO* gene expression due to inoculation with PM causing fungi is often regarded as putative evidence for a role as susceptibility gene, one might argue that this suggests that *CsaMLO1* is a functional susceptibility gene, and *CsaMLO8* or *CsaMLO11* are not. However we would like to point out that even though *CsaMLO1* expression in leaves is significantly induced upon PM inoculation relative to the expression before inoculation (Figure 3C), the expression of

CsaMLO1 before inoculation is much lower than that of *CsaMLO8* which is constitutively higher expressed (Figure 2), so consequently even after inoculation the transcript abundance of *CsaMLO8* is still higher than that of *CsaMLO1*.

Previously we observed that inoculation of cucumber with *P. xanthii* led to upregulation of *CsaMLO8* in hypocotyl tissue, but not in leaf or cotyledon [13]. To our surprise we could not reproduce this result in our experiments described here, even though using the same cucumber genotype, *P. xanthii* isolate, climatic conditions and inoculation protocol, and using the same qRT-PCR primers and protocol (Figure 3A). Even though we did observe a small induction of *CsaMLO8* expression in hypocotyl at four and six hours post inoculation, differences in *CsaMLO8* transcript abundance in hypocotyl tissue between time points were far from significant (ANOVA, P = 0.389). It should be noted that the variation in transcript abundances between different biological replicates is quite high, both in our experiments described here and in our previously published results. As the current experiment has a larger sample size (four to eight independent biological replicates per time point instead of three independent biological replicates in [13]) and was repeated with similar results (Additional file 3), we conclude that upregulation of *CsaMLO8* in hypocotyl tissue [13] was probably an artefact caused by a low number of biological replicates.

We previously described that a loss-of-function mutant allele of *CsaMLO8* leads to hypocotyl-specific resistance towards PM, with partial resistance in leaf tissue, and attributed this tissue specificity to the supposed tissue-specific upregulation of *CsaMLO8* [13]. Now that we have shown that *CsaMLO8* is in fact not upregulated in hypocotyl tissue, we have to come up with a different explanation for the observed tissue specificity of *Csamlo8*-based resistance. It is in this sense interesting to note that *CsaMLO1* basal expression is very low (Figure 2) whereas it is induced upon PM inoculation in leaf tissue but not in hypocotyl tissue (Figure 3). Assuming that both *CsaMLO1* and *CsaMLO8* are functional susceptibility genes, we can hypothesize that in a *Csamlo8* loss-of-function mutant, which expresses functional *CsaMLO1*, there is hardly any expression of a functional *MLO* susceptibility gene in hypocotyl tissue, whereas there is induced expression of *CsaMLO1* in leaf tissue, resulting in complete resistance in hypocotyl tissue and only partial resistance in leaf tissue. If this would be true, a double *Csamlo1*/*Csamlo8* loss-of-function mutant would be expected to have complete resistance in both leaf and hypocotyl tissue.

No putative loss-of-function *CsaMLO1* or *CsaMLO11* mutants could be identified in resequenced cucumber germplasm

As loss-of-function mutations in functional susceptibility genes can lead to durable resistance [40], it would be worthwhile to obtain cucumber lines with mutations in clade V *MLO* genes. It has previously been described that a natural mutant allele in *CsaMLO8*, caused by insertion of a retrotransposable element, leads to partial resistance to *P. xanthii*. This mutant allele has a rather high frequency in breeding material, probably because of its beneficial effect on PM resistance [13]. Furthermore, several other mutant alleles of *CsaMLO8* were identified in resistant cucumber genotypes [33]. It is therefore reasonable to assume that if loss-of-function mutations in *CsaMLO1* and/or *CsaMLO11* would contribute to PM resistance, they also would have been selected for during cucumber breeding. Therefore we decided to screen a publicly available dataset of SNPs and indels in a collection of 115 cucumber genotypes [37] for putative loss-of-function alleles in the coding regions of *CsaMLO1* and *CsaMLO11* (Additional file 4 and 5). We did not find any evidence for variant alleles with a large effect on the amino acid sequence in either of the genes (e.g. a SNP leading to gain of an early stop codon or a frameshift indel), although we observed three SNPs in *CsaMLO1* leading to amino acid substitutions (Table 2). Of these three SNPs, two were predicted to cause an amino acid substitution from valine to isoleucine, two amino acid residues with very similar physiochemical properties. Furthermore, those amino acid residues were in the C-terminal domain of the CsaMLO1 protein, a region which is not conserved compared to other clade V MLO proteins (Additional file 6). A third SNP was predicted to lead to a substitution of the 170th amino acid residue, a valine, into a glycine residue, at a location conserved to be either a valine, a leucine or an isoleucine in other clade V MLO proteins (Additional file 6). As glycine and valine are both relatively small, aliphatic, non-polar amino acids, this substitution can be considered a rather conservative mutation. Without further evidence it does not seem very likely that this SNP represents a loss-of-function allele of *CsaMLO1*. In conclusion, we did not find strong evidence in this dataset for possible loss-of-function alleles of *CsaMLO1* and/or *CsaMLO11*, although it should be noted that by focussing on SNPs and indels we could have overlooked mutant alleles that are harder to find by short-read resequencing, such as the transposable element characterised in *CsaMLO8*, which can have a profound effect on the function of the genes.

In another approach to try to identify possible mutant alleles of *CsaMLO1* or *CsaMLO11* we resequenced two additional cucumber genotypes, CS-PMR1 and cv. Santou, which were previously mentioned to have QTLs for PM resistance colocalizing with the genomic positions of *CsaMLO1* and *CsaMLO11* [31, 35]. Although we identified several SNPs, indels and structural variations at the *CsaMLO1* and *CsaMLO11* loci, especially in

the more PM resistant genotype CS-PMR1, none of these are predicted to lead to any change in the encoded CsaMLO1 or CsaMLO11 proteins (Additional files 7-10). In addition, we verified whether the transcript abundances of *CsaMLO1* and *CsaMLO11* were different between these cucumber genotypes (Additional file 11), but we concluded that there were no significant differences among them. Therefore, we concluded that the observed resistance by Fukino et al. (2013) is likely caused by other genes rather than *CsaMLO1* or *CsaMLO11*.

The fact that we could not find convincing loss-of-function alleles of *CsaMLO1* or *CsaMLO11* in a diverse panel of cucumber genotypes implies that loss-of-function mutations in either of these genes have apparently not been selected for in cucumber breeding. An explanation for this finding could be that *Csamlo1* and *Csamlo11* knockout mutations could have only a small, additive effect on PM resistance in *Csamlo8* mutant background, and not have any effect in *CsaMLO8* background, comparable to the situation in *Arabidopsis* [10]. Furthermore it could be possible that loss-of-function mutations in *CsaMLO1* and *CsaMLO11* have pleiotropic effects on plant fitness, and are therefore selected against.

It would in our opinion be interesting to study the effect of knock-out mutants of *CsaMLO1* and *CsaMLO11*, for instance by targeted mutation using the increasingly popular CRISPR-*Cas9* technology [49], such as was already done in the bread wheat *TaMLO-A1* gene [50]. This might lead to a new, durable source of PM resistance in cucumber, especially when combined with the already existing *Csamlo8* partial resistance [13].

CONCLUSIONS

In this study we analysed the role of cucumber clade V *MLO* genes in susceptibility to PM. We showed by means of heterologous overexpression of the cucumber *MLO* genes *CsaMLO1*, *CsaMLO8* and *CsaMLO11* that all three genes are able to restore susceptibility in *mlo* tomato, although the effect of *CsaMLO11* overexpression was weaker compared to *CsaMLO1* or *CsaMLO8* overexpression. Additionally, we studied the transcription levels of these genes in different tissues of cucumber, both with and without inoculation with a PM causing fungus, *P. xanthii*, showing that *CsaMLO8* is higher expressed compared to *CsaMLO1* and *CsaMLO11* in all aerial tissues, although *CsaMLO1* expression in leaves is induced by inoculation with *P. xanthii*. We discuss that *CsaMLO8* is therefore likely to be the major clade V *MLO* gene in cucumber concerning PM susceptibility, with a potential minor role for *CsaMLO1* and *CsaMLO11*, comparable to earlier findings for *mlo* genes in *Arabidopsis* and tomato. In roots, however, *CsaMLO1* and *CsaMLO11* appeared to be

much higher expressed, which might have implications on the interactions of cucumber with root pathogens or beneficial microbes. No potential natural loss-of-function mutations in either *CsaMLO1* or *CsaMLO11* have been found so far. It would be interesting to generate *Csamlo1* and *Csamlo11* mutants, for instance by CRISPR-*Cas9* technology, to investigate whether such mutations have an added effect on top of PM resistance caused by *Csamlo8*.

METHODS

Cloning of *CsaMLO1* and *CsaMLO11*

A homozygous cucumber breeding line derived from a parental line of cv. Anaxo was grown in a greenhouse in Wageningen, the Netherlands. Growing conditions were 20°C (day) and 16°C (night), with a 16 h/8 h day/night cycle, and a relative humidity of 70%. RNA isolation and cDNA synthesis were performed as previously described [13].

The coding sequence of *CsaMLO1* was amplified from cDNA with primers 5′-caccTTCCTTC-CACACCCCTAAGA-3′ (Forward) and 5′-TGAATGGTGTAAACGAGATTGC-3′ (Reverse). As template 50 ng cDNA was used in a 50 µl reaction using Advantage 2 polymerase (Takara Bio, U.S.A.). Cycling conditions were: 1 min initial denaturation at 95 °C, followed by 35 cycles of 30 s denaturation at 95 °C and 3 min annealing and extension at 68 °C. Reactions were finished by 3 min incubation at 68 °C. The PCR product was subsequently diluted 100 times, and used as template for a 50 µl reaction using Phusion high-fidelity polymerase (ThermoFisher Scientific, U.S.A.). Cycling conditions were: 30 s initial denaturation at 98 °C, followed by 25 cycles of 20 s denaturation at 98 °C, 30 s annealing at 55 °C, and 30 s extension at 72 °C. Reactions were finished by 10 min incubation at 72 °C.

The coding sequence of *CsaMLO11* was amplified from cDNA as previously described [13], using primers 5′-caccTTTGTTTCCCTACGCGTTCT-3′ (Forward) and 5′-TATAC-CAACCCCCAACCTCA-3′ (Reverse).

Cloning of *CsaMLO1* and *CsaMLO11* PCR products, through the Gateway-compatible vector pENTR/D-TOPO (ThermoFisher Scientific, U.S.A.) to binary vector pK7WG2, which harbours the constitutively active 35S Cauliflower Mosaic Virus promotor and the *nptII* selectable marker gene for kanamycin resistance [51], was done as previously described [13].

Complementation of *ol-2* tomato with cucumber *MLO* genes

Cotyledon explants of *ol-2* mutant tomato seedlings were transformed with *CsaMLO1* and *CsaMLO11* overexpression constructs as previously described [13]. The *ol-2* mutant carries a loss-of-function mutation in the *SlMLO1* gene [11]. Obtained tomato transformants were assessed for presence of the transgenes by PCR using the same primers as used for the cloning of *CsaMLO1* and *CsaMLO11* (see above) and for presence of the *nptII* marker gene with primers 5′-GAAGGGACTGGCTGCTATTG-3′ (*nptII* forward) and 5′-AATATCACGGGTAGCCAACG-3′ (*nptII* reverse). For each of the two transformations with a different construct, seven (*CsaMLO11*) or eight (*CsaMLO1*) independent transgenic plants were selected, and were assessed for transgene expression by qRT-PCR using primer pairs specific for *CsaMLO1*: 5′-TGAAAGTTTCCGGCGGAGTT-3′ (*CsaMLO1*-Forward) and 5′-AGGAAGCTTTACCCTTGGCG-3′ (*CsaMLO1*-Reverse) or specific for *CsaMLO11*: 5′-GCGACGGCGTTGAGAAATTG-3′ (*CsaMLO11*-Forward) and 5′-GGGTGACAAGTGGTGGGAGG-3′ (*CsaMLO11*-Reverse). As housekeeping gene for normalization of *CsaMLO1* or *CsaMLO11* expression in tomato, *SlEF*-α was used, with primer pair 5′-ATTGGAAACGGATATGCCCCT-3′ (SlEF-α forward) and 5′-TCCTTACCTGAACGCCTGTCA-3′ (SlEF-α reverse). qRT-PCR was performed as previously described [13].

Evaluation of PM resistance of *CsaMLO* overexpressing *ol-2* tomato

From each of the individual transformants of both *CsaMLO1* and *CsaMLO11* overexpressing *ol-2* plants, two cuttings were inoculated with an isolate of *Oidium neolycopersici* maintained on susceptible tomato plants in a climate chamber in Wageningen, the Netherlands, as previously described [13].

For two individual T1 transformants per overexpression construct, seeds of self-pollinated plants were harvested. In principle the T1 transformants with highest transgene expression were chosen for generation of T2 families, although as the *CsaMLO11* transformant with the highest expression did not give viable seeds another T1 transformant with enough viable seeds was randomly selected. In addition, seeds of two self-pollinated *CsaMLO8* overexpressing plants described in [13] were harvested. Twenty-two to thirty seeds of each of the six T2 families were sown on soil, plantlets were assayed for presence of the *nptII* marker gene by PCR as described above. As susceptible control, 12 seeds of cultivar Moneymaker (MM) were sown. As resistant control, 12 seeds of resistant mutant line *ol-2*, the background used for the complementation, were sown. For five plants per T2 family and for five plants of the susceptible and resistant controls, leaf samples were harvested and immediately frozen in liquid nitrogen. Transgene expression efficiency in these plants was determined by means of qRT-PCR using primers and conditions as described above. To analyse differences in transgene expression efficiency between the T2 families a one-way ANOVA test was performed

on the dCt values, followed by Dunnet's T3 post hoc tests. Homogeneity of the variances was tested using Levene's test. All statistical analyses were performed using SPSS v23 software (IBM). T2 families and controls were inoculated with *O. neolycopersici* as described above. The susceptibility/resistance of plants was scored after two weeks on a 0-3 scale as described earlier [11]. Differences in PM susceptibility between the T2 families and controls were analysed with a nonparametric Kruskal-Wallis test followed by Stepwise Stepdown Multiple Comparison post hoc tests to identify homogeneous subsets, using SPSS v23 software (IBM).

Expression analysis of *MLO* genes in cucumber using qRT-PCR

For expression analysis of *MLO* genes in PM-inoculated cucumber tissues, PM susceptible cucumber cultivar 'Sheila' was grown and inoculated with a *P. xanthii* isolate as previously described [13]. Prior to inoculation and at 4, 6, 8 and 24 h post inoculation (hpi), from eight individual plants per time point hypocotyl, cotyledon and (first) true leaf samples were harvested separately, and were immediately frozen in liquid nitrogen. RNA isolation, cDNA synthesis and qRT-PCR were performed as previously described [13]. For quantification of *CsaMLO1* and *CsaMLO11* expression, respectively, we used the primer sequences *CsaMLO1*-Forward and *CsaMLO1*-Reverse and *CsaMLO11*-Forward and *CsaMLO11*-Reverse, as described above. Primer pairs specific for the cucumber housekeeping genes *TIP41* and *EF-*α, as described by Warzybok et al. [52], were used for normalization of expression.

Samples for which the difference in Ct value between the technical replicates was larger than 1.0, or for which one or both of the technical replicates did not reach the detection threshold were excluded from the analysis. Ct values per sample were normalised by subtracting the geometric mean of the Ct values for the two housekeeping genes, giving deltaCt, abbreviated as dCt. In the time series of inoculated cucumber tissues, dCt values were subsequently normalized by the average dCt value for each gene/tissue combination at 0 hpi, giving ddCt. Averages and standard errors of ddCt values were calculated over four to eight biological replicates per gene/tissue/time point combination. Normality of ddCt distributions was tested using Shapiro-Wilk tests ($P > 0.05$). Differences in ddCt value between time points were analysed with ANOVA tests. Homogeneity of variances were tested using Levene's test. If ANOVA tests showed a significant effect of time points ($P < 0.05$), Bonferroni post hoc tests were performed to analyse which time points were significantly different from one another. All statistical analyses were performed using SPSS v23 software (IBM). Relative transcript abundances were calculated as 2^{-ddCt}.

In a second experiment, PM susceptible cucumber cultivar 'Sheila' was grown and inoculated with *P. xanthii* under the same conditions as described above. Samples of leaf and hypocotyl tissue were harvested prior to inoculation and at 4, 6, 8, 12 and 24 h post inoculation (hpi), from eight individual plants per time point. Total RNA was isolated using a phenol-based protocol described by [53]. cDNA synthesis and qRT-PCR were performed as described above.

In another experiment, cucumber genotypes 'Sheila', 'Santou' and 'CS-PMR1' were grown in a greenhouse in Wageningen, the Netherlands. Five weeks post seeding, leaf samples were harvested and were immediately frozen in liquid nitrogen. Total RNA was isolated using the RNeasy plant mini kit (Qiagen, Germany). cDNA was synthesised using 500 ng of RNA samples with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, U.S.A.). Before use in qRT-PCR, cDNA samples were diluted 2-fold. To quantify the expression of *CsaMLO1* and *CsaMLO11*, qRT-PCR was performed with conditions and subsequent data analysis as described above, normalizing dCt values using the average dCt value of the 'Sheila' samples.

Analysis of *MLO* data in RNA-seq datasets

Cucumber genotype 'Chinese long' inbred line 9930 was cultivated under standard greenhouse conditions (20°C (day) and 16°C (night), with a 16 h/8 h day/night cycle, and a relative humidity of 70%). Separate samples of roots, root tips, hypocotyls, cotyledons, stems, leaves and fruit were harvested and immediately frozen in liquid nitrogen, using one (hypocotyls, cotyledons and stems), two (roots and root tips) or three (leafs and fruit) individual samples per tissue. Material was sent to KeyGene B.V., The Netherlands, for RNA-seq. Total RNA from each sample was isolated using the RNeasy plant mini kit (Qiagen, Germany). Subsequently, RNA-seq libraries were made following the TruSeq RNA Sample Preparation v2 Guide protocol. After concentration measurement by qPCR (LightCycler 480; Roche), PhiX (~0.6 %) was spiked as a control according to the manufacturer's recommendations. The libraries were pooled, and sequenced using a Illumina HiSeq 2000 sequencer. The resulting reads were sorted into single fasta files per sample based on the sample tag sequences. The obtained read length was approximately 100 nt at a minimal read length of 36 nt. Reads were aligned to the reference genome ('Chinese long' inbred line 9930, version 2 [30]). The transcript abundance per sample was assessed on the basis of the number of sequenced fragments, normalised by the length of the coding sequence of the gene, per million of total reads sequenced (fragments per kilobase of transcript per million sequenced reads, FPKM). We extracted FPKM values per sample from the total dataset for each of the three clade V *MLO* genes, using Excel. For samples with more than one biological replicate, differences in FPKM values were analysed with ANOVA tests, followed by Bonferroni post hoc tests if vari-

ance was homogeneous, or Dunnet T3 post hoc tests if variance was not homogeneous. Homogeneity of variances was tested using Levene's test. All statistical analyses were performed using SPSS v23 software (IBM).

RNA-seq data for Arabidopsis were analysed using the Gene Expression Atlas from EMBL-EBI [54]. Baseline expression values in Arabidopsis tissues as quantified by [36] were filtered for clade V *MLO* genes *AtMLO2* [AT1G11310], *AtMLO6* [AT1G61560] and *AtMLO12* [AT2G39200] and downloaded as a tabular file.

In silico screening of *MLO* sequence variants in 115 lines

Total lists of SNPs and indels identified in 115 cucumber genotypes by [37] were downloaded from the Cucurbit Genomics Database [38]. SNPs and indels were filtered by Excel based on the genomic positions of *CsaMLO1* (Chr1: 8,159,427..8,165,253, negative strand) or *CsaMLO11* (Chr6: 14,120,024..14,125,039, positive strand). Sequence variants were manually annotated based on their genomic location to see whether they were located in introns or exons, when they were located in exons the effect on the coding sequence was scored using CLC Main Workbench v. 7.6.4.

Resequencing of CS-PMR1 and Santou

Seeds of cucumber genotypes CS-PMR1 and Santou were ordered from the Genetic Resources Center, NARO (National Agriculture and Food Research Organization), Japan. Young leaves of both genotypes were harvested and immediately frozen in liquid nitrogen. DNA was isolated from leaves as described by [55]. Total DNA was send for library preparation and whole genome sequencing with an average coverage of 25 reads per base pair using Illumina Hiseq PE150 technology with insert size of 350 bp, by Novogene Company Limited, Hong Kong, People's Republic of China. Resulting reads were aligned to the cucumber reference genome ('Chinese long' inbred line 9930, version 2 [30]) using Bowtie, version 2.2.6 [56]. Filtering the reads for the genomic location of *CsaMLO1* and *CsaMLO11* and SNP/indel calling were performed using the SAMtools software package, version 0.1.18 [57].

To inspect a region with low read coverage in intron 6 of *CsaMLO1,* this region was amplified by PCR from DNA of genotype CS-PMR1 using primers 5'-CCTGCCTTGATGTGGATCGT-3' (Forward) and 5'-AGTGCCTTCTTCTGACCGTT-3' (Reverse). To inspect a region with low read coverage in intron 12 of *CsaMLO11,* this region was amplified by PCR from DNA of genotype CS-PMR1 using primers 5'- AGCACACAGAGGATTTGGTCA-3' (Forward) and 5'-TGAACGAGAACCCTGATGCA-3' (Reverse). For both PCR reactions, as template 2 µl DNA was used in a 50 µl reaction using DreamTaq DNA polymerase (ThermoFisher Scientific, U.S.A.). Cycling conditions were: 1 min initial denaturation at 95 °C, followed by

40 cycles of 30 s denaturation at 95 °C, 30 seconds annealing at 60 °C and 1 min annealing at 72 °C. Reactions were finished by 7 min incubation at 72 °C. PCR products were visualised by staining with GelRed and electrophoresis on agarose gels. Sequencing reactions were performed in triplicate, using the same primers used for amplification (GATC Biotech, Germany). Obtained sequences were aligned using CLC Main Workbench v. 7.6.4. The consensus sequence for the amplified region was extracted from the alignment. This consensus sequences were then aligned to the genomic reference sequences of *CsaMLO1* and *CsaMLO11*, respectively.

To verify whether the observed sequence variants in intron 6 of *CsaMLO1* and intron 12 of *CsaMLO11* in genotype CS-PMR1 cause any effect on splicing, the corresponding regions were amplified from cDNA. RNA was isolated from young leaves of cucumber genotypes CS-PMR1 and Santou using the RNeasy Kit (Qiagen, Germany). Possible DNA contamination of RNA samples was removed by treatment with DNase I, Amp. Grade (Invitrogen life technologies, U.S.A.). cDNA was synthesised using 2 µg of RNA samples with an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, U.S.A.). A *CsaMLO1* coding sequence fragment was amplified from cDNA using primers *CsaMLO1*-Forward (as above) and 5'-ATGGCAGCCATAGATACGCC (Reverse). A *CsaMLO11* coding sequence fragment was amplified from cDNA using primers 5'-GTGGTGGTCAGTATCAGCCC-3' (Forward) and 5'-CGTCGAACCCATCTGTGTGA-3' (Reverse). PCR reactions were performed using DreamTaq DNA polymerase (ThermoFisher Scientific, U.S.A.), with cycling conditions as described above. PCR products were visualised by staining with GelRed and electrophoresis on agarose gels. Sequencing reactions were performed in triplicate, using the same primers used for amplification (GATC Biotech, Germany). Obtained sequences were aligned using CLC Main Workbench v. 7.6.4. The consensus sequence for the amplified region was extracted from the alignment. These consensus sequences were then aligned to the cDNA reference sequences of *CsaMLO1* and *CsaMLO11*, respectively.

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AUTHORS' CONTRIBUTIONS

JAB, HJS and YB designed the experiments. JAB, MA and GB performed the experiments. JAB drafted the manuscript. MA, RGFV, HJS and YB critically revised the manuscript. All authors read and approved the manuscript.

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ADDITIONAL DATA

All figures and additional datafiles described in this chapter were deposited online, and can be accesed through doi:10.4121/uuid:9b31358d-b8da-449a-8ae8-0fea922bc186

Additional file 1

Format: PDF

The relative transcript abundances of *CsaMLO1*, *CsaMLO8* and *CsaMLO11* in T2 families of a tomato *mlo* mutant overexpressing *CsaMLO1*, *CsaMLO8* and *CsaMLO11*, were determined by qRT-PCR. Data were normalised relatively to the reference gene *SlEF-* . Average transcript abundances of four or five randomly selected individuals are shown on a logarithmic scale. Error bars represent standard error of the mean. Different letters above the bars indicate statistical significance of Ct-values (One-way ANOVA with Dunnet's T3 post hoc test, $P < 0.05$). *CsaMLO1*, *CsaMLO8* or *CsaMLO11* expression was not detectable in non-transformed tomato *mlo* mutant or Moneymaker.

Additional file 2

Format: PDF

Data on the transcript abundance in four tissues of Arabidopsis thaliana, determined using RNA-seq was investigated and downloaded using the Expression Atlas of EMBL-EBI (https://www.ebi.ac.uk/gxa/home). The FPKM values (Fragments Per Kilobase of transcript per Million mapped fragments) for *AtMLO2*, *AtMLO6* and *AtMLO12* in each of the tissues is shown on a logarithmic scale.

Additional file 3

Format: PDF

Relative transcript abundances in two tissues of PM susceptible cucumber cultivar 'Sheila' (**A**) hypocotyl and **B**) leaf, before and at 4, 6, 8, 12 and 24 hours post inoculation with *P. xanthii* were determined using qRT-PCR. Data were normalized relative to the geometric average of the Ct values of reference genes *Ef-* and *CACS*, and subsequently normalized relative to the average dCt value at 0 hpi for both tissues. Each bar shows the relative expression of five to eight biological replicates, as indicated above the bars, on a logarithmic scale. Error bars indicate standard error of the mean.

Additional file 4

Format: XLSX

The list of SNPs and indels in 115 resequenced cucumber accessions, available from the Cucurbit Genomics Database [38], was filtered for the genomic region of *CsaMLO1* (Chr1:8159428..8165253). The genic location of each SNP/Indel was determined manually (exon/intron), for exonic SNPs the effect on the predicted amino acid sequence was scored (synonymous/nonsynonymous).

Additional file 5

Format: XLSX

The list of SNPs and indels in 115 resequenced cucumber accessions, available from the Cucurbit Genomics Database [38], was filtered for the genomic region of *CsaMLO11* (Chr6:14,120,024..14,125,039). The genic location of each SNP/Indel was determined manually (exon/intron), for exonic SNPs the effect on the predicted amino acid sequence was scored (synonymous/nonsynonymous).

Additional file 6

Format: PDF

Protein alignment of clade V MLO proteins of *Arabidopsis thaliana* (AtMLO2, 6 and 12), *Medicago trunculata* (MtMLO1), *Pisum sativum* (PsMLO1), *Lotus japonicus* (LjMLO1), *Cucumis sativus* (CsaMLO1, 8 and 11), *Solanum lycopersicum* (SlMLO1), *Capsicum annuum* (CaMLO2) and *Nicotiana tabacum* (NtMLO1). A bar graph shows the conservation of the individual residues. Colours indicate amino acid residues with similar physiochemical properties according to the RasMol colour scheme. Locations of amino acid substitutions in CsaMLO1 due to SNPs listed in Table 3 are indicated by a red arrow.

Additional file 7

Format: PDF

Resequencing data of the genomic region of *CsaMLO1* in cucumber genotypes CS-PMR1 and Santou. The location of the gene on the chromosome is indicated by a red cursor. For each of the two genotypes, the total reads mapping to the location and the coverage per base pair are given. SNPs are indicated by coloured stripes (green for A, red for T, blue for C, brown for G), indels are indicated by black stripes. Read pairs with a small (smallest 0.5%) or large (largest 0.5%) insert size are coloured dark blue or dark red, respectively. Reads for which the other mate in the mate pair was not mapped are indicated by a bright red outline.

Additional file 8

Format: PDF

Resequencing data of the genomic region of *CsaMLO11* in cucumber genotypes CS-PMR1 and Santou. The location of the gene on the chromosome is indicated by a red cursor. For each of the two genotypes, the total reads mapping to the location and the coverage per base pair are given. SNPs are indicated by coloured stripes (green for A, red for T, blue for C, brown for G), indels are indicated by black stripes. Read pairs with a small (smallest 0.5%) or large (largest 0.5%) insert size are coloured dark blue or dark red, respectively. Reads for which the other mate in the mate pair was not mapped are indicated by a bright red outline.

Additional file 9

Format: XLSX

The list of SNPs and indels identified in the genomic sequence of *CsaMLO1* and *CsaMLO11* in the resequenced cucumber genotypes CS-PMR1 and Santou. The reference allele (in cucumber reference genome Chinese Long 9930) and the alternative allele are given. Furthermore, the genic location of each SNP/Indel was determined (exon/intron), for exonic SNPs the effect on the predicted amino acid sequence was scored (synonymous/nonsynonymous).

Additional file 10

Format: PDF

Intron 6 of *CsaMLO1* **(A)** and intron 12 of *CsaMLO11* **(D)** were amplified from genomic DNA isolated from the cucumber genotype CS-PMR1, and subsequently sequenced in triplicate by Sanger sequencing. The obtained sequences were aligned to the reference cucumber genome (Chinese long inbred 9930, v2). Numbers above the alignment are relative to the start codon of the respective genes.

The region surrounding intron 6 of *CsaMLO1* **(B and C)** and the region surrounding intron 12 of *CsaMLO11* **(E and F)** were amplified from cDNA of cucumber genotypes CS-PMR1 and Santou, and subsequently sequenced in triplicate by Sanger sequencing. Amplified products were analysed on 1.25% agarose gels. It was found that for both amplified regions, the products amplified from CS-PMR1 and Santou were of similar sizes. Sequences of cDNA were identical to one another and to the reference cDNA sequence (Chinese long inbred 9930, v2).

Additional file 11

Format: PDF

Relative transcript abundances of **CsaMLO1** and **CsaMLO11** in leaf tissues of cucumber genotypes Sheila, Santou and CS-PMR1 were determined using qRT-PCR. Data were normalized relative to the geometric average of the Ct values of reference genes **Ef-α**, **TIP41** and **CACS**, and subsequently normalized relative to the average dCt value of Sheila. Each bar shows the relative expression of three biological replicates, on a logarithmic scale. Error bars indicate standard error of the mean.

Chapter 4

Analysis of QTL DM4.1 for downy mildew resistance in cucumber reveals multiple subQTL: a novel *RLK* as candidate gene for the most important subQTL

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ABSTRACT

Background: One of the biggest problems in cucumber cultivation is cucurbit downy mildew (DM), caused by the obligate biotroph *Pseudoperonospora cubensis*. Whereas DM in cucumber was previously efficiently controlled by the *dm-1* gene from Indian cucumber accession PI 197087, this resistance was broken by new strains of the pathogen, prompting the search for novel sources of resistance. A promising source of resistance is the accession PI 197088. It was previously shown that DM resistance in this genotype inherits polygenically. In this paper we put the focus on one of the QTL, located on chromosome 4.

Results: We performed QTL mapping in recombinant populations derived from an introgression line, which had a 12 Mb introgression from PI 197088 on chromosome 4 in a susceptible (HS279) background. QTL mapping based on different disease phenotypes (i.e. chlorosis, sporulation, and necrosis) gave markedly different results, leading to the identification of three subQTL within the previously established DM4.1 interval. Detailed observations on families segregating for the second and third subQTL, DM4.1.2 and DM4.1.3, respectively, confirmed the effects of these two subQTL. Transcriptomic analysis of near-isogenic lines (NILs) with either subQTL DM4.1.2 or subQTL DM4.1.3 was carried out. We found that whereas inoculation with *P. cubensis* in general had large effects on gene expression, this effect was stronger in NIL DM4.1.2 compared to both NIL DM4.1.3 and susceptible parent HS279. Furthermore several defence pathway genes were analysed in more detail, and found to be more strongly upregulated in NIL DM4.1.2 compared to the other genotypes. Fine-mapping of subQTL DM4.1.2 combined with differential gene expression analysis identified a *Receptor-like Kinase* (*CsLRK10L2*) as the most likely causal gene. A 551 bp deletion in this gene was identified in the susceptible genotypes, including the reference genome (Chinese Long 9930 v2), whereas a presumably functional copy of the gene was present in NIL DM4.1.2. Heterologous expression of this gene in *N. benthamiana* resulted in a strong necrotic response.

Conclusions: The previously identified major QTL DM4.1 from PI197088, was shown to consist of multiple subQTL: DM4.1.1 affected pathogen-induced necrosis, DM4.1.2 was shown to have an additive effect on sporulation, and DM4.1.3 had a recessive effect on chlorosis as well as an effect on sporulation. Transcriptomic analysis revealed that many genes in general, and defence pathway genes in particular, were differentially expressed in NIL DM4.1.2 compared to NIL DM4.1.3 and the susceptible parent HS279. This indicates that the resistance from subQTL DM4.1.2 likely involves defence signalling pathways, whereas resistance due to subQTL DM4.1.3 is more likely to be independent of known defence pathways. Based on fine-mapping data we identified the

RLK gene *CsLRK10L2* as a likely candidate for subQTL DM4.1.2, as this gene was found to have a loss-of-function mutation in the susceptible parent HS279, and was strongly upregulated by *P. cubensis* inoculation in NIL DM4.1.2. Heterologous expression of this gene triggered necrosis, providing further evidence that this gene is indeed causal for subQTL DM4.1.2.

Keywords: Downy mildew (*Pseudoperonospora cubensis*), Cucumber (*Cucumis sativus*), Plant-pathogen interactions, PI 197088, QTL mapping, Transcriptomics, *Receptor-like Kinase (RLK)*, *Leaf rust kinase 10-like* (*LRK10L*), *Wall-associated kinase-like* (*WAKL*)

BACKGROUND

The oomycete *Pseudoperonospora cubensis* [(Berk. & Curt.) Rost.] belongs to the family Peronosporaceae. Obligate biotrophs within the Peronosporaceae, such as *P. cubensis*, are commonly referred to as Downy Mildew (DM) pathogens [1]. Although DM pathogens share a biotrophic lifestyle and morphological similarities with fungi causing powdery mildew (PM), the two groups of pathogens are unrelated. The host range of *P. cubensis* includes ca. 20 genera and at least 50 species within the Cucurbitaceae family, including economically important crops such as cucumber (*Cucumis sativus*), melon (*Cucumis melo*), watermelon (*Citrullus lanatus*), and squash (*Cucurbita* spp.) [2]. DM is considered to be the most important disease in cucumber worldwide, as it causes up to 100% of yield loss, and strains of *P. cubensis* have become resistant against fungicides as well as have overcome resistance in cucumber germplasm [3].

Cucumber is thought to have been domesticated ca. 3000 years ago in India, the centre of origin for this species. Wild cucumber (*C. sativus* var. *hardwickii*) still occurs in northern India as well as southern China [4]. During domestication, cucumber went through several genetic bottlenecks, causing a strong reduction in genetic diversity, likely due to a small initial population size, combined with a very strong selection pressure, e.g. for absence of bitterness and presence of longer fruit [5]. The primary source of disease resistance is in (semi)wild cucumber accessions, maintained by gene banks, as these often carry resistance alleles that might have been lost during cucumber domestication. For over four decades, DM in cucumber was efficiently controlled by the recessive *dm-1* gene, introgressed in modern cultivars from Indian *C. sativus* var. *hardwickii* accession PI 197087 [6, 7]. However, after massive epidemics following a new, more virulent, strain of *P. cubensis*, *dm-1* resistance is no longer effective in controlling cucumber DM, although it still provides some level of intermediate resistance [8, 9].

In order to identify novel sources of DM resistance, 1300 cucumber cultigens (accessions, breeding lines as well as elite cultivars) in the USDA Agriculture Research Service collection were evaluated in multi-year, multi-location experiments. After scoring these 1300 genotypes for three years in test fields in North Carolina (U.S.A.) and Poland, the 40 most resistant as well as the ten most susceptible cultigens were retested for three more years in both greenhouse and open field experiments in North Carolina and Bangalore (India). The consistently most resistant genotypes were accessions PI 197088 and PI 605996 (both of Indian origin) and PI 330628 (originating from Pakistan) [10]. Interestingly, in a screening ten years earlier the most DM resistant cultigens were all derived from PI 197087, even though PI 197087 itself only had an intermediate score [11]. However, in the new screening, none of those previously DM resistant lines were found to be very resistant, demonstrating that current strains of *P. cubensis* have overcome PI 197087-derived resistance [10].

It was shown that in F2 populations derived from crosses among accessions PI 197088, PI 605996 and PI 330628 significant numbers of plants scored as susceptible, indicating that the resistance in these three highly resistant lines are likely conferred by different genes [12]. Subsequently mapping studies were performed aimed at unravelling the genetic architecture of DM resistant accessions. Several groups mapped QTL in accession PI 197088. The overall conclusion is that resistance in PI 197088 is polygenic, and that some QTL (notably QTL on chromosome 5 and 4) were identified by most groups as having the largest effect. However, the contribution of the different identified QTL to overall DM resistance varied greatly from study to study, possibly reflecting differences in inoculum strains in different parts of the world, and/or differences in experimental design and environmental conditions between studies [13–16]. DM resistance was also mapped in PI 330628, again identifying loci on chromosomes 4 and 5 as major QTL, at similar intervals compared to those of PI 197088 [17].

In our study, we focus on a QTL on chromosome 4 from PI 197088 (DM4.1), which was found by most previously published QTL mapping studies as having large or moderate effects. In order to identify the causal gene(s) for a QTL, it is advisable to reduce genetic variation due to other QTL by creating near-isogenic lines (NIL) in a uniform genetic background, which turns the quantitative effect of the QTL in a more discretely inherited Mendelian trait [18]. Recently, two QTL for DM resistance on chromosomes 4 and 5 from resistant cucumber accession PI 330628 were fine-mapped to intervals containing only thirteen and three predicted genes, respectively, by developing NIL-derived segregating families [19].

Traditionally, plant breeding has focused on dominant resistance (R) genes, conferring qualitative resistance against pathogens. Since the advent of molecular techniques such as the polymerase chain reaction (PCR) and gene/genome sequencing, many R genes were cloned and characterized. It was found that the majority of the cloned R genes (80%) encode either intracellular proteins with nucleotide-binding and leucine rich repeat domains (NLRs) or plasma membrane-bound receptor-like kinases (RLKs) [20]. NLRs trigger immune signalling by either direct recognition of cognate pathogen-encoded effector proteins, or indirect recognition of effector-mediated alterations of host proteins. NLR mediated defence signalling leads to effector-triggered immunity (ETI) which often involves the hypersensitive response (HR) leading to programmed cell death [21]. Interestingly, whereas most plant genomes encode hundreds of predicted *NLR* genes, the cucumber genome was found to encode only 57 predicted *NLR* genes, and similarly low numbers were found in other cucurbit species [22]. Whereas some of these *NLR* genes might indeed confer resistance against pathogens, it is likely that cucumber relies on other types of genes more than other plant species for conferring resistance.

The second largest group of cloned resistance genes encode plasma membrane localized receptor-like kinases (RLKs) and receptor-like proteins (RLPs) [20]. RLKs are proteins with a single transmembrane helix, a variable extracellular domain, and a rather conserved intracellular kinase domain. RLPs are essentially RLKs without a (functional) kinase domain, and were shown to be independently evolved from RLKs on multiple occasions [23]. RLKs play important roles not only in disease resistance, but also in growth and development [24]. The extracellular domains of RLKs/RLPs involved in resistance recognize either apoplastic pathogen effectors or conserved microbe-associated and damage associated molecular patterns (MAMPs and DAMPs, respectively). Traditionally, a distinction was made between ETI and PAMP-triggered immunity (PTI), in the sense that a weaker PTI response confers basal resistance against large groups of pathogens (e.g. fungal resistance by chitin perception or bacterial resistance by flagellin perception) whereas a stronger ETI response confers specific resistance against adapted pathogen species [21]. However, the identification of broadly conserved effectors and narrowly conserved PAMPs have shown that this dichotomy is an oversimplification [25].

RLK genes form one of the most abundant gene families in plant genomes, with model organism *Arabidopsis thaliana* having over 600 predicted *RLK* genes. RLKs have diverse extracellular domains, and RLKs with similar extracellular domains are usually also more similar to one another regarding kinase domains, indicating that they form monophyletic subfamilies. Based on both the kinase-phylogeny of RLKs and their extracellular domains, 46 different RLK subfamilies were proposed [23], although for the far majority

of RLKs, both the recognized extracellular stimulus as well as the downstream targets of the kinase domain are still unknown. The most expanded and therefore well-known subfamily is that of the LRR-RLKs, which have a leucine rich repeat domain similar to that of the NLRs, allowing them to bind and recognize a wide variety of proteins and peptides. It was found that the cucumber genome encodes 178 to 192 LRR-RLKs as well as 42 to 56 LRR-RLPs, several of which are encoded by genes located within resistance QTL [26]. The second-most well-known RLK subfamily is that of L-type lectin RLKs (LecRKs), the extracellular domains of which resemble soluble legume lectins which are involved in oligosaccharide binding. Several LecRKs are involved in plant defence [27, 28], whereas others are involved in plant growth and development, or differentially expressed upon abiotic stresses [29]. The cucumber genome was found to encode 25 LecRKs, several of which were found to be induced by the pathogens *Phytophthora melonis* and *P. capsici* [30].

In this report, we fine-mapped a QTL for DM resistance from PI 197088, and studied a novel *RLK* as a candidate gene for this QTL.

RESULTS

Distinguishing different disease symptoms revealed three subQTL within the DM4.1 interval

In order to fine-map QTL DM4.1, a QTL isogenic introgression line was selected originating from a cross between the DM resistant cucumber accession PI 197088 and the susceptible line HS279, followed by repeated backcrossing with HS279 as the recurrent parent. Using marker assisted selection (MAS), this plant was selected because it had a (heterozygous) 12 Mb introgression on chromosome 4 corresponding to QTL DM4.1, in a homozygous, uniform HS279 background. After two generations of selfing, 27 plants with recombinations between markers at Chr4:11.479.953 and Chr4:20.438.834 were selected to develop families. Recombinant families were inoculated with *P. cubensis* in a controlled climate chamber experiment. Phenotypic data were collected on the DM inoculation response of these 27 families using three criteria: chlorosis, sporulation, and necrosis, each on a 1-9 scale with 1 being completely susceptible and 9 being completely resistant. Individuals were genotyped using nine SNP markers (Additional File 1), seven within the QTL interval and two flanking the interval. It was found that of the 27 families, eight were fixed for all nine markers, whereas the other 19 families were segregating. We conducted QTL analysis with R/qtl using the "scanone" procedure for all three phenotypes individually, correcting for the presence of sub-populations by a population co-factor (Figure 1).

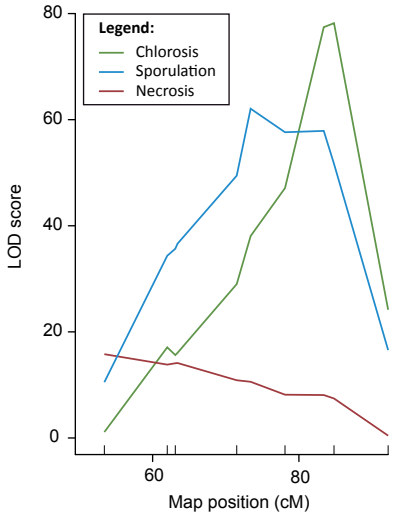

Figure 1 – QTL analysis DM resistance within the DM4.1 interval based on multiple disease phenotypes
QTL analysis was carried out in a population consisting of 27 recombinant families derived from a QTL isogenic introgression line. The mapping population was scored for chlorosis, sporulation and pathogen-induced necrosis, and genotypes with seven SNP markers in the DM4.1 interval. Two SNP markers flanking this interval were not polymorphic, as the progenitor of the mapping population was fixed for these alleles.

Although the three QTL for the different symptoms overlapped, we found that the peak positions were markedly different, indicating the potential existence of multiple causal genes, each with a different effect on the disease phenotype. Additional Data 1 gives physical locations (based on the cucumber reference genome, Chinese Long 9930 v2 [31]) of peak- and flanking markers for the QTL detected for each of the three phenotypes, which we will refer to as QTL DM4.1.1, DM4.1.2 and DM4.1.3 hereafter.

Disease tests on segregating populations confirm the presence of subQTL DM4.1.2 and DM4.1.3

As the QTL data indicated the potential existence of multiple subQTL within the greater DM4.1 locus (Figure 1, Additional Data 1), we selected two heterozygous individuals from recombinant families segregating for subQTL DM4.1.2 and DM4.1.3, respectively (Figure 2A). Both individuals were selfed in order to develop segregating residual heterozygous lines (RHLs). Additionally, one individual homozygous for the full DM4.1 introgression was selfed in order to develop a near isogenic line (NIL DM4.1). Each of the two RHLs was inoculated with *P. cubensis* in a controlled climate chamber experiment, using resistant donor PI 197088, partial resistant NIL DM4.1, and susceptible recurrent parent HS279 as controls. Phenotypic data were collected seven (chlorosis) and twelve (sporulation and necrosis) days post inoculation on a 1-9 scale (Figure 2B-E). Individuals were genotyped using SNP markers.

Significances of differences in disease phenotypes for both of the populations were determined using Kruskal-Wallis tests ($p<0.05$). Stepdown post hoc analysis revealed that in the RHL segregating for QTL DM4.1.2, significant differences ($p<0.05$) were

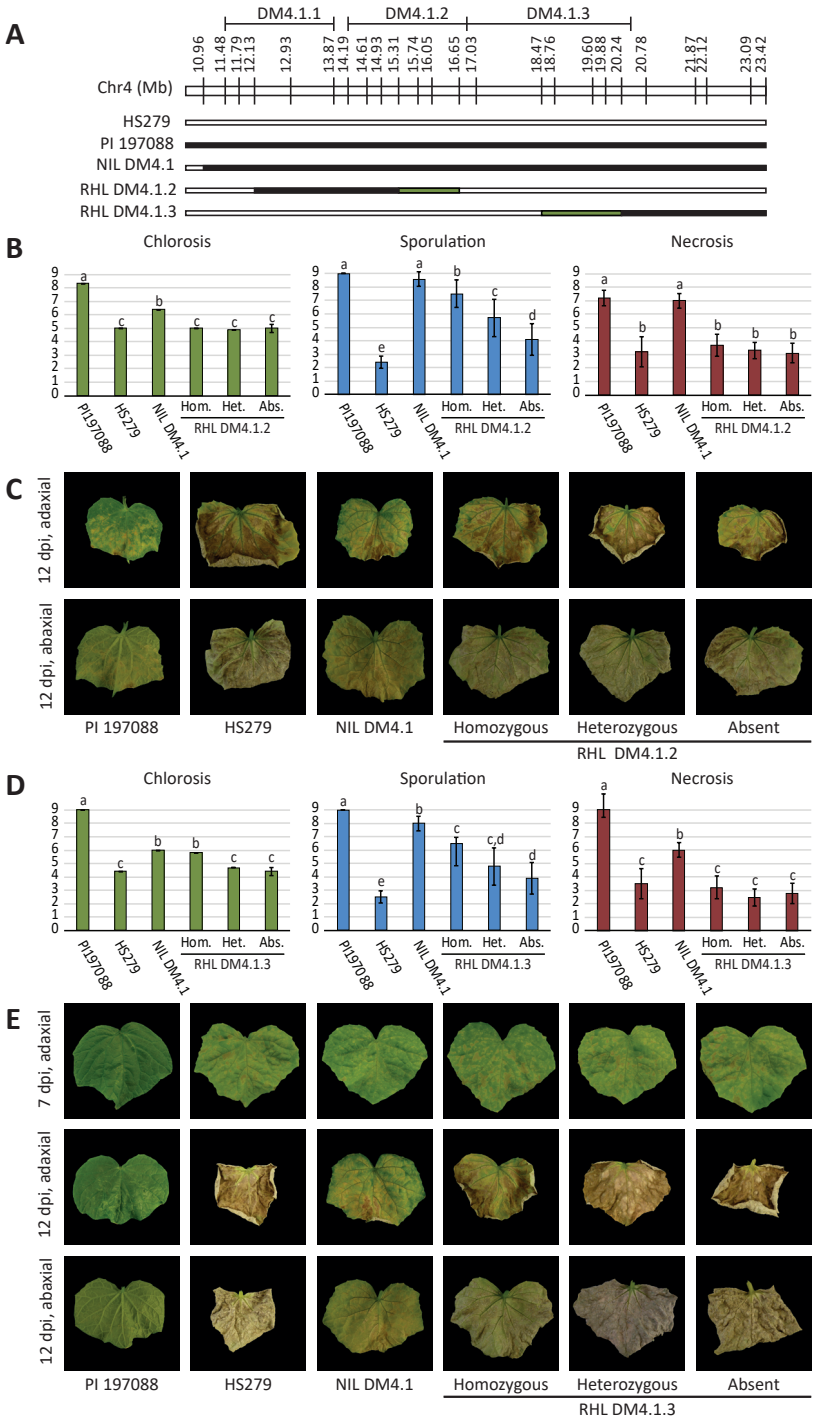

Figure 2 – *P. cubensis* disease test on RHLs segregating for subQTL DM4.1.2 and DM4.1.3

Figure 2 – *P. cubensis* disease test on RHLs segregating for subQTL DM4.1.2 and DM4.1.3
A) Residual heterozygous lines (RHLs) were developed from individuals in the mapping population heterozygous for partial introgressions individuals homozygous for the full introgression. Additionally, a homozygous individual was selected to develop a near isogenic line (NIL DM4.1). Bars represent the allele of genotypes at marker locations on the DM4.1 interval. Black bars indicate the PI 197088 allele, white bars indicate the HS279 allele, green bars represent heterozygosity in the individuals and thus segregation in the RHLs.
B-E) RHLs segregating for subQTL DM4.1.2 **(B-C)** or DM4.1.3 **(D-E)** were inoculated with *P. cubensis* and subsequently scored for chlorosis (7 dpi), sporulation (12 dpi) and necrosis (12 dpi). Eight individuals per genotype (homozygous, heterozygous or azygous) were scored. Bars represent average phenotype scores on a 1-9 scale ranging from susceptible to resistant. Error bars represent standard deviation. Bars with different letters are statistically significant from one another (Kruskal-Wallis, $p<0.05$). Representative photographs per genotype are shown.

found regarding sporulation (Figure 2B): plants homozygous for QTL DM4.1.2 showed less sporulation than heterozygous plants, which in turn sporulated less than plants homozygous for absence of the QTL. Homozygous plants for QTL DM4.1.2 sporulated significantly more than NIL DM4.1, indicating that the DM4.1.2 introgression does not completely explain the loss-of-sporulation due to locus DM4.1. Plants homozygous for absence of QTL DM4.1.2 still sporulated significantly less than the susceptible control HS279, potentially indicating a background effect of the part of the DM4.1 introgression which was fixed in this population (Figure 2A). No significant differences were found in the population segregating for QTL DM4.1.2 regarding either chlorosis or necrosis.

In the RHL segregating for QTL DM4.1.3, significant differences ($p<0.05$) were found regarding chlorosis (Figure 2D): plants homozygous for QTL DM4.1.3 showed significantly less chlorosis than either heterozygous plants or plants homozygous for absence of DM4.1.3, whereas there were no significant differences in chlorosis between heterozygous plants and plants homozygous for absence of DM4.1.3, indicating a recessive effect of subQTL DM4.1.3 regarding chlorosis. Homozygous plants were not significantly more chlorotic compared to NIL DM4.1, indicating that subQTL DM4.1.3 fully explains the loss-of-chlorosis effect of QTL DM4.1. There was a significant difference between on the one hand homozygous individuals and NIL DM4.1 and on the other hand resistant control PI 197088, confirming that other QTL of PI 197088 also contribute to loss-of-chlorosis. Plants homozygous for subQTL DM4.1.3 sporulated significantly less than plants homozygous of absence of DM4.1.3, indicating that subQTL DM4.1.3 also partially contributes to loss-of-sporulation, additional to its effect on chlorosis. Heterozygous individuals had scores in between those of plants homozygous for presence and plants homozygous for absence of QTL DM4.1.3, but were not significantly different to either of them. Individuals in the RHL homozygous for absence of DM4.1.3 showed less sporulation than susceptible control HS279, suggesting a background effect of the part of the DM4.1 introgression which was fixed in this population (Figure 2A).

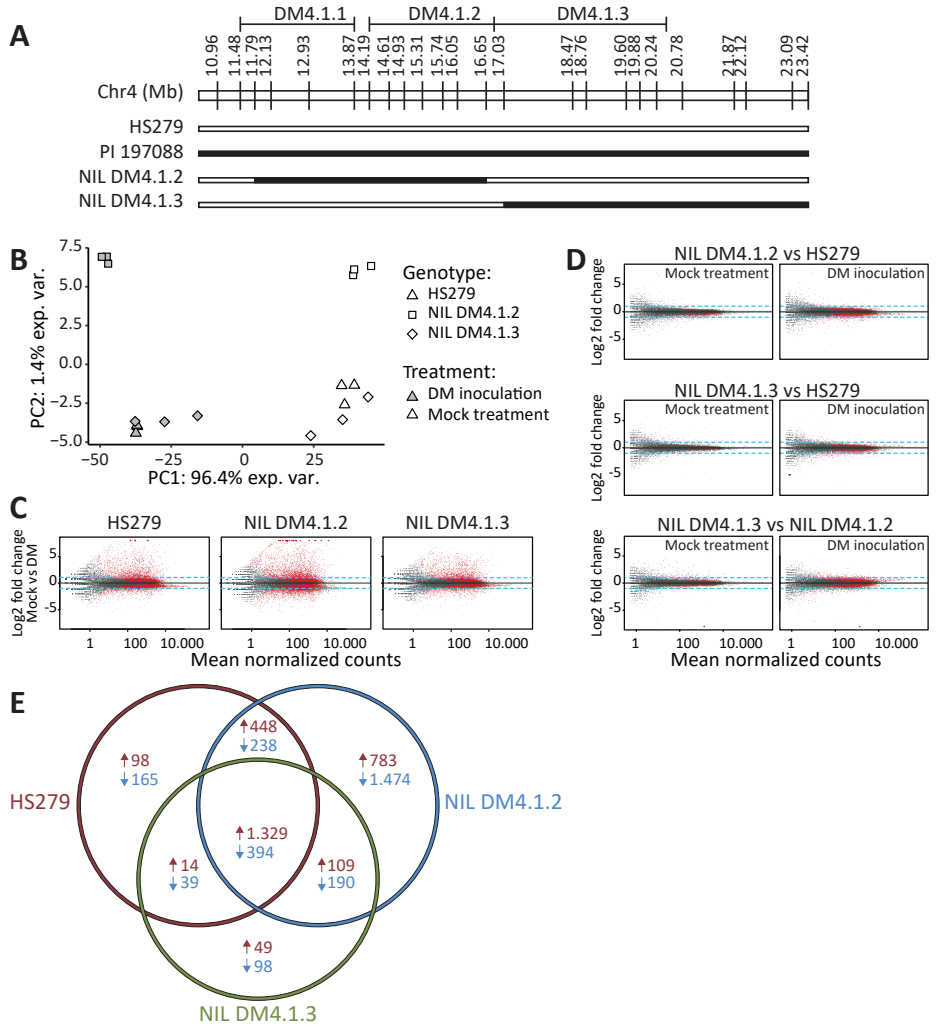

Figure 3 – Transcriptome analysis of NILs with either subQTL DM4.1.2 or DM4.1.3

Analysis of transcriptome data from leaves of three cucumber genotypes (HS279, susceptible, and NILs DM4.1.2 and DM4.1.3, both partially resistant) three days post *P. cubensis* inoculation or mock control, with three independent samples per genotype x treatment combination.

A) Individuals homozygous for partial introgressions corresponding to subQTL DM4.1.1 and DM4.1.2 (NIL DM4.1.2) or DM4.1.3 (NIL DM4.1.3) were selected to develop near-isogenic lines (NILs). Bars represent the allele of genotypes at marker locations on the DM4.1 interval. Black bars indicate the PI 197088 allele, white bars indicate the HS279 allele.

B) Principle component analysis of transcriptome data.

C) MA plots for pairwise differential expression analysis contrasts between mock-treated and *P. cubensis* inoculated samples. Each point represents a detected gene. The X-axis represents the mean normalized counts per gene under both conditions, whereas the Y-axis represents the Log_2 fold change in *P. cubensis* inoculated samples compared to mock-treated samples. Differentially expressed genes (adjusted $p < 0.05$) are represented in red. Blue lines represent a 2-fold change threshold.

Figure 3 – Transcriptome analysis of NILs with either subQTL DM4.1.2 or DM4.1.3
D) MA plots for pairwise differential expression analysis contrasts between genotypes under mock-treated (left column) or P. cubensis inoculated (right column) conditions.
E) Venn-diagram representing differentially expressed upregulated (in red) and downregulated (in blue) genes in *P. cubensis* inoculated samples compared to mock-treated samples. Differentially expressed genes are here defined as statistically significant (adjusted p <0.05) and >2-fold up- or downregulated.

Neither subQTL DM4.1.2 nor subQTL DM4.1.3 were found to have an effect on necrosis, as plants in both RHLs were not significantly different from susceptible control HS279, regardless of whether they were homozygous for presence of the subQTL, heterozygous, or homozygous for absence of the subQTL (Figure 2B-E). An attempt was made to develop a RHL segregating for subQTL DM4.1.1, but no effect of presence of the introgression was found (Additional Data 2), which might either be explained by the fact that the segregating introgression in this family did not completely cover the previously detected DM4.1.1 interval (Additional Data 2A), or the effect of this subQTL is only expressed in presence of the other subQTL, potentially due to interaction of the underlying causal genes.

Transcriptomics indicates that subQTL DM4.1.2 is associated with increased differential gene expression upon *P. cubensis* inoculation, in contrast to subQTL DM4.1.3

Two homozygous individuals were selected for selfing to develop near isogenic lines (NILs), one (NIL DM4.1.2) with an introgression corresponding to both subQTL DM4.1.1 and DM4.1.2, the other (NIL DM4.1.3) with an introgression corresponding to subQTL DM4.1.3 (Figure 3A). RNA was isolated from leaves of both NILs as well as from susceptible parent HS279, three days post inoculation with *P. cubensis* or a mock treatment, with three biological replicates. RNAseq yielded ca. 50M clean, trimmed 100 bp paired-end reads per sample, of which ca. 90% mapped to the cucumber reference genome (Chinese Long 9930 v2 [31]).

Principle component analysis (PCA) of the RNAseq data revealed that the treatment (*P. cubensis* inoculation versus mock treatment) accounted for 96.4% of the observed variance in gene expression (Figure 3B). A genotype effect accounted for 1.4% of the observed variance, which separated NIL DM4.1.2 from the other two genotypes. Biological replicates within each of the six genotype-treatment combinations clustered together in the PCA plot, although there was some variation between biological replicates of genotype NIL DM4.1.3 under both treatments (Figure 3B). Differential gene expression was determined based on an adjusted p-value <0.05 and a fold-change >2. Pairwise contrasts were established between treatments (Figure 3C), as well as between geno-

types (Figure 3D). Consistent with greater PCA separation based on treatment, many more genes were differentially expressed in treatment comparisons (Figure 3C) than in genotype comparisons (Figure 3D). Additionally, more genes were differentially expressed between genotypes after *P. cubensis* inoculation (Figure 3D, right column) than after mock-treatments (Figure 3D, left column).

Cross-listing of differentially expressed genes due to the treatment effect in the three genotypes revealed that many more genes were uniquely up- and downregulated in NIL DM4.1.2 compared to both NIL DM4.1.3 and the susceptible control HS279 (Figure 3E).

Genes upregulated by subQTL DM4.1.2 include defence pathway genes

To test whether subQTL DM4.1.2 and DM4.1.3 differentially influence known defence pathways, expression patterns of cucumber homologs of known defence pathway genes were studied in more detail. Cucumber homologs of SA-inducible genes *PR1*, *PR2*, *EDS1*, *PAD4* and *NPR1* as well as JA-inducible genes *PR3*, *PGIP2* and *RST1* were identified by BLAST searches of the published *Arabidopsis thaliana* protein sequences against the translated cucumber reference genome (Chinese Long 9930 v2).

All examined defence pathway genes were significantly upregulated in *P. cubensis* inoculated plants compared to mock-treated plants for all three genotypes (with the exception of *CsPR3A*) (Figure 4, Additional Data 3). Generally, there were no significant differences in defence pathway gene expression between the genotypes in mock-treated plants (with the exception of *CsPR2*).

However, in *P. cubensis* inoculated plants, expression of defence pathway genes was generally higher in NIL DM4.1.2 compared to the other genotypes: Five out of the thirteen studied defence genes were significantly higher expressed in NIL DM4.1.2 compared to HS279, and eight out of the thirteen genes were significantly higher expressed in NIL DM4.1.2 compared to NIL DM4.1.3 (Figure 4, Additional Data 3), indicating that subQTL DM4.1.2 is likely to harbour a causal gene triggering defence responses. In contrast, there were no significant differences in expression between NIL DM4.1.3 and HS279 for any of the thirteen defence pathway genes, indicating that the causal gene underlying subQTL DM4.1.3 does not involve increased upregulation of defence pathways.

Fine-mapping and identification of candidate genes for subQTL DM4.1.2 for reduced sporulation

To narrow down the subQTL DM4.1.2 interval, additional plants were selected having recombinations within the interval, and lacking subQTL DM4.1.3 (Figure 5A). These recombinants were selfed in order to create populations, which were tested for DM re-

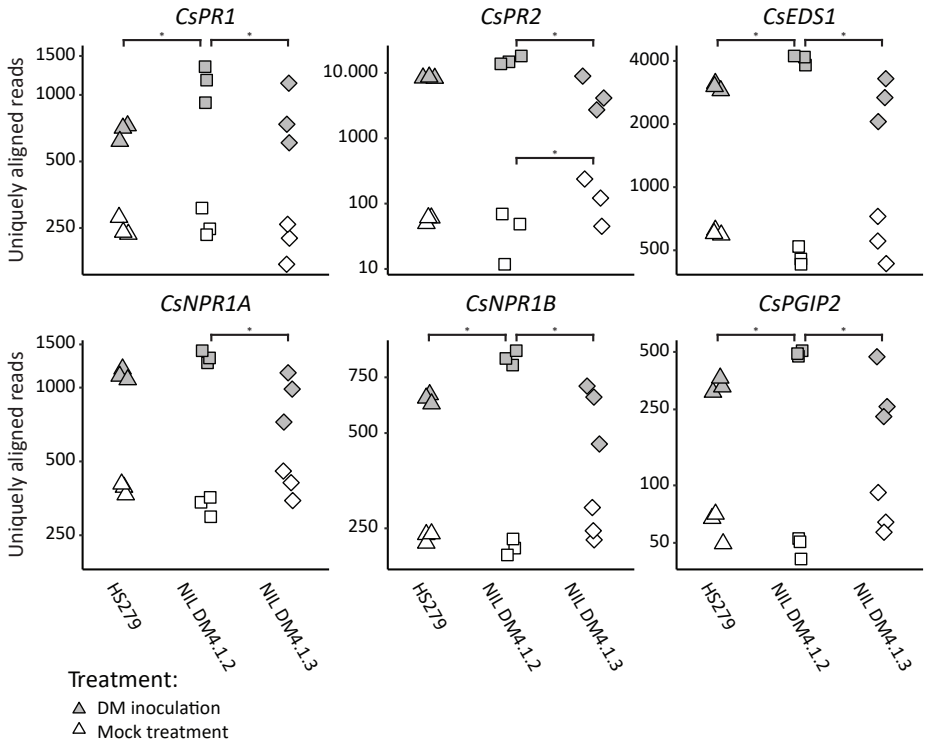

Figure 4 – Expression analysis of defence pathway genes in the susceptible genotype HS279, a NIL with the sporulation reducing subQTL 4.1.2, and a NIL with the cholorosis reducing subQTL 4.1.3.

Expression data of cucumber homologs of known defence pathway genes were extracted from the RNAseq dataset, and plotted per sample on a logarithmic scale. Asterisks represent statistically significant differences between genotypes (adjusted $p < 0.05$). For all genes, inoculation induced significant upregulation in all genotypes ($p < 0.05$). SubQTL 4.1.2 led to significantly higher expression of several defence pathway genes compared to subQTL 4.1.3, or the susceptible genotype HS279.

sistance. Four recombinant populations were used to fine-map subQTL DM4.1.2, as two families segregated for the decrease in sporulation, previously associated with subQTL DM4.1.2, whereas one family was fixed for the susceptible allele and one family was fixed for the resistant allele. Analysis of these four recombinant populations allowed fine-mapping of subQTL DM4.1.2 to the interval Chr4:15.309.857-15.738.683 (Figure 5A), containing 40 predicted genes in the cucumber reference genome (Chinese Long 9930 v2).

In order to identify potential candidate genes for subQTL DM4.1.2, we investigated the expression of the 40 genes within the fine-mapped interval in our RNAseq dataset (Additional Data 4). No genes were differentially expressed between the two genotypes

in mock-treated samples, whereas only one gene (Csa4M410850) was differentially expressed between genotypes in *P. cubensis* inoculated samples. We found that Csa4M410850 was not detected at all in mock-treated HS279 plants, and at a very low level in mock-treated NIL DM4.1.2 plants. However, the gene was upregulated in both genotypes after *P. cubensis* inoculation, although to a ca. tenfold higher level in NIL DM4.1.2 compared to HS279 (Figure 5B). As Csa4M410850 is annotated as a *Receptor-like Kinase* (*RLK*) gene, which are frequently involved in pathogen detection, we selected it as an interesting candidate gene for subQTL DM4.1.2.

Additionally, we identified four non-synonymous polymorphisms (SNPs) between HS279 and NIL DM4.1.2 in predicted genes within the fine-mapped interval Chr4:15.309.857-15.738.683 (Additional Data 5). However, based on the annotation of Csa4M416480 and Csa4M416990, we do not consider these two genes as candidates.

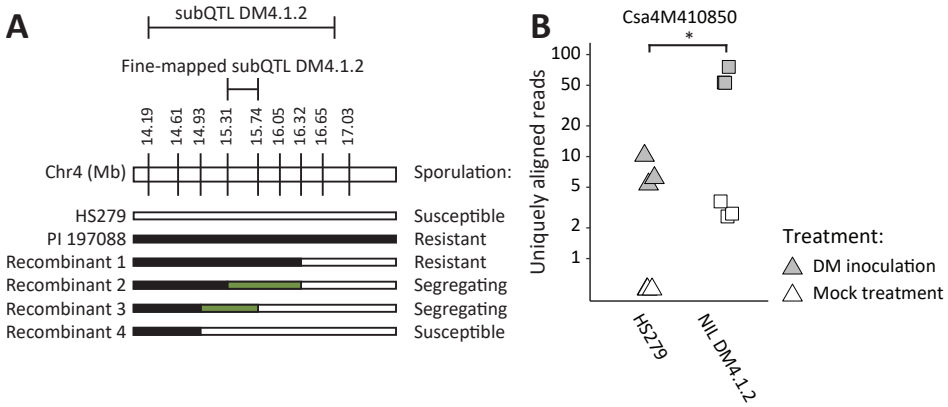

Figure 5 – Fine-mapping and candidate gene expression subQTL DM4.1.2
A) Screening of individuals derived from the mapping population allowed identification of additional informative recombinants within the DM4.1.2 interval. Bars represent genotypes at marker locations. Black bars indicate the PI 197088 allele, white bars indicate the HS279 allele, green bars represent heterozygosity. Individuals were self-fertilized to develop populations, which were phenotyped regarding sporulation.
B) Expression data of gene Csa4M410850, encoding an RLK gene within the fine-mapped interval of DM4.1.2 interval, was extracted from the RNAseq dataset and plotted per sample on a logarithmic scale. No expression of this gene was detected in any of the mock-treated samples of the susceptible genotype HS279, but for visualization purposes a 0.5 pseudocount was added. The asterisk denotes a significant difference between both genotypes in *P. cubensis* inoculated samples (adjusted $p < 0.05$). Differences between mock-treated and *P. cubensis* inoculated samples were also statistically significant (adjusted $p < 0.05$).

Genomic analysis of *RLK* locus QTL DM4.1.2 indicates a structural variation

As we selected two *RLK* genes (Csa4M410830 and Csa4M410850) as the most likely candidates for subQTL DM4.1.2, we decided to study the genomic context of these genes. To this end, we visually inspected the alignment of sequencing reads in the RLK locus (Ch4:15.413.000-15.435.000).

The cluster of predicted RLK genes in the DM4.1.2 interval consists of three genes: Csa4M410830, Csa4M410840 and Csa4M410850 (Figure 6A). Visual inspection of aligned RNAseq reads revealed that reads aligning to the Csa4M410850 locus actually form a longer transcript than predicted, consisting of three exons, one of which was predicted to be a separate gene (Csa4M410860) without any annotation (Additional Data 6A). The first *RLK* gene in the cluster (Csa4M410830) was abundantly transcribed, whereas there were no indications of transcription of Csa4M410840 in our RNAseq data (Additional Data 6A).

To clarify the RLK locus structure we performed whole genome sequencing (WGS) of NIL DM4.1. Sequencing reads were aligned to the cucumber reference genome (Chinese Long 9930 v2), reads aligning to the RLK locus were extracted. Visual inspection of WGS reads aligning to the *RLK* locus indicated a structural variation in Csa4M410860, characterized by a local, drastic decrease in coverage at the interval Chr4:15.434.200-15.434.350 and deviating insert sizes of mate pairs flanking this interval (Figure 6B, Additional Data 6B).

To validate the suspected structural variation in Csa4M410860, primers were developed flanking the locus. PCR on DNA isolated from susceptible recurrent parent HS279 amplified the ca. 250 bp product identical to the reference genome, whereas the PCR product from NIL DM4.1.2 was longer, ca. 700 bp (Figure 6C). Sanger sequencing of the amplicon revealed the presence of a 551 bp insertion in NIL DM4.1.2 compared to HS279 and the reference genome. Realignment of NIL DM4.1.2 RNAseq reads to the *RLK* cluster including the newly discovered 551 bp indel demonstrated the presence of an apparently intact 1.905 bp long gene, comprising Csa4M410850 and Csa4M410860 including the newly identified 551 bp indel (Figure 6D). The sequence of this correctly annotated novel gene was deposited to NCBI GenBank [MK936607].

Characterizing the *RLK* genes in the DM4.1.2 locus reveals that *CsLRK10L2* can trigger necrosis

To verify the expression of the newly identified *RLK* gene and the sequence of the mRNA, we sequenced the cDNA from the newly assembled RLK gene and from the other expressed *RLK* gene in the cluster (Csa4M410830). Sanger sequencing of both alleles of both *RLK* genes confirmed the expected sequences based on the assembled genes.

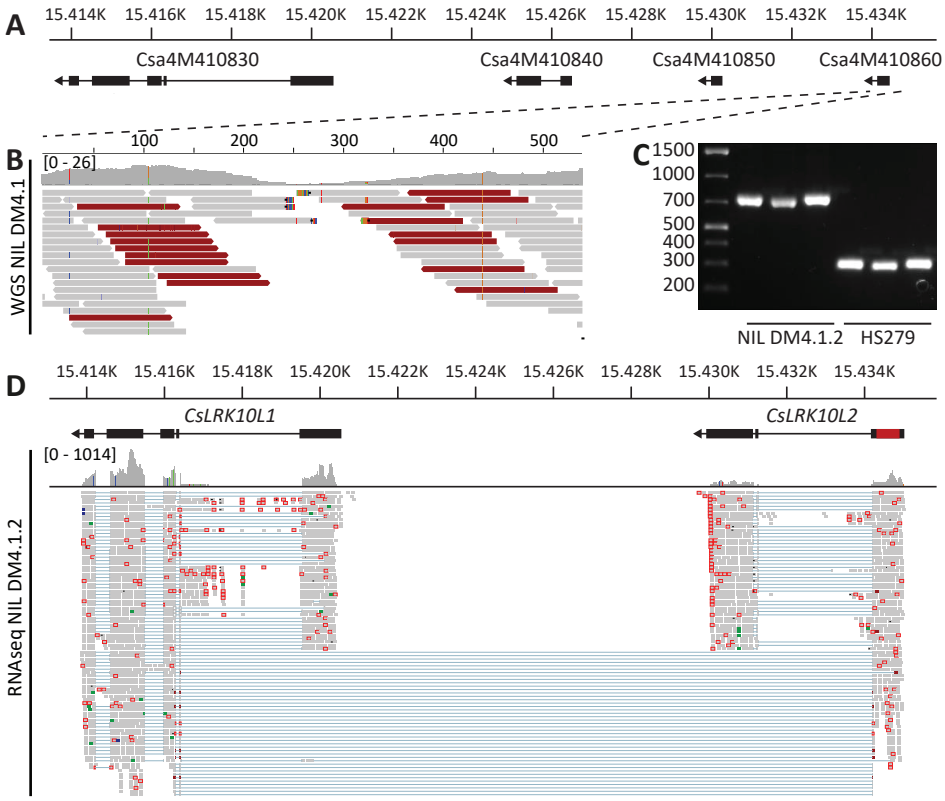

Figure 6 – Genomic analysis of the *RLK* cluster DM4.1.2, indicating structural variation

A) Predicted gene models of genes in the *RLK* cluster within the interval of subQTL DM4.1.2. Black boxes indicate predicted exons, lines represent predicted introns. Arrowheads indicate the orientation of the reading frame. Physical locations on chromosome 4 of the cucumber reference genome (Chinese Long 9930 v2) are indicated.

B) Whole genome sequencing reads of NIL DM4.1 aligning to predicted gene Csa4M410860 are visualized using the Integrative Genomics Viewer (IGV). A coverage graph is given above the aligned reads. Reads pairs with larger than expected or smaller than expected insert sizes are indicated in dark red and dark blue, respectively.

C) PCR was performed using primers designed to amplify the predicted gene Csa4M410860 on DNA samples isolated from the partially resistant NIL DM4.1.2 and the susceptible recurrent parent HS279.

D) RNAseq reads from *P. cubensis* inoculated NIL DM4.1.2 were re-aligned to the RLK cluster including the 551 bp insertion found in the predicted gene Csa4M410860. Gene models of *CsLRK10L1* (Csa4M410830) and *CsLRK10L2* (novel gene consisting of both Csa4M410850 and Csa4M410860) are indicated above, including the 551 bp insertion in red. Numbers indicate physical location on chromosome 4 of the cucumber reference genome (Chinese Long 9930 v2).

In order to functionally characterize the *RLK* genes, both alleles amplified from HS279 as well as NIL DM4.1.3 were cloned, and transiently overexpressed in leaves of *Nicotiana benthamiana*. Three days post infiltration, a necrotic reaction was observed in the leaf area infiltrated with the NIL DM4.1.3 allele of the novel *RLK* gene, but not in the parts of the leaves infiltrated with the HS279 allele of the novel *RLK*, nor with either allele of Csa4M410830 (Figure 7A). Leaves were stained with trypan blue to visualize the necrotic tissue (Figure 7B).

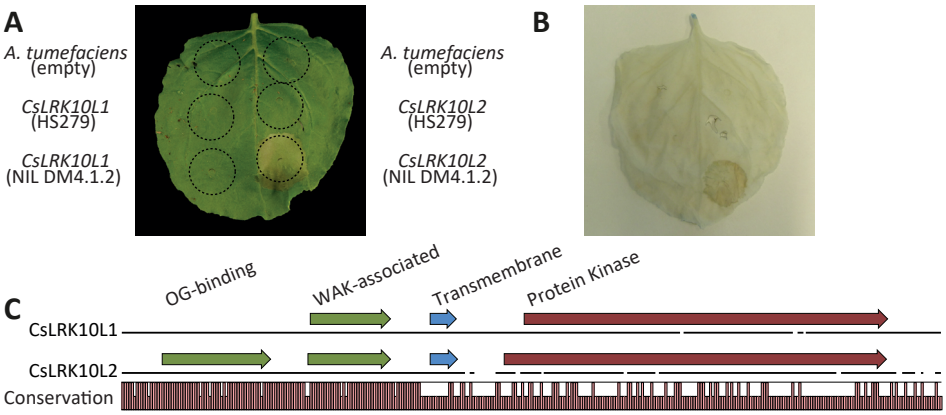

Figure 7 – Functional characterization of *CsLRK10L* genes
A) Alleles of *CsLRK10L1* and *CsLRK10L2* cloned from cucumber genotypes HS279 and NIL DM4.1.2 were transiently expressed in *Nicotiana benthamiana* leaves by agroinfiltration. Empty *A. tumefaciens* cultures were used as a negative control. *CsLRK10L2* cloned from NIL DM4.1.2 consistently triggered a necrotic reaction in >20 individual plants.
B) Leaves of *N. benthamiana* infiltrated with *CsLRK10L* overexpression constructs as described in **(A)** were stained using Trypan blue to visualize dead cells.
C) Multiple protein sequence alignment of predicted proteins encoded by *CsLRK10L1* and *CsLRK10L2*. A graph indicates conservation per amino acid. Pfam domains as identified by InterProScan v5.27 are indicated by arrows.

Phylogenetic analysis of *RLK* genes based on multiple domains

We found that the N-terminal parts of the predicted proteins of both *RLK* genes were conserved (>90% identical), whereas the C-terminal parts were less conserved (<30% identical) (Figure 7C). InterPro domain annotation of the predicted protein sequences indicated that the conserved, N-terminal parts of both proteins contain predicted WAK-associated domains (IPR032872), characteristic for *Wall-Associated RLK* (*WAKL*) genes (Figure 7C). The novel *RLK* gene also contained a predicted oligogalacturonan-binding domain (IPR025287), which is also commonly found in *WAKL* genes. The non-conserved C-terminal part of the proteins both contained predicted transmembrane helices and protein kinase domains (IPR000719), but whereas the predicted active site of the pro-

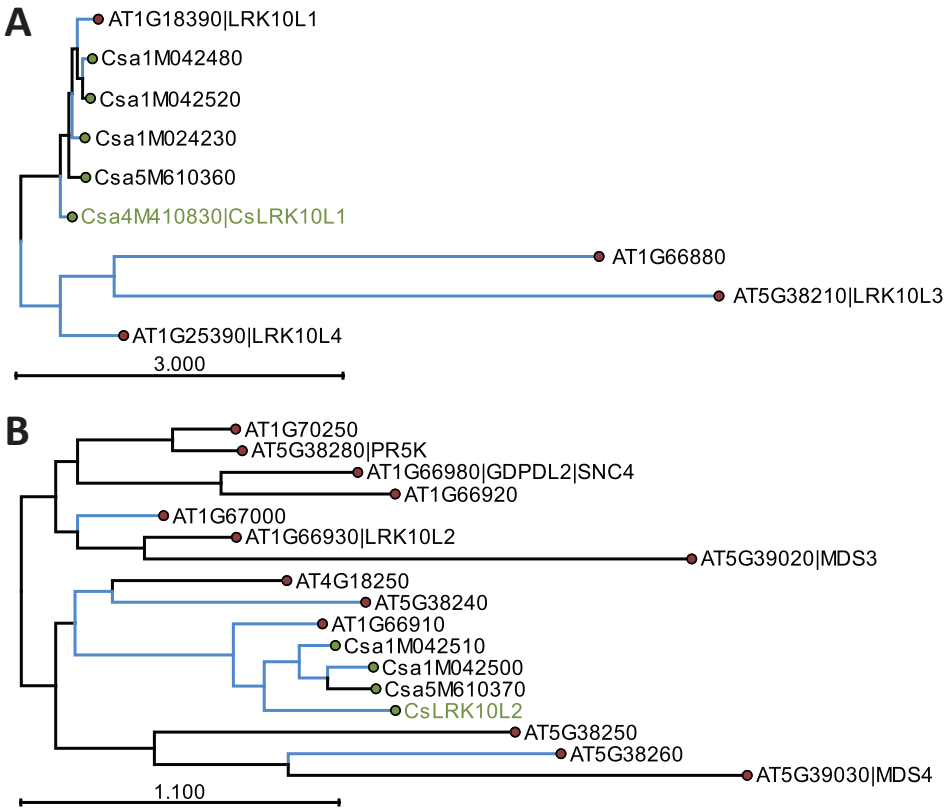

Figure 8 – Phylogenetic analysis CsLRK10L genes

tein encoded by *RLK* gene Csa4M410830 contained the conserved arginine-aspartate (RD)-motif, this motif was lost in the protein encoded by the novel *RLK* gene.

To find more evidence for the function of the identified RLK gene we compared the gene family in cucumber with the better functionally annotated *RLK* gene family in *Arabidopsis thaliana*. We performed BLASTp queries against both translated genomes of cucumber (Chinese Long 9930 v2) and *Arabidopsis* (TAIR 10), using the oligogalacturonan-binding, WAK-associated and Protein Kinase domains of both genes. As was expected, BLAST results using the extracellular domains of both proteins identified the same set of homologs. There was however no overlap between the BLAST output regarding the protein kinase domains of both RLKs (Additional Data 7). Kinase domain homologs of the protein encoded by *RLK* Csa4M410830 belong to RLK subfamily LRK10-like 1, according to the nomenclature proposed by Shiu and Bleecker (2003), whereas many of the kinase domain homologs of the protein encoded by the novel *RLK* gene belong to subfamily

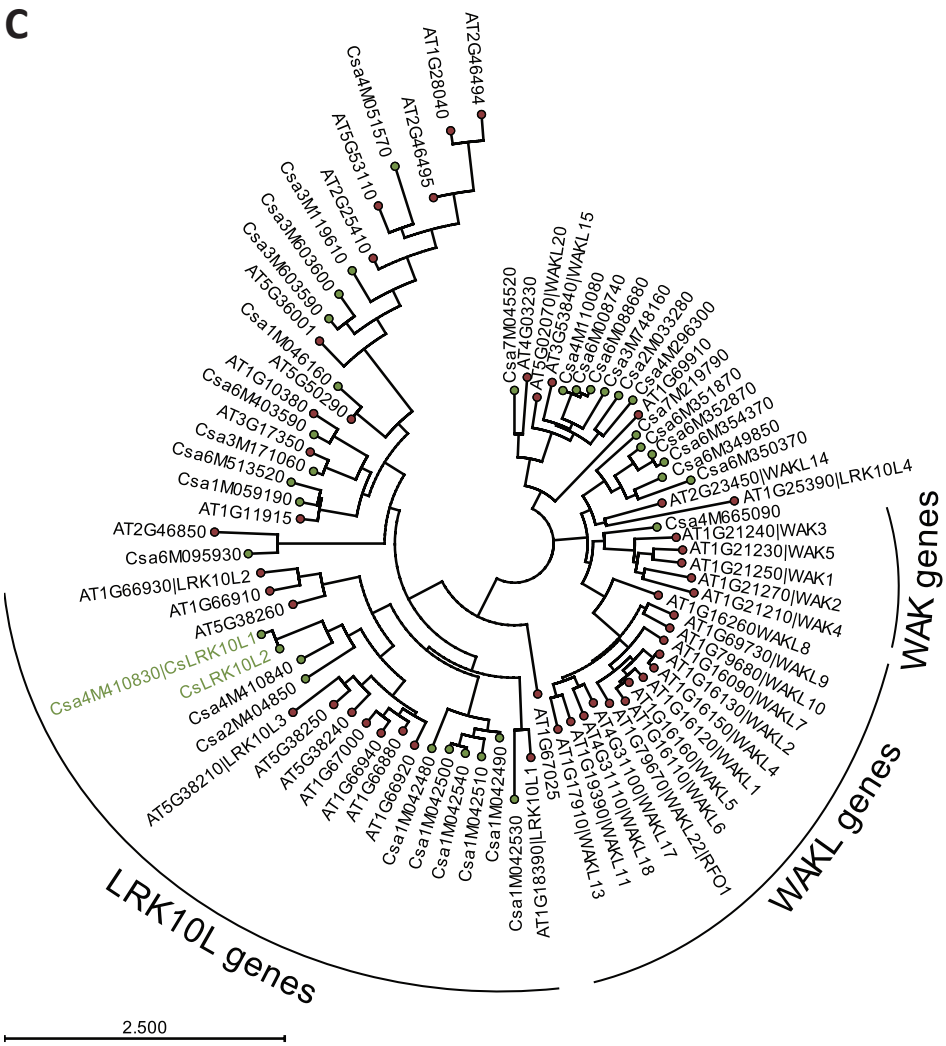

Figure 8 – Phylogenetic analysis CsLRK10L genes

Homologs of the kinase domains of *CsLRK10L1* **(A)** and *CsLRK10L2* **(B)** were identified using BLASTp against cucumber and *Arabidopsis* translated reference genomes, and used to construct phylogenetic (maximum likelihood) trees. Cucumber and *Arabidopsis* homologs are indicated with green and red circles, respectively. Branches leading to homologs with predicted oligogalacturonan (OG) binding and/or WAK-associated domains are coloured blue.

C) A phylogenetic (maximum likelihood) tree was constructed based on predicted OG-binding domains of *Arabidopsis* and cucumber proteins. Clades containing the majority of previously annotated WAK, WAKL and LRK10L proteins are indicated. CsLRK10L1 and CsLRK10L2 are indicated in green.

LRK10-like 2. The majority of the homologs of both genes regarding the extracellular domains also belonged to the LRK10-like 1 and 2 subfamilies. Therefore, we will further refer to these genes as *CsLRK10L1* (Csa4M410830) and *CsLRK10L2* (the novel *RLK* gene corresponding with Csa4M410850 and Csa4M410860).

In order to verify whether identified homologs in cucumber and *Arabidopsis* had predicted oligogalacturonan-binding and/or WAK-associated domains, we scanned the complete predicted proteomes of cucumber (Chinese Long 9930 v2) and *Arabidopsis* (TAIR 10) using InterProScan v5.27 for presence of oligogalacturonan-binding (IPR025287) and/or WAK-associated (IPR032872) domains. We identified 35 cucumber and 42 Arabidopsis proteins with such domains (Additional Data 8). Phylogenetic (Maximum likelihood, ML) trees were constructed based on the kinase domains of BLAST-identified homologs of both CsLRK10L1 (Figure 8A) and CsLRK10L2 (Figure 8B), as well as on the extracellular domains of all identified proteins with OG-binding and/or WAK-associated domains (Figure 8C).

As the gene identifiers of predicted WAK-domain genes indicated that many of them were closely together on the cucumber and *Arabidopsis* genomes (i.e. consecutive gene IDs), we mapped the location of each of the WAK-domain genes on their respective genomes (Additional Data 9) and found that indeed the majority of genes with predicted WAK domains were part of clusters in the genome.

DISCUSSION

QTL DM4.1 consists of multiple subQTL contributing to DM resistance

Previously, several groups have mapped DM resistance inherited from accession PI 197088. Whereas the previous publications describing a single QTL on chromosome 4 all mapped QTL for DM resistance in populations also segregating for other loci in the genome, we developed populations segregating for loci in the DM4.1 interval only, in a homozygous, susceptible, background, aiming at greater resolution. Furthermore, we distinguished different symptoms (i.e. chlorosis, sporulation and necrosis) whereas other groups used "overall" DM disease indices. The combination of these two factors enabled us to discover three separate subQTL in the DM4.1 locus.

Recently another group also scored their segregating populations using multiple disease phenotype criteria, i.e. yellowing (chlorosis), collapsing (necrosis) and "general impression" [32]. These populations were derived from other sources of resistance, i.e. cucumber genotypes Gy14 and WI 2757. They found that whereas a delay in chlorosis

was highly correlated with the score based on general impression, there was no strong correlation between scores for yellowing and collapsing, indicating that anti-chlorosis and anti-necrosis in these populations had a different genetic basis. Both this study and our results demonstrate that it can be wise to score multiple aspects of disease progression separately, as this might give a more complete picture of disease resistance, potentially enabling the identification of QTL which might be overlooked by a simpler scoring.

It is rather unusual to find multiple QTL for disease resistance in close genetic proximity of one another, probably because closely linked QTL will easily be overlooked, as they might appear as one QTL, similarly to how cucumber DM4.1 was initially considered to be a single QTL. This scenario is reminiscent of the findings of den Boer et al. [33], who found that several QTL for DM resistance in lettuce all fell apart in multiple subQTL.

SubQTL DM4.1.2 upregulates defence pathway genes upon inoculation, in contrast to subQTL DM4.1.3

Recently, Burkhardt and Day [34] investigated transcriptomic trends of resistant cucumber accession PI 197088 and a susceptible control (cv. Vlaspik) in a time course after inoculation with *P. cubensis*. They found that thousands of genes were differentially expressed between mock-treated and *P. cubensis* inoculated plants, and that this response was stronger and faster in PI 197088 compared to the susceptible control. As resistance in PI 197088 is highly polygenic [14–16], we were interested to find out which transcriptomic changes could be attributed to the QTL we are studying.

Our RNAseq results on NILs with either subQTL DM4.1.2 or DM4.1.3, and the susceptible parent HS279, indicated that in agreement with the previous findings [34], *P. cubensis* inoculation drastically alters gene expression (Figure 3B,C). In contrast, gene expression differences between the three studied genotypes was rather subtle (Figure 3B,D), as was to be expected based on the similar genetic background of these genotypes. However, by comparing the differentially expressed genes due to *P. cubensis* inoculation in the three genotypes, we found that 56.7% of all downregulated genes and 27.7% of all upregulated genes were uniquely upregulated in NIL DM4.1.2, whereas only low amounts of genes were uniquely up- or downregulated in HS279 and NIL DM4.1.3 (Figure 3E). This indicated that the partial resistance conferred by subQTL DM4.1.2 is associated with rather large differences in gene expression, in contrast to partial resistance conferred by subQTL DM4.1.3. More detailed analysis of cucumber homologs of known SA- as well as JA-induced defence pathway genes indicated that several of these were stronger upregulated in NIL DM4.1.2 compared to the other two genotypes after *P. cubensis* inoculation (Figure 4), implying that DM4.1.2-associated resistance is linked to

known defence pathways, whereas DM4.1.3-associated resistance apparently depends on other mechanisms.

The *RLK*-gene *CsLRK10L2* is likely the causal gene for subQTL DM4.1.2

By combining fine-mapping (Figure 5A) with differential gene expression analysis (Additional Data 4, Figure 5B), we selected the *CsLRK10L2* gene (Csa4M410850) as the most likely candidate gene for subQTL DM4.1.2. This fitted our previous observation that resistance conferred by this subQTL is associated with increased expression of defence pathway genes (Figure 4), as *RLK* genes are commonly known to be frequently involved in pathogen signalling [24].

Interestingly, the 551 bp insertion that was found in the DM4.1.2 allele of *CsLRK10L2* led to a predicted gene of normal size. It is likely that the DM4.1.2 allele represents the functional allele of the *RLK* gene, whereas the deletion-allele of HS279, which is also present in the cucumber reference genome Chinese Long 9930, represents a loss-of-function mutation. The question is then why domesticated cucumber genotypes such as HS279 and Chinese Long 9930 have lost this gene, as this would decrease their resistance to *P. cubensis*. A further study on the working mechanism of the *CsLRK10L2* gene might shed more light on answering this question.

Based on the presence of WAK-like and oligogalacturonan-binding domains in the extracellular domains of *CsLRK10L1* and *CsLRK10L2* (Figure 7C), we speculate that these genes might be involved in signalling of loss of cell wall integrity, similar to *WAKL* genes in which such domains are usually found [35–37]. Many *WAKL* genes have been found to contribute to quantitative resistance against various fungal and bacterial pathogens in multiple plant species [38–44]. Our working hypothesis is that *CsLRK10L2* contributes to quantitative disease resistance against *P. cubensis* through OG-perception. This fits with the observation that the gene is able to trigger necrosis in *N. benthamiana* leaves without supplying an external ligand, as OG is normally present in small concentrations due to plant-encoded polygalacturonases.

Other putative candidate genes for subQTL DM4.1.2

We selected *CsLRK10L2* as the most likely candidate gene for subQTL DM4.1.2. However, there were other potential candidate genes as well. Our results showed that there were non-synonymous SNPs in three genes within the DM4.1.2 interval (Additional Data 5).

One of those genes was *CsLRK10L1* (Csa4M410830), located in the same cluster as *CsLRK10L2*. Contrary to our findings regarding CsLRK10L2, overexpression of neither the susceptible nor the resistant allele of *CsLRK10L1* had any effect in *N. benthami-*

ana. However we cannot rule out the possibility that this gene could have an effect on DM resistance in cucumber. The second gene in the interval with a non-synonymous SNP was Csa4M416480. This gene has high homology (82% identical amino acid sequences) to the *Arabidopsis CBR1* gene, which is involved in fatty acid desaturation in developing seeds and male gametophytes. *cbr1* loss of function mutants were found to have defects in male fertility, seed setting and seed germination whereas vegetative growth was unaffected [45], but to our knowledge no effects of *CBR1* or related genes on pathogen resistance are known. Additionally, the substitution in the encoded protein in the NIL DM4.1.2 allele of this gene was conservative, as valine and isoleucine have rather similar physiochemical properties.

The third gene in the interval with (two) non-synonymous mutations is Csa4M416990, encoding a cucumber homolog (53% identical amino acid sequences) of *Arabidopsis* F-box protein SKIP24. F-box proteins are part of the SCF complex, which is involved in protein ubiquitination leading to subsequent proteolysis. The F-box protein subunit of this complex is thought to grant substrate specificity to the complex and as such F-box genes form a very diverse and abundant gene family [46, 47]. The specificity of SKIP24 and closely related F-box proteins is to our knowledge unknown, and no mutant phenotypes are available, leading us to focus on the *CsLRK10L* genes in this publication instead. However, Wang [19] reported fine-mapping QTL DM4.1 from cucumber accession WI 7101 (PI 330628) to a 82 kb interval, containing 13 predicted genes, including *CsSKIP24* (Csa4M416990) as the most likely candidate gene. Whereas the causal genes of QTL DM4.1 from PI 330628 and DM4.1.2 from PI 197088 are not by definition allelic, we found mutations in the same gene in our genotype. It is in principle possible that subQTL DM4.1.2 in PI 197088 has two causal genes, one of which is shared with PI 330628. Functional studies regarding candidate genes *CsLRK10L2* and *CsSKIP24*, e.g. by complementation in DM susceptible cucumber, are needed in order to verify whether either or both genes are involved in DM resistance. Furthermore, additional fine-mapping experiments in our PI 197088 derived NILs might enable us to verify whether this subQTL can either be further divided in two subQTL, or delimited to a region excluding one or both of the candidate genes. However, such experiments were outside the scope of the current publication.

Phylogenetic analysis of *CsLRK10L* genes reveals patterns of domain reshuffling

In a phylogenetic analysis of the predicted extracellular domains of RLK proteins of cucumber and *A. thaliana* with OG-binding and/or WAK-associated domains, we found that the extracellular domains of CsLRK10L1 and CsLRK10L2 were close homologues of one another, being more closely related to one another than to any other protein (Figure

8C). This indicates that the two genes likely arose due to tandem duplication, as is often the case for *RLK* genes. However, our results showed that regarding the intracellular kinase domains, each of the two genes has a distinct set of homologues (Figure 8A-B), indicating a unique evolutionary history for both of the two genes. Apparently the extracellular domain of one of the two genes, encoded by the first exon, was duplicated, but was subsequently fused to an alternative intracellular kinase domain. Previously it was reported that in general RLKs with similar extracellular domains also have similar kinase domains, with the exception of LRK10L, CrRLK1-like and S-domain RLKs, which can have several different kinase domains [23].

Several kinase domain homologs of CsLRK10L2 are involved in disease resistance

Arabidopsis homologs of the CsLRK10L2 kinase domain included eight genes not previously described in literature, as well as five previously described genes, some of which were found to be involved in plant-pathogen interactions. One CsLRK10L2 kinase domain homolog was the *Arabidopsis LRK10L2* gene, which gives its name to the LRK10L2 subfamily of *RLK* genes. This gene was named after the wheat *LRK10* gene due to sequence homology of the extracellular domain [23]. The wheat *LRK10* gene was found to be a candidate gene for the *lr10* locus, contributing to resistance to leaf rust caused by *Puccinia recondita* [48]. Another CsLRK10L2 homologue is *Arabidopsis* PR5K, which has an extracellular domain homologous to pathogenesis related PR5 proteins [49], and overexpression of which in creeping bentgrass (*Agrostis palustris*) led to increased resistance to *Sclerotinia homoeocarpa*. Yet another homolog in the CsLRK10L2 clade is SNC4, an RLK with two predicted extracellular glycerophosphoryl diester phosphodiesterase domains. An auto-active mutant allele of *SNC4* obtained in an EMS screen had increased resistance against DM caused by *Hyaloperonospora arabidopsidis*, as well as elevated expression of SA-marker genes *PR1* and *PR2* and JA-marker gene *PDF1.2* [50]. Finally, two other CsLRK10L2 kinase domain homologs were MDS3 and MDS4, which have malectin-like extracellular domains classifying them as *CrRLK1L* family members. CrRLK1L malectin-like domains are thought to bind to pectin in the cell wall, similar to WAK proteins, even though the sequences of CrRLK1L and WAK proteins are not very homologous [51]. MDS3 and MDS4 were found to be involved in growth regulation under heavy metal ion stress [52].

Conclusion and future perspectives

We have shown that QTL DM4.1 from PI 197088 consists of three subQTL, each with different effects on disease phenotype. One subQTL, DM4.1.2, caused a decrease in sporulation and was associated with increased expression of defence pathway genes. We further focussed on this subQTL, and identified a candidate gene, *CsLRK10L2*. A 551

bp deletion, leading to a loss-of-function allele, was found in susceptible genotype HS279, similar to the reference genome Chinese Long 9930 v2. It was found that the intracellular kinase domain of the encoded protein was homologous to several *Arabidopsis* RLKs with known functions in plant defence against several unrelated pathogen. Furthermore, we found that heterologous overexpression of the gene in *N. benthamiana* triggered a necrotic response, in contrast to the susceptible allele. We consider the possibility that *CsLRK10L2* plays a role as a receptor of the DAMP oligogalacturonan, the breakdown product of pectin in the plant cell wall. More experimental work is needed to confirm whether CsLRK10L2 is indeed the causal gene for subQTL DM4.1.2, and if so, to identify the mechanisms by which this protein leads to increased DM resistance. Furthermore, three additional candidate genes were identified based on non-homologous polymorphisms between genotypes with and without the genotypes, of which *CsSKIP24* (Csa4M416990) stood out based on the finding that mutations in this gene also occurred in another DM resistant genotype [19].

Future work will hopefully lead to fine-mapping and identification of candidate genes for the other subQTL as well, especially regarding DM4.1.3 which had a recessive effect on both chlorosis as well as sporulation. Furthermore the DM resistant accession PI 197088 in which these subQTL were identified contained several other QTL, for which fine-mapping and identification of candidate genes can be performed.

METHODS

Plant materials and growing conditions

Plant introduction line PI 197088, highly resistant to DM caused by *P. cubensis* [10], was originally collected in Assam, India on April 16th, 1951 and is maintained by the United States National Plant Germplasm System (NPGS). Homozygous breeding line HS279 is a pickling type cucumber, susceptible to DM, with good horticultural characteristics.

A marker-assisted backcrossing strategy was employed in order to generate NILs and segregating populations. In an F3 population derived from a PI 197088 x HS279 cross, a partially resistant individual with a recombination event close to QTL DM4.1 was selected. This F3 individual was backcrossed to recurrent parent HS273 for three generations, using marker assisted selection with SNP markers for background selection of HS279 alleles and foreground selection of PI197088 alleles in the DM4.1 interval. A resulting F3BC3 individual fixed for HS279 alleles at all markers except for the DM4.1 introgression was self-fertilized for two generations to generate F3BC3S2 populations, which were genotyped with SNP markers within the DM4.1 interval in order to select

27 individuals with recombination events. Recombinant F3BC3S2 individuals were self-fertilized in order to generate both fixed and segregating F3BC3S3 families, which were used as fine-mapping populations. Several informative heterozygous and homozygous F3BC3S3 individuals were selected to generate segregating RHLs and fixed NILs, respectively.

Unless otherwise indicated plants were grown on blocks of rockwool in climate chambers with temperatures of 22 °C (day) and 17 °C (night), with a 16/8h day/night cycle, and a relative humidity of 80%.

P. cubensis inoculum maintenance, disease tests and phenotyping

An isolate of *P. cubensis* obtained from an infected cucumber field in Haelen, the Netherlands, was maintained on fully expanded cucumber leaves, healthy in appearance before inoculation. For pathogen maintenance, detached leaves were kept in closed boxes containing water-soaked paper towels, and inoculated with a spore suspension developed as described below. Boxes containing inoculated cucumber leaves were kept in a climate chamber under 18 °C (day) and 15 °C (night), with a 16/8h day/night cycle for ten days. Heavily infected detached leaves were preserved at -20 °C as inoculum source for <6 months. Spore suspensions were produced by washing spores from frozen infected leaves using tap water, and filtering through cheesecloth. The spore concentration was measured using a haemocytometer, and adjusted to $1x10^4$ spores/ml.

Cucumber plants for *P. cubensis* disease tests were grown in plastic tents, which were closed the day before inoculation to ensure a high relative humidity. Both sides of cucumber leaves were sprayed with spore suspension prepared as described above. After inoculation, plants were left in darkness at 18/15°C (day/night) for 24 hours in closed plastic tents. Starting from seven days post inoculation, yellowing (chlorosis), sporulation, and collapsing (necrosis) of leaves were assessed by eye on a 1-9 scale, 9 being fully resistant and 1 being fully susceptible.

QTL analysis and statistical analysis disease scores

For QTL mapping, phenotypic data were collected on 27 $F_3BC_3S_3$ families, of which 19 were segregating and 8 were uniformly homozygous. For homozygous families, average phenotypic scores of 20 individual plants were used. For segregating families, individual scores of 20-91 plants were used. All plants were genotyped using 7 SNP markers within the DM4.1 interval as well as 2 SNP markers flanking the interval. QTL were mapped for each of four phenotypes, i.e. chlorosis, sporulation, pathogen-induced necrosis, and autonecrosis, using the "scanone" procedure from the R/qtl package [53], and including family identifiers as a covariable to correct for the population structure.

For analysis of DM resistance in segregating RHLs, 94 plants per family were sown, of which based on SNP genotyping eight plants each of homozygous, heterozygous and azygous individuals were selected. Plants were phenotyped, average scores and standard deviations were determined for the three symptoms (chlorosis, sporulation and necrosis). Statistical analysis of phenotypic data was performed using SPSS v23 software (IBM), with non-parametric Kruskal-Wallis tests and stepwise step-down post hoc analysis.

RNA extraction, sequencing and differential expression analysis

For RNA extractions, plants of genotypes HS279, NIL DM4.1.2 and NIL DM4.1.3 were inoculated with *P. cubensis* as described above, or a mock treatment consisting of spraying leaves with tap water. Three days post inoculation, leaves of three biological replicates per genotype x treatment combination were sampled and immediately frozen in liquid N_2. Leaf samples were stored at -80°C. Leaf samples were ground in liquid N_2, and total RNA was isolated by using the RNeasy Kit (Qiagen, Germany). Possible DNA contamination of RNA samples was removed by treatment with DNase I, Amp Grade (Invitrogen life technologies, U.S.A.). RNA samples were shipped on dry-ice to BGI Tech Solutions (Denmark) for RNA sequencing using a BGISEQ-500 platform, resulting in ca. 50 million fastq reads (100 bp PE) per sample. Fastq reads were aligned to the cucumber reference genome (Chinese Long 9930 v2) using TopHat (v2.1.1) [54]. Uniquely aligned read counts per gene per sample were determined using HTSeq-count [55]. Differential expression analysis was performed in R using package DEseq2 (v3.8) [56]. Principal component analysis (PCA) was performed on regularized log values of read counts. As suggested in the DESeq2 vignette, treatment and genotype were combined as a single factor for the analysis of contrasts between genotypes and treatments. Differentially expressed genes were called significant using an adjusted P-value (Benjamini–Hochberg adjustment) and a false discovery rate of < 0.05. As a threshold for biological significance a two-fold change in expression was used. Lists of up- and downregulated genes from the contrasts of the conditions were compared and used to create the Venn diagram with the overLapper function from R package systemPipeR [57].

SNPs in coding regions were called using Samtools mpileup (v1.3.1) [58], and the effect on coding sequences of predicted genes were annotated using SnpEff [59].

Identification of cucumber orthologues of defence pathway genes

Protein sequences of *A. thaliana* defence pathway genes *PR1* (AT2G14610), *PR2* (AT3G57260), *PR3* (AT3G12500), *PAD4* (AT3G52430), *EDS1* (AT3G48090), *NPR1* (AT1G64280), *RST1* (AT3G27670) and *PGIP2* (AT5G06870) were retrieved from The Arabidopsis Information Resource (TAIR) on www.arabidopsis.org [accessed on 13-03-

2019]. Protein sequences were used as queries for BLASTp searches against reference genomes of cucumber (Chinese Long 9930 v2) and *Arabidopsis* (TAIR10) in order to identify homologous genes in both species. Multiple sequence alignments and neighbour-joining phylogenetic trees were constructed using CLC Genomics Workbench v11, with standard settings. Putative cucumber defence pathway genes were selected based on orthology with the *Arabidopsis* gene. In cases where multiple putative cucumber orthologues of an *Arabidopsis* defence pathway gene were identified, capital letters (A-D) were added to the gene names in order to discriminate between orthologues. Additional Data 10 gives an overview of identified putative cucumber defence pathway genes.

Identification and sequencing of *RLK* structural variation

For genomic inspection of the *RLK* region, DNA was isolated from a leaf sample obtained from genotype NIL DM4.1, using a CTAB protocol [60]. DNA was shipped on dry-ice to Novogene Bioinformatics Technology Co. (Hongkong) for Illumina HiSeqX resequencing. Obtained clean reads (100bp PE) were aligned to the reference genome (Chinese Long 9930 v2) using Bowtie2 (v2.2.6) [61]. Reads mapping to the *RLK* locus were manually inspected using IGV (v2.3.32) [62].

For PCR amplification of the suspected structural variation in gene Csa4M410860, DNA was isolated from three independent individuals of genotypes NIL DM4.1.2 and HS279 as described above. PCR reactions were performed using DreamTaq DNA polymerase (Thermo Fisher Scientific) according to manufacturer's protocol, with primers F 5'-TTCCCCGCGGACATCTCTA-3' and R 5'-AGGTCAACTTTCACACAGTCCA-3'. PCR products were sent for Sanger sequencing (GATC Biotech, Germany) using the same primers.

Cloning and transient overexpression of *CsLRK10L* genes

RNA was isolated from genotypes NIL DM4.1.2 and HS279, as described above. cDNA was synthesized using Superscript III reverse transcriptase (Thermo Fisher Scientific) with oligo-dT primer, following the manufacturer's protocol. *CsLRK10L1* and *CsLRK10L2* were amplified from both cDNA samples using Phusion high-fidelity polymerase (Thermo Fisher Scientific) according to manufacturer's protocol with primers F 5'-CACCATGGATTCCCCAATTTCCTC-3' (both genes) and R 5'-GGAGCTGTCTGCTATTGATGG-3' (*CsLRK10L1*) or R 5'-AACCACAACAATCCTTAACAACC-3' (*CsLRK10L2*). PCR products were run on agarose gels and subsequently purified from gel using the QIAquick Gel Extraction Kit (Qiagen, Germany).

Purified products were cloned into Gateway-compatible vector pENTR D-TOPO (Thermo Fisher Scientific) and transformed to chemically competent *Escherichia coli* strain One

Shot TOP10. Presence of the right fragment was assessed by colony PCR using primers. Plasmids were recovered using the Qiaprep spin miniprep kit (Qiagen, Germany). Sequencing reactions were performed in duplo using pUC/M13 forward and reverse sequencing primers (GATC Biotech, Germany).

Entry plasmids were transferred using LR clonase II (Thermo Fisher Scientific) into binary vector pK7WG2, which harbours the constitutively active 35S Cauliflower Mosaic Virus promotor and the *nptII* marker gene for kanamycin resistance [63]. Recombinant plasmids were transformed to chemically competent *E. coli* strain dh5α. Positive recombinant bacterial colonies were screened by colony PCR using *CsLRK10L* specific primers as described above, and sequenced. Recombinant plasmids were recovered using the Qiaprep spin miniprep kit (Qiagen, Germany). Binary vectors were transformed to electrocompetent cells of *Agrobacterium tumefaciens* strain AGL1-virG by electroporation.

N. benthamiana plants were grown in a greenhouse under standardized conditions for 5 weeks. *A. tumefaciens* strains harbouring the binary vectors were cultured in LB medium with appropriate antibiotics for 18 h at 28°C. Cells were collected by centrifugation, resuspended in agroinfiltration medium and adjusted to the desired concentration. To increase expression efficiency, *A. tumefaciens* strains were mixed in a 1 : 1 ratio with a strain expressing silencing suppressor *P19* [64]. *A. tumefaciens* cultures were infiltrated in *N. benthamiana* leaves with a needleless syringe.

Phylogenetic analysis

Partial protein sequences of CsLRK10L1 and CsLRK10L2 predicted oligogalacturonan-binding, WAK-associated and protein kinase domains were used as BLASTp queries against translated genomes of cucumber (Chinese Long 9930 v2) and *Arabidopsis* (TAIR 10) in order to select homologous genes. Regarding kinase domain homologues of both proteins, multiple sequence alignments were performed, and maximum-likelihood phylogenetic trees were constructed using CLC Genomics Workbench v11.

Predicted proteomes of cucumber (Chinese Long 9930 v2) and *Arabidopsis* (TAIR 10) were scanned using InterProScan v5.27 against the Pfam database for presence of oligogalacturonan-binding (IPR025287) and/or WAK-associated (IPR032872) domains. Hits were extracted from the proteomes using the –getfasta option from Bedtools v2.27.1 [65], and used to construct multiple sequence alignments and maximum-likelihood phylogenetic trees using CLC Genomics Workbench v11.

AVAILABILITY OF DATA AND MATERIAL

RNAseq data of cucumber genotypes HS279, NIL DM4.1.2 and NIL DM4.1.3 analysed in this paper were submitted to NCBI SRA under accession number PRJNA544259 (to be released on publication). All other data generated or analysed during this study are included in this published article and its additional data files.

AUTHORS' CONTRIBUTIONS

FWKH, FB and WHF developed plant materials, performed QTL mapping and (fine) mapping. JAB designed, performed and analysed the rest of the experiments, with valuable suggestions by HJS and YB as well as assistance by FB and LL. JAB drafted the manuscript. WHV, RGFV, YB and HJS gave critical feedback on the draft manuscript. All authors read and approved the manuscript.

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ADDITIONAL DATA

All figures and additional datafiles described in this chapter were deposited online, and can be accesed through doi:10.4121/uuid:9b31358d-b8da-449a-8ae8-0fea922bc186

Additional Data 1

Format: XLSX

Physical locations of nine SNP markers used in QTL analysis are given based on the cucumber reference genome (Chinese Long 9930 v2). Furthermore, flanking and peak markers for each of the QTL detected in Figure 1 are given.

Additional Data 2

Format: PDF

Similarly as described for Figure 2, a family was developed segregating for a partial introgression corresponding to part of subQTL DM4.1.1. **(A)** Bars represent the allele of genotypes at marker locations on the DM4.1 interval. Black bars indicate the PI 197088 allele, white bars indicate the HS279 allele, green bar represents heterozygosity. **(B)** The RHL was inoculated with P. cubensis, chlorosis was scored at 7 dpi whereas sporulation and necrosis were scored at 12 dpi. Bars represent average phenotype scores on a 1-9 scale ranging from susceptible to resistant. Error bars indicate standard deviations. Bars with different letters indicate statistically significant differences (Kruskal-Wallis test, $p < 0.05$)

Additional Data 3

Format: PDF

Expression data of cucumber homologs of known defence pathway genes were extracted from the RNAseq dataset, and plotted per sample on a logarithmic scale. Asterisks represent statistically significant differences between genotypes (adjusted $p < 0.05$). For all genes except *CsPR3A*, differences between treatments within genotypes were statistically significant ($p < 0.05$).

Additional Data 4

Format: XLSX

Expression data of genes within the physical interval of the fine-mapped subQTL DM4.1.2 were analysed. Pairwise contrasts between genotypes NIL DM4.1.2 and HS279

under both conditions as well as between both conditions for both genotypes are given. Both the Log_2 of the fold change and the adjusted P value are given. Additionally, physical locations and annotations per gene are indicated.

Additional Data 5

Format: XLSX

SNPs and indels in the fine-mapped DM4.1.2 interval were determined using the SAMtools mpileup command, and annotated using SnpEff. Non-synonymous SNPs are given.

Additional Data 6

Format: PDF

A) RNAseq reads from *P. cubensis* inoculated NIL DM4.1.2 aligning to the *RLK* cluster are visualized using the Integrative Genomics Viewer (IGV). Split reads are indicated with blue lines. A coverage graph is given above the aligned reads.

B) Whole genome sequencing reads of NIL DM4.1 aligning to the *RLK* cluster are visualized using the Integrative Genomics Viewer (IGV). A coverage graph is given above the aligned reads. Reads pairs with larger than expected or smaller than expected insert sizes are indicated in dark red and dark blue, respectively. Dotted lines denote the interval shown at greater resolution in Figure 6B, corresponding to predicted gene *Csa4M410860*.

Additional Data 7 – BLAST output CsLRK10L protein domains

Format: XLSX

Galacturonan binding (GUB), WAK-associated (WAK) and protein kinase domains of CsLRK10L1 and CsLRK10L2 were used as BLASTp queries against translated *Arabidopsis* and cucumber genomes. Tabular BLAST output is given.

Additional Data 8 – InterProScan analysis WAK domains

Format: XLSX

Predicted proteins in the translated cucumber and *Arabidopsis* genomes were scanned using InterProScan v 5.27 for presence of galacturonan binding and WAK-associated domains. Start and end locations of predicted domains are indicated.

Additional Data 9 – Chromosomal locations WAKL genes

Format: PDF

Genomic positions of *Arabidopsis* and cucumber genes encoding proteins with predicted galacturonan-binding and/or WAK-associated domains were retrieved and visualized.

Additional Data 10 – Predicted cucumber defence pathway genes

Format: XLSX

Cucumber orthologues of known *Arabidopsis* defence pathway genes were identified by BLASTp search followed by phylogenetic analysis. When multiple orthologues were found, capital letters were added as a suffix.

Chapter 5

A transposon insertion in susceptibility gene *Amino Acid Permease 2A* (*CsAAP2A*) contributes to quantitative resistance to downy mildew in cucumber

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ABSTRACT

Background: Cucurbit Downy Mildew (DM), caused by the obligate biotroph *Pseudoperonospora cubensis*, is one of the most destructive diseases in cucumber. In the last two decades existing resistance in the cucumber genepool, derived from Indian accession PI 197087, has been overcome by the pathogen. The related cucumber accession PI 197088 was found to be a promising source of novel resistance to DM. Resistance in PI 197088 was previously shown to inherit quantitatively, with 3-11 QTL contributing to overall resistance. We recently fine-mapped one of the QTL, previously shown to explain ca. 14 to 27% of the genetic variation, on chromosome 4.

Results: By means of RNAseq we identified differentially expressed genes in the near isogenic line (NIL) DM4.1.3, harbouring a DM resistance QTL on chromosome 4 from PI 197088 in the susceptible HS279 background. The gene *Amino Acid Permease 2A* (*CsAAP2A*) within the QTL interval was found to be ca. 100-fold downregulated in NIL DM4.1.3 compared to the susceptible recurrent parent HS279, and therefore selected as a candidate gene. Whole genome sequencing indicated a large structural variation between the two genotypes in exon four of *CsAAP2A*. PCR and Sanger sequencing revealed the insertion of a *Cucumis* Mu-like element (CUMULE) transposon. This insertion-allele of *CsAAP2A* was not observed in a re-sequenced core collection of 115 cucumber genotypes. In view of the loss of the putative function as amino acid transporter, we measured free amino acids in leaves infected with *P. cubensis*. In both genotypes HS279 and NIL DM4.1.3 the amino acid concentration was increased in inoculated leaves compared to mock-treated leaves, but the amino acid concentration in NIL DM4.1.3 (with *csaap2a* mutation) was significantly lower than in HS279.

Conclusions: *CsAAP2A* was identified as a novel candidate susceptibility gene to DM. A loss-of-function mutation in *CsAAP2A* due to the insertion of a transposable element was observed in the fine-mapped DM4.1.3 locus originating from cucumber accession PI 197088. Studying the amino acid dynamics of the cucumber-*P. cubensis* interaction revealed that the pathogen induces an increase of the concentration of amino acids in infected leaves. The identified *csaap2a* loss of function mutation partially prevents the increase in amino acids, possibly limiting the flow of nutrients towards the pathogen and thereby the pathogen's fitness.

Keywords: Downy mildew (*Pseudoperonospora cubensis*), Cucumber (*Cucumis sativus*), Plant-pathogen interactions, Nutrient transport, *Amino Acid Permease* (*AAP*), Susceptibility gene, Transposable element, CUMULE

BACKGROUND

One of the most destructive cucurbit diseases is downy mildew (DM), caused by the obligate biotrophic oomycete *Pseudoperonospora cubensis* [(Berk. & Curt.) Rost.]. *P. cubensis* can infect over 50 different plant species in the family Cucurbitaceae, including several economically important crops such as melon (*Cucumis melo* L.), watermelon (*Citrullus lanatus* (Thunb.) Matsum & Nakai) and squash (*Cucurbita* spp.), and is especially a limiting factor in cucumber (*Cucumis sativus* L.) cultivation [1, 2].

Cucumber DM is characterized by the appearance of chlorotic lesions on leaves, which appear angular as they are restricted by leaf veins. Brown to black sporulation can be observed on the abaxial side of the lesions. As the disease progresses, lesions become necrotic and multiple lesions will coalesce, leading to wilting and necrosis of the leaf and, eventually, death of the plant [3, 4].

Whereas chemical control by means of fungicide application can play a role in disease management [5], breeding for resistance to DM is the most economic as well as environmentally friendly method to control the disease and prevent DM outbreaks. Modern systematic DM resistance breeding in cucumber started in the 1930s and 1940s, using 'Bangalore' and 'Chinese Long' as sources of resistance [6], eventually leading to the DM resistant cultivar 'Palmetto' [7]. However, the resistance of 'Palmetto' was broken within two years after its commercial release [8]. Hereafter, a new source of resistance was found in the Indian semi-wild cucumber accession PI 197087, characterised by small brown (necrotic) lesions instead of larger yellow (chlorotic) lesions [9]. DM resistance from PI 197087 was found to inherit as a single recessive gene, named *p* or *dm-1* [7, 10, 11] and consequently this gene was introgressed into many cucumber cultivars. DM resistance due to *dm-1* was effective for nearly 50 years [12]. Recently, a missense mutation in the gene *CsSGR* (*Staygreen*) was found to be causal for *dm-1* resistance [13]. Since the year 2004 however, new virulent strains of *P. cubensis* emerged which have partially overcome *dm-1* based resistance [12, 14], prompting the search for novel sources of DM resistance.

In a large multi-location germplasm screening, wild cucumber accession PI 197088 came up as one of the most resistant genotypes [15]. Interestingly, PI 197088 was originally collected on the same date and location as PI 197087 indicating that the two accessions might be closely related. However PI 197088 was found to be resistant to post-2004 strains of *P. cubensis* which are virulent on PI 197087. In contrast to PI 197087-based resistance, which is presumably caused by a single recessive gene, several groups discovered that DM resistance in PI 197088 is polygenic, with multiple QTL contributing

to overall disease resistance. In 2010, Caldwell et al. filed a patent describing three QTL derived from PI 197088 on chromosomes 2, 4 and 5 in one F3 and two F5 mapping populations with three different susceptible parents [16]. Yoshioka et al. mapped seven QTL on chromosomes 1, 3 and 5 in a collection of 111 RILs derived from a cross between the PI197088 inbred line CS-PMR1 and the intermediate susceptible line Santou [17]. Li et al. mapped five QTL on chromosomes 1, 3, 4 and 5 in a set of 183 F3 families originating from a cross between PI 197088 and susceptible parent Changchunmici [18]. Wang et al. identified 11 QTL on chromosomes 1 to 6 in a set of 148 RILs derived from a cross between PI 197088 and Coolgreen [19]. Whereas these four groups all found that DM resistance in PI 197088 is polygenic, it is striking that there is no consensus at all on the genetic architecture of DM resistance in PI197088, as each of the groups reached different conclusions on the amount of QTL and the contributions of the different QTL on overall DM resistance. However, one or more QTL on chromosome 5 were usually found to have the largest effect on resistance, often followed by a QTL on chromosome 4. Different mapping outcomes are probably caused by differences in experimental set-up, such as the usage of different susceptible parents, different mapping population structures, different inoculation methods and sources, and different ways of scoring.

Plant breeding has often focussed on monogenic resistance, usually conferred by resistance (*R)* genes in a gene-for-gene interaction with cognate *Avr* genes of the pathogen [20]. However, employing *R*-gene expressing crops puts selective pressure on pathogen populations, leading to new virulent strains of the pathogen which are not detected by the *R*-gene. A potentially more durable form of resistance is polygenic resistance. Polygenic resistance refers to the concept that several genes, each with a partial effect, can contribute to overall resistance [21]. As the underlying causal genes for each of the loci contributing to polygenic resistance might work by different mechanisms [22], it is to be expected that it is harder for a pathogen to break polygenic resistance in comparison to monogenic resistance conferred by a single *R* gene. However, to acquire a satisfying level of resistance in a commercially acceptable cultivar by use of a source of resistance which is inherited quantitatively, such as cucumber accession PI 197088, poses difficulties in breeding, as it requires the introgression of multiple small-effect loci from a resistant progenitor. This in turn can cause significant linkage drag, as resistant progenitors will likely have many undesirable traits. Identification of causal genes underlying loci for polygenic resistance will therefore greatly facilitate the usage of polygenic resistance in plant breeding, as it enables the development of molecular markers perfectly linked to the causal quantitative resistance gene [21]. Identification of causal genes with small effects by fine-mapping in a complex polygenic background is rather complicated, therefore it is helpful to first introgress individual medium-effect or small-effect loci in a homogeneous susceptible background, after which the partial

resistance conferred by the locus will segregate according to Mendelian ratios [22] and can thus be mapped and identified more easily.

Recently we fine-mapped a QTL from cucumber accession PI 197088 for DM resistance on chromosome 4 (DM4.1), which was previously shown to explain 13.7 to 27% of the phenotypic variance in DM resistance [18, 19] and is therefore one of the most promising QTL for DM resistance in PI 197088. By repeated backcrossing using marker assisted selection (MAS) we obtained near isogenic lines (NILs), each having small PI 197088-derived introgressions on chromosome 4, in a susceptible background genotype. To our surprise, we found that instead of a single QTL (DM4.1) three individual loci could be distinguished within a 12 Mb interval, which we named DM4.1.1, DM4.1.2 and DM4.1.3. The individual effects of each of these three QTL on the disease phenotype is markedly different: the first QTL, DM4.1.1, decreased the amount of disease-induced necrosis, or "collapsing", although this effect was not possible to score in absence of the two other DM resistance QTLs. The second QTL, DM4.1.2, decreased the amount of sporulation of the pathogen. The third QTL, DM4.1.3, had a recessively inherited effect on pathogen-induced chlorosis (yellowing), especially at early time points after inoculation, indicating that it probably slows the growth of the pathogen. The combined effect of the three loci is markedly stronger than the sum of the separate effects of the individual loci (**Chapter 4**).

Biotrophic plant pathogens require long-lasting interactions with living host cells to complete their life cycle, during which pathogens must avoid recognition by the host and/or suppress defence responses, as well as alter host cellular processes in order to facilitate pathogen growth and reproduction by providing nutrients [23]. For these reasons, pathogens such as oomycetes secrete a plethora of effector proteins in order to hijack the host [24]. In order to establish a compatible interaction, pathogens also depend on host-encoded susceptibility (*S*) genes to facilitate infection, to suppress defence responses and to support the uptake of nutrients by the pathogen [25, 26]. Loss-of-function mutations in *S* genes decrease the susceptibility of the plant, and thereby contribute to resistance. Whereas *R* genes are usually dominantly inherited, loss-of-function alleles of *S* genes confer recessive resistance. Even though recessive resistances have been used in plant breeding for a long time, the concept of *S* genes was introduced in 2002 [27] after the discovery of *pmr6*, which confers powdery mildew resistance in *Arabidopsis* [28]. As exemplified by the barley *mlo* gene, which confers powdery mildew resistance for over seven decades [29], resistance due to *S* genes can be very durable. Whereas some *S* genes confer (near) complete resistance (e.g. barley *mlo* [29] and *Arabidopsis dmr1-dmr6* [30]), the majority of identified *S* genes contributes to partial resistance [25]. In cucumber, recessively inherited "hypocotyl" resistance to

powdery mildew was recently shown to be due to a loss-of-function mutation in the susceptibility gene *CsaMLO8* [31], whereas *dm-1* resistance to DM which was effective for over 40 years was shown to be likely due to a loss-of-function mutation in susceptibility gene *Staygreen* (*CsSGR*) [13].

Here, we report how we found a natural loss-of-susceptibility mutation in a cucumber *Amino Acid Permease* gene (*CsAAP2A*) within the fine-mapped DM4.1.3 locus as a likely candidate gene contributing to quantitatively inherited DM resistance in PI 197088. *AAP* genes were previously found to be transcriptionally upregulated in roots of *Arabidopsis thaliana* infected with plant parasitic nematodes [32], and subsequently it was discovered that mutations in several *AAP* genes contributed to nematode resistance [33, 34]. Although there are no experimental data to prove it, it was postulated that *AAP* genes play a role in transport of amino acids to nematode feeding sites, and hence that mutations in *AAP* genes limit the availability of amino acids for the parasite, thereby decreasing the nematode's fitness [34]. The current report is the first indication that *AAP* genes also contribute to susceptibility to other pathogens.

RESULTS

Fine-mapping and identification of candidate genes for subQTL DM4.1.3 for reduced sporulation

We previously developed a near isogenic line (NIL DM4.1.3) with partial DM resistance due to an introgression on chromosome 4 from PI 197088 in the susceptible HS279 background. Initial QTL mapping results delimited subQTL DM4.1.3 to the ca. 3.6 Mb interval Chr4:16,876,817-20,438,834 (**Chapter 4**). To narrow down this interval, we selected fourteen cucumber plants with recombinations within the DM4.1.3 interval, from the population used for QTL mapping. These recombinants were selfed in order to develop families, which were tested for DM resistance. Eight of these families were fixed for the resistant allele of subQTL DM4.1.3, characterised by a uniform low level of chlorosis. Three families were fixed for the susceptible allele of the subQTL, characterised by a uniform high level of chlorosis. The remaining three families were segregating, in ratios not significantly deviating from the expected 1:3 ratio of resistant versus susceptible individuals based on recessive resistance (X^2 test, $p>0.05$). Analysis of these recombinant populations allowed fine-mapping of subQTL DM4.1.3 to the interval Chr4:18,842,456-19,596,926 (Figure 1), containing 80 predicted genes in the cucumber reference genome (Chinese Long 9930 v2).

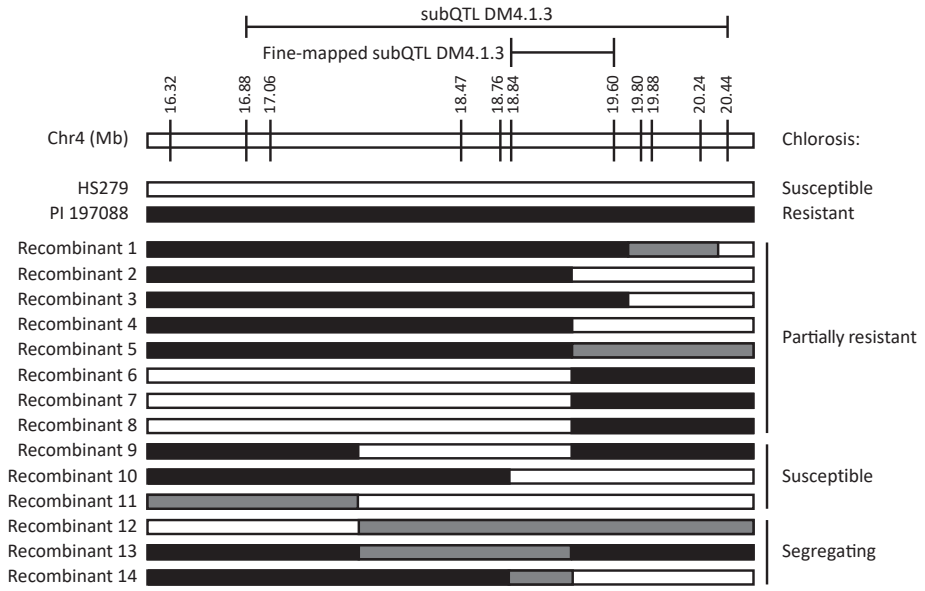

Figure 1 – Fine-mapping subQTL DM4.1.3
Screening of individuals derived from the mapping population allowed identification of additional informative recombinants within the DM4.1.3 interval. Bars represent genotypes at marker locations. Black bars indicate the PI 197088 allele, white bars indicate the HS279 allele, grey bars represent heterozygosity. Individuals were self-fertilized to develop families, which were inoculated with *P. cubensis*. Chlorosis was scored one week post inoculation on a 1-9 scale, with 1 being completely yellow and 9 being fully green. Plants with scores of 6 or higher were designated (partially) resistant.

In order to identify candidate genes for QTL DM4.1.3, we investigated the expression of the 80 predicted genes within the fine-mapped interval in a previously generated RNAseq dataset, obtained by sequencing RNA isolated from *P. cubensis* inoculated cucumber leaves of NIL DM4.1.3 and the susceptible recurrent parent HS279 (**Chapter 4**). Thirteen genes within the interval were found to be differentially expressed between genotypes NIL DM4.1.3 and HS279 (Table 1, Additional Data 1). Furthermore, non-synonymous polymorphisms between the two genotypes in the interval of QTL DM4.1.3 were determined (Additional Data 2).

An *Amino Acid Permease* (*AAP*) gene (gene ID: Csa4M573860) was selected as a promising candidate gene, based on the finding that this gene was downregulated by a factor 100 in NIL DM4.1.3 compared to HS279, which was highly significant (Benjamini-Hochberg corrected p value = 3.07×10^{-28}) (Table 1).

Table 1 – Differential expression analysis of genes in the DM4.1.3 interval
Differentially expressed genes (Benjamini-Hochberg adjusted p<0.05) within the fine-mapped DM4.1.3 interval (Chr4:18,842,456-19,596,926) between DM susceptible cucumber genotype HS279 and partially resistant NIL DM4.1.3, three days post inoculation with *Pseudoperonospora cubensis*. Average expression values (AE) of three biological replicates of both genotypes are given, as determined using HTSeq [71]. Differential expression was determined using R package DEseq2 [72].

Gene ID	Annotation	AE HS279	AE DM4.1.3	Fold change	p value
Csa4M573860.1	Amino acid permease	213	2	0.01	$3.07x10^{-28}$
Csa4M575860.1	Unknown protein	140	87	0.62	$8.35x10^{-04}$
Csa4M580380.1	Protein pns1	91	50	0.55	$1.16x10^{-04}$
Csa4M593900.1	C2 domain-containing protein	42	22	0.52	$1.58x10^{-02}$
Csa4M594450.2	Mediator of RNA polymerase II transcription subunit 11	13	2	0.18	$2.22x10^{-03}$
Csa4M598010.1	Putative GTP-binding protein	78	112	1.44	$4.40x10^{-02}$
Csa4M608070.1	Triacylglycerol lipase, putative	27	82	3.09	$1.34x10^{-06}$
Csa4M608110.1	3-hydroxyisobutyryl-CoA hydrolase-like protein	859	644	0.75	$1.31x10^{-05}$
Csa4M608130.1	Probable aspartic protease	2358	1578	0.67	$2.63x10^{-08}$
Csa4M608160.1	4-hydroxyphenylpyruvate dioxygenase	2834	3853	1.36	$1.45x10^{-02}$
Csa4M608170.1	NAC domain protein	25	62	2.46	$6.81x10^{-03}$
Csa4M614170.1	Putative MYB DNA-binding domain superfamily protein	198	89	0.45	$9.24x10^{-08}$
Csa4M614200.1	Kinase	82	150	1.82	$4.40x10^{-05}$

Lack of *AAP* expression is due to the insertion of a CUMULE transposon

In order to investigate the reason why the *AAP* gene was very lowly expressed in NIL DM4.1.3, genomic DNA was isolated from both genotypes NIL DM4.1 and susceptible parent HS279. Whole genome sequencing data were obtained using BGISEQ-500 technology, generating 100 bp paired end reads. The alignment of reads to the reference genome in the genomic locus of *AAP* (Chr4:18,882,370-18,886,704) was manually inspected (Additional Data 3). Several indications were found of a structural variation in the fourth exon of the *AAP* gene in genotype NIL DM4.1: double coverage of an 11 bp stretch of the exon, alignment problems of reads at either side of this 11 bp sequence, and flanking reads with mate pairs aligning to different regions of the cucumber reference genome (Figure 2). These three observations led us to hypothesize that there might be an insertion of a transposable element at this location in the DM4.1.3 allele of the *AAP* gene.

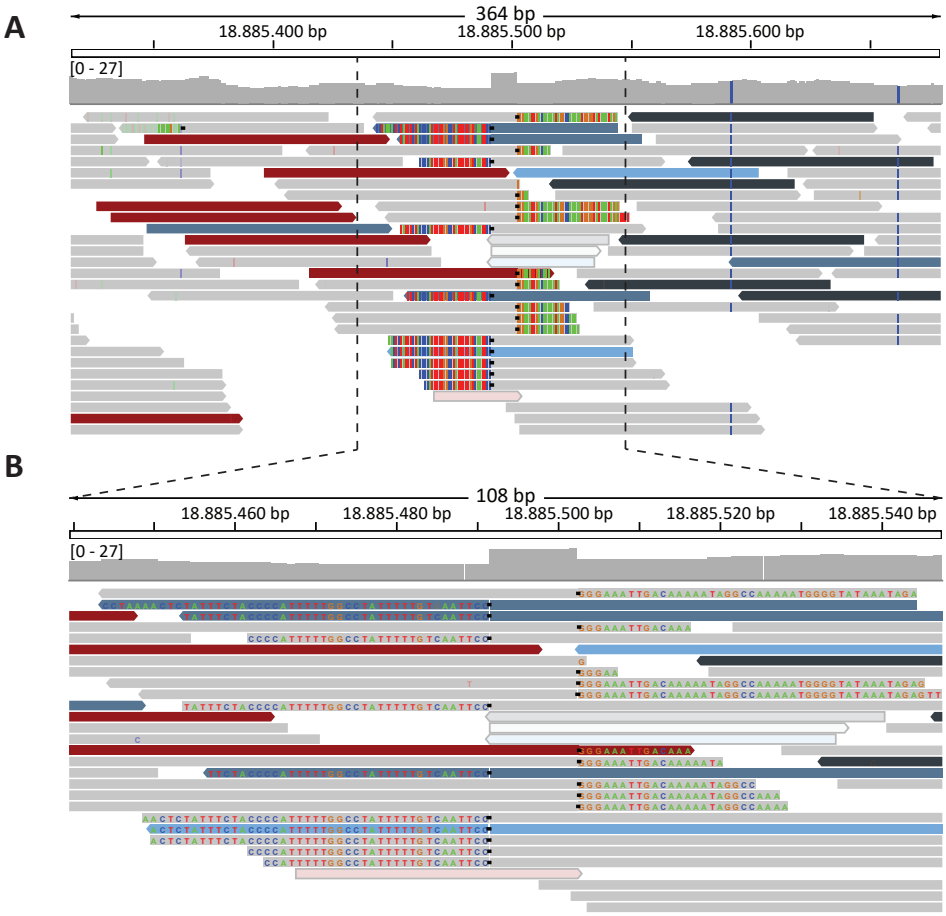

Figure 2 – Genomic analysis of *AAP* locus in NIL DM4.1 reveals a large structural variation
Whole genome resequencing data (Paired-end reads, 100 bp) of cucumber genotype NIL DM4.1 were manually inspected using Integrative Genomics Viewer software [69]. A scale bar indicates the genomic position on the reference genome (Chinese Long 9930 v2). Coverage per bp of the reference genome is indicated by the bar graph. Nucleotides in resequencing reads not identical to the reference genome are indicated with colours (green for A, red for T, blue for C, brown for G), indels are indicated by black horizontal stripes. Reads for which the other mate in the mate pair was mapped on a different chromosome or with deviating insert sizes are indicated by non-grey colours.
A) Local alignment of reads to genomic locus Chr4:18,885,310-18,885,674 (corresponding to the fourth exon of *AAP* gene Csa4M573860.1 plus surrounding introns) indicated an 11 bp stretch of the reference genome with double coverage compared to the rest of the reference genome; reads with alignment problems before as well as after this eleven bp stretch; and flanking reads with mate pairs mapping to other genomic loci.
B) Local alignment of reads to genomic locus Chr4:18,885,439-18,885,547 showing the findings in **A)** in more detail.

To test this hypothesis, PCR reactions were performed on DNA isolated from resistant donor PI 197088, susceptible recurrent parent HS279, partial DM-resistant NIL DM4.1 and partial resistant NIL DM4.1.3 (Figure 3A,B), using a primer pair flanking exon four of the *AAP* gene (Figure 3C). Cloning and Sanger sequencing of resulting PCR products revealed the presence of a 7,688 bp insertion in the resistant genotypes compared to the susceptible HS279 (Figure 3C). This insertion had all the hallmarks of an autonomous Mu-like DNA transposon (Figure 3D):

- The insertion was flanked by a duplication of 11 bp of the *AAP* gene (Target site duplication, TSD).
- The first and the last 118 bp of the insert are inverted copies of one another (terminal inverted repeats, TIR) (Additional Data 4).
- The transposon appeared to contain a 2206 bp long *MuDRA transposase* gene consisting of a single exon, with high sequence homology (90% identical nucleotide sequences) to the *MuDRA transposase* characterized by van Leeuwen et al. in the melon CUMULE (*Cucumis* Mutator-Like Element) transposon [35].

Furthermore we identified a gene consisting of five exons with a cumulative length of 1,599 bp with homology to *Ubiquitin-like peptidase* genes (*Ulp1*), which was also identified in the melon CUMULE transposon [35]. By re-aligning RNAseq reads obtained from NIL DM4.1.3 to the 7.688 bp insertion (Additional Data 5), it was found that whereas there was no evidence of expression of the *MuDRA* gene, the *Ulp1* gene was found to be abundantly expressed.

Upstream of this *Ulp1* gene, another short ORF of 408 bp was found to be expressed, with no apparent homology to any described gene. Furthermore an extra tandem repeat region was identified, located between the short expressed ORF and the *Ulp1* gene. This 1682 bp long tandem repeat region consists of nine repeats of a 178 bp segment plus a tenth 80 bp partial repeat, with 0-5 SNPs between each of the repeats (Additional Data 6). According to RNAseq data, this tandem repeat region is transcribed to RNA, although no ORFs could be identified within the region. The annotated DNA sequence of the CUMULE transposable element was deposited in the NCBI GenBank database [GenBank: MN062013].

A BLASTn search was performed screening nucleotide sequences of the *MuDRA transposase* gene, the *Ulp1* protease gene and the TIR domains of the newly identified CUMULE transposon as queries against the cucumber reference genome (Chinese long 9930 v2). We identified 67 regions with homology to the *MuDRA* transposase, 61 regions with homology to the *Ulp1*, and 94 regions with homology to the TIR domains, scattered over all seven cucumber chromosomes and often in close proximity to one

another (Additional Data 7), indicating that CUMULE transposons are rather common in the cucumber genome.

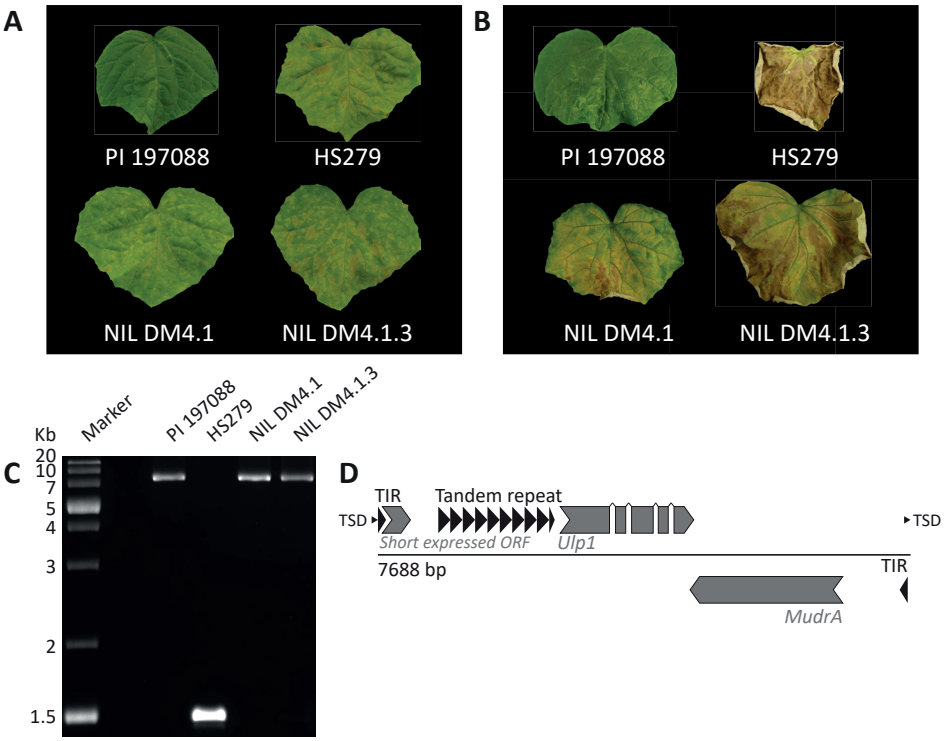

Figure 3 – PCR Amplification of *AAP* alleles from resistant and susceptible cucumber genotypes lead to the identification of a CUMULE transposon

A) Representative DM phenotypes of cucumber genotypes PI 197088 (resistant donor), HS279 (susceptible recurrent parent) and partially resistant NILs DM4.1 and DM4.1.3 with PI 197088 introgressions in HS279 background, seven days post spray inoculation with *Pseudoperonospora cubensis*.

B) Representative DM phenotypes of the four genotypes described in **A)**, fourteen days post spray inoculation with *P. cubensis*.

C) DNA isolated from the cucumber genotypes described in **A)** was used as template for PCR with *AAP* specific primers. Amplified products were analysed with gel electrophoresis. Whereas the product amplified from HS279 DNA gave a band of expected size (1.5 Kb), products amplified from (partially) resistant genotypes resulted in larger (ca. 8 Kb) fragments.

D) The large fragment amplified in **C)** was cloned and sequenced, which revealed the presence of a 7688 bp insertion with hallmarks of a *Cucumis* Mu-like element (CUMULE) in the DM4.1.3-allele of the AAP gene. The CUMULE transposon is schematically represented. Repeat regions (TSD: target site duplication, TIR: Terminal Inverted Repeat) are indicated in black. Putative coding sequences (Short expressed ORF, *Ulp1*: *Ubiquitin-like protease 1*, *MudrA*: *transposase*) are indicated in grey. Elements on the negative strand are indicated in opposite direction, below the black line. The full CUMULE sequence plus annotation has been deposited in GenBank [MN062013].

A recently described script [36] was used to perform an in silico search for occurrence of the CUMULE-allele of the *AAP* gene in a resequenced core collection of 115 divergent cucumber accessions [37] (Additional Data 8). In 107 out of the 115 accessions, only reads indicating the WT *AAP* allele were observed. In the remaining eight accessions, no reads indicative of either presence or absence of the insertion were found by the script, due to low coverage at this location. However, in seven out of the eight accessions at least one read could be observed consistent with the WT allele of the *AAP* gene by manual inspection of aligned reads at the locus.

Whereas the insertion of the CUMULE transposon apparently led to abolishment of *AAP* expression (Table 1), we determined the effect of the insertion on the predicted protein sequence (Additional Data 9). We found that the insertion of the TE led to truncation of the last 306 amino acid residues out of the total 466, replacing them with 19 other amino acid residues. This indicates that even residual *AAP* expression will presumably not encode a functional AAP protein, and the TE-allele of the *AAP* gene is a bona fide loss-of-function allele.

Amino acid profiling of cucumber leaves with and without QTL DM4.1.3

As we identified an amino acid transporter gene (*AAP*) as a likely candidate susceptibility gene for QTL DM4.1.3, we hypothesized that impairment of this gene may lead to lower amino acids levels in leaves, thereby reducing the feeding of the pathogen. In view of this hypothesis, we extracted total free amino acids from leaves of eight individual plants of NIL DM4.1.3, as well as from eight individuals of susceptible parent HS279, seven days after inoculation with *P. cubensis*. For both genotypes, eight mock-inoculated plants were included as controls. Free amino acid concentrations in leaf extracts were quantified using GC-FID (Figure 4).

Generally, it was found that leaves of both genotypes inoculated with *P. cubensis* had higher amino acid concentrations compared to mock-inoculated leaves. In *P. cubensis* inoculated leaves of genotype NIL DM4.1.3, the concentration of several amino acids was found to be lower than in inoculated leaves of the susceptible parent, indicating that the QTL partially prevents the increase in amino acids. Differences due to genotype, treatment and the interaction were all found to be statistically significant (Two-way MANOVA, $p<0.05$). Between subject effects were tested *post hoc* for each of the individual amino acids, revealing that for nine out of the 19 quantified amino acids (Ala, Gly, Val, Leu, Ile, Thr, Ser, Pro, Gln) there was a significant genotype x treatment interaction ($p<0.05$). For five other amino acids (Asn, Met, Glu, Phe, Trp) there was a significant main effect of the treatment ($p<0.05$) but no significant effect of the genotype ($p>0.05$).

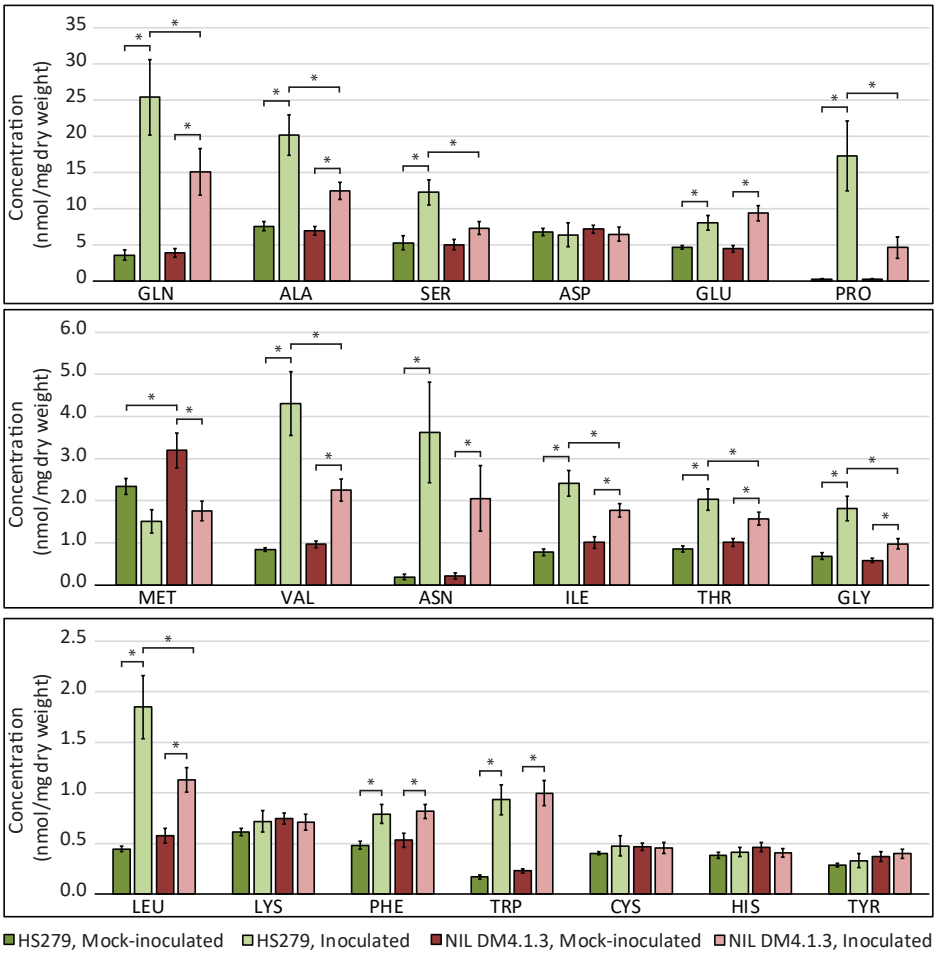

Figure 4 – Amino acid profiling of cucumber leaf extracts
Amino acids were extracted from leaves of cucumber genotypes HS279 (DM susceptible) and NIL DM4.1.3 (partially DM resistant), seven days post inoculation with *P. cubensis* or mock treatment. Free amino acid concentrations were quantified using GC-FID. Coloured bars indicate the average amino acid concentration per amino acid (in nmol per mg dry weight) in each of the genotype-treatment combinations (N = 8 in each group). Error bars indicate standard error of the mean. Differences due to genotype, treatment and the interaction were all found to be statistically significant (Two-way MANOVA, $p<0.05$). Asterisks indicate significant between-subject differences ($p<0.05$).

For the remaining five amino acids (Asp, Cys, Lys, His, Tyr), no significant differences in concentration were observed ($p>0.05$).

Phylogenetic analysis of the *AAP* gene family in several plant species

AAP genes form a gene family with multiple copies in all land plant species characterized so far [37]. To study the evolutionary relationship amongst *AAP* homologs we

constructed a phylogenetic tree using 93 *AAP* homologs identified in ten plant species. AAP protein sequences were identified in the genomes of four cucurbit species (cucumber [*Cucumis sativus*], melon [*Cucumis melo*], watermelon [*Citrullus lanatus*] and zucchini [*Cucurbita pepo*]), as well as the non-cucurbit eudicots *Arabidopsis thaliana*, barrel clover (*Medicago trunculata*) and tomato (*Solanum lycopersicum*), the monocot rice (*Oryza sativa*) and the lower plants *Selaginella moellendorffi* and *Physcomitrella patens.* Additional Data 10 gives an overview of the identified *AAP* proteins.

In the resulting maximum likelihood phylogenetic tree (Figure 5), the grouping of identified AAP homologs is largely consistent with a previously published phylogeny of *AAP* genes [37], as four clades can be observed which can each be subdivided in two subclades. According to the nomenclature proposed by Tegeder & Ward [37], newly identified *AAP* gene homologs are named based on their position in the phylogenetic tree. The candidate gene Csa4M573860 will hereafter be referred to as *CsAAP2A*.

Four out of the five cucumber *AAP* genes, including our candidate gene *CsAAP2A*, belong phylogenetically to subclade 3A, whereas the *CsAAP6* gene belongs phylogenetically to subclade 4B. Clade 1, which includes the *Arabidopsis AtAAP7* gene, does not have any cucumber *AAP* homologs. *Cucurbita pepo* (zucchini) has one Clade 1 orthologue (*CpAAP7*) but apparently the orthologue in this clade was lost in the lineage comprising cucumber, melon, and watermelon.

In the phylogenetic tree (Figure 5) four orthologous groups of cucurbit *AAP* genes can be observed. In three of these orthologous groups, two or three zucchini (*C. pepo*) homologs are basal to the other cucurbit *AAP* genes. Therefore, one melon (*CmAAP2A*) and one watermelon (*ClAAP2A*) orthologue of candidate gene *CsAAP2A* can be identified from the phylogenetic tree, but in other plant species no single *CsAAP2A* orthologue can be inferred, indicating separate gene/genome duplications and/or gene deletion events in the evolutionary branches leading to these species.

Transcript abundance profiling of cucumber *AAP* genes

A previously described RNA-seq dataset consisting of gene expression data from a variety of cucumber tissues of reference accession 'Chinese Long 9930' [38] was analysed to study tissue-specific expression patterns of *AAP* genes in cucumber (Figure 6A). It was found that our candidate gene *CsAAP2A* and *CsAAP2B* were abundantly transcribed in stem tissue, at a slightly lower level in root and hypocotyl tissue, and were barely detectable in cotyledons, leaves, flowers, and fruit. In contrast, *CsAAP3A* was highly expressed in leaf tissue, flowers, fruits, and cotyledon tissue, whereas *CsAAP3B* was generally highly expressed compared to the other *CsAAP* genes in all examined tissues,

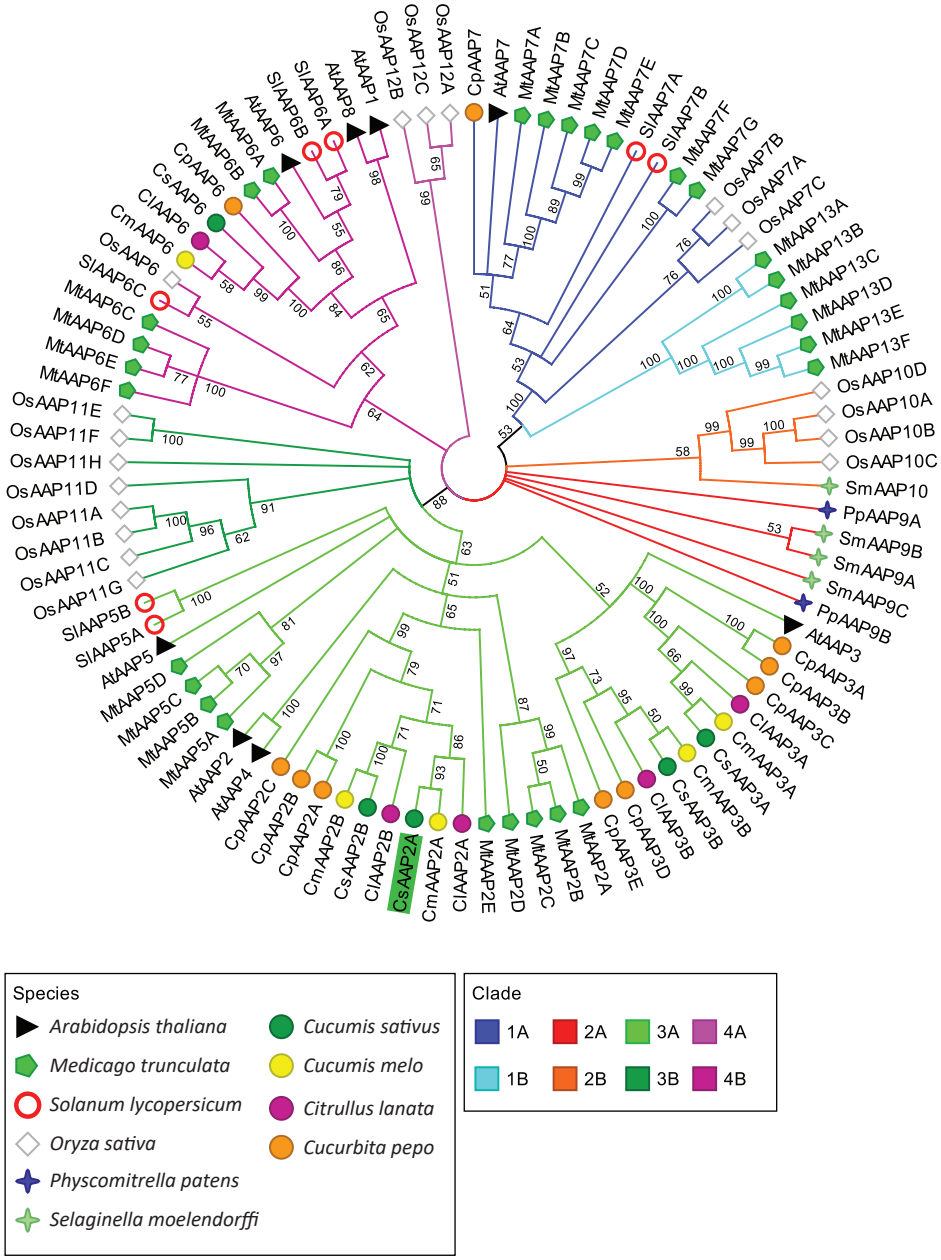

Figure 5 – Phylogenetic analysis of *AAP* genes in ten plant species

A multiple protein sequence alignment was made of putative AAP proteins identified in the genomes of ten plant species. A maximum-likelihood tree was constructed using CLC Genomics Workbench 11, represented here as a cladogram. Numbers at nodes represent bootstrap values in percentages (1000 bootstrap replications). Nodes with bootstrap support values <50% were collapsed. Candidate gene *CsAAP2A* (Csa4M573860.1) is highlighted in green. Accession numbers of sequences in the phylogenetic tree are available in Additional Data 9.

and was especially abundant in root tissue. *CsAAP6* had relatively high expression values in hypocotyl, cotyledon and leaf tissue.

To determine the effects of DM on *AAP* gene expression, cucumber leaves of both genotypes HS279 and NIL DM4.1.3 inoculated with *P. cubensis* or mock-treated leaves were sampled at 1, 3, 5 and 7 days post inoculation (dpi). RNA was isolated, and qRT-PCR was performed on cDNA samples using primers specific for each of the *CsAAP* genes (Figure 6B, Additional Data 11). In genotype HS279, significant treatment x time point interaction effects were found for all *AAP* genes except *CsAAP3A* (Two-way ANOVA, $p<0.05$). For the four other *AAP* genes, a significant reduction in expression (One-way ANOVA, $p<0.05$) was found in *P. cubensis* inoculated leaves compared to mock-treated leaves,

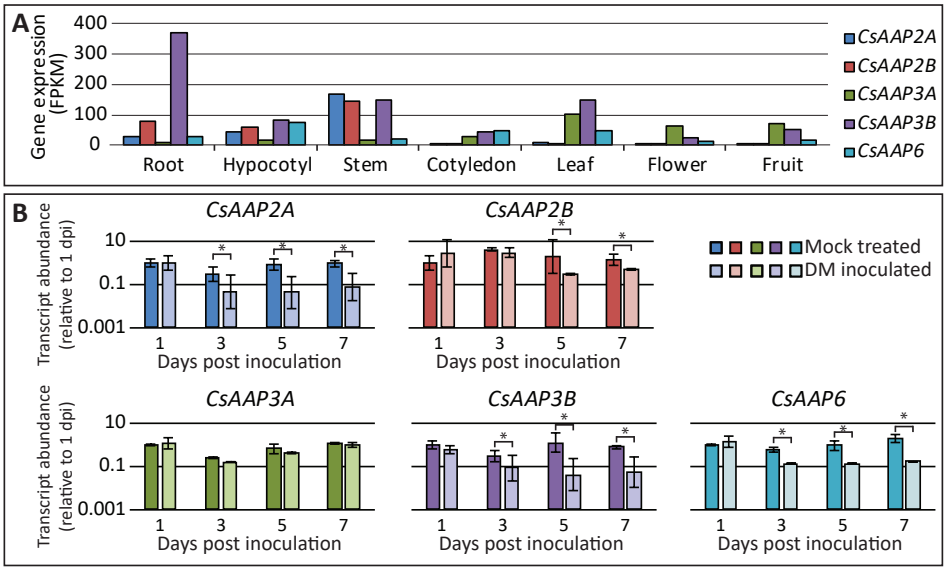

Figure 6 – Expression profile of cucumber *AAP* genes in different tissues and in *Pseudoperonospora cubensis* inoculated leaves

A) The transcript abundance (in number of fragments per kilobase of transcript per million mapped reads, FPKM) of cucumber *AAP* genes in different tissues of 'Chinese Long' inbred line 9930. The amount of independent biological replicates per tissue was either one (hypocotyl, stem and cotyledon), two (root) or three (leaf and fruit). Data from [39].

B) Relative expression level of cucumber *AAP* genes in leaves of DM susceptible cucumber genotype HS279 in response to inoculation with *P. cubensis* or a mock treatment. Relative transcript abundances at 1, 3, 5 and 7 days post inoculation with *P. cubensis* were determined using qRT-PCR. Data were normalized relative to reference gene *TIP41*, and subsequently normalized relative to the average dCt value of mock-treated plants at 1 dpi for each gene. Relative transcript abundances were calculated as 2^{-ddCt}. Each bar shows the relative expression of three biological replicates on a logarithmic scale. Error bars indicate standard deviation. Asterisks indicate significant differences between mock treated and inoculated plants (ANOVA, $P < 0.05$).

starting at 3 dpi for genes *CsAAP2A, CsAAP3B* and *CsAAP6* or at 5 dpi for *CsAAP2B* (Figure 6B). In genotype NIL DM4.1.3, significant treatment x time point interaction effects were again found for all *AAP* genes except for *CsAAP3A* (Two-way ANOVA, $p<0.05$), and a significant reduction in expression expression (One-way ANOVA, $p<0.05$) was found for the other *AAP* genes at several time points (Additional Data 11). Expression of *CsAAP2A* was at every time point and in mock treated as well as *P. cubensis* inoculated leaves reduced by 100-1000 fold in NIL DM4.1.3 compared to HS279, consistent with RNAseq data (Table 1).

DISCUSSION

csaap2a is a likely candidate gene for DM4.1.3 contributing to DM resistance

We have shown here that a loss-of-function allele of the cucumber amino acid transporter *csaap2a* (Csa4M573860) is the most likely candidate gene for the DM4.1.3 locus, contributing to quantitative downy mildew resistance. DM4.1.3 was previously found as a QTL for reduced chlorosis due to *P. cubensis* (**Chapter 4**), and in this study fine-mapped to an interval containing 80 predicted genes (Figure 1).

By means of transcriptomic analysis we identified *CsAAP2a* as a candidate gene for DM4.1.3, as this gene was significantly (ca. 100-fold) downregulated in NIL DM4.1.3 compared to HS279. Our results indicated the presence of a 7,688 bp insertion corresponding to a Mu-like transposon in the DM4.1.3 allele of *CsAAP2A*. Presumably the insertion of this transposon silences expression of the gene, for example by changing the methylation status of the gene and/or by post-transcriptional breakdown of aberrant transposon-containing primary transcripts [39]. Even if the DM4.1.3 allele of *CsAAP2A* would be expressed (e.g. in other tissues or other circumstances than the *P. cubensis* inoculated leaves studied here), the integration of a 7,688 bp insertion in the fourth exon of the gene would truncate the protein, removing over two-thirds of the amino acid sequence, presumably rendering the remaining protein non-functional (Additional Data 9). The finding that a loss-of-function mutation in the *CsAAP2A* gene leads to (partial) resistance leads to the conclusion that *CsAAP2A* should be considered to be a susceptibility gene (S-gene) for downy mildew. This is in agreement with the finding that the DM4.1.3 locus has a recessive effect on disease resistance (**Chapter 4**). To our knowledge, this is the first example of an effect of mutations in amino acid transporter genes on downy mildew resistance.

Whereas *CsAAP2A* appears to be the most likely candidate gene for QTL DM4.1.3, we cannot exclude the possibility that other genes within the fine-mapped interval might

(also) contribute to DM resistance. Twelve other genes within the interval were found to be significantly differentially expressed between genotypes with and without DM4.1.3 (Table 1), although the difference in expression between the two genotypes was several orders of magnitude smaller than the difference observed for *CsAAP2A*. Furthermore five genes within the interval had non-synonymous polymorphisms between genotypes with and without QTL DM4.1.3 (Additional Data 2). Each of these SNPs could potentially have an effect on the function of the encoded proteins, although none of them were predicted to have large effects (e.g. early stop codon). However, further studies would be needed to exclude the possibility that either of these mutations could contribute to DM resistance.

Further fine-mapping experiments could decrease the interval of DM4.1.3, excluding genes as candidate for DM4.1.3. Alternatively, reverse genetic approaches could be helpful to functionally characterize *CsAAP2A*, for example by making knock-out mutants in a DM susceptible genotype using CRISPR-*Cas9* [40] to verify whether this confers partial resistance. Alternatively, (over)expressing *CsAAP2A* in DM4.1.3 background is possible, verifying whether this restores full susceptibility. CRISPR-*Cas9* technology was recently used to generate cucumber mutants with broad virus resistance due to mutations in the *eIF4E* gene [41], exemplifying the applicability of this technique in cucumber resistance breeding. However, such experiments were outside the scope of this current publication.

Amino acid transporters as broadly conserved susceptibility genes

Obligate parasitic organisms are by definition fully dependent on their host to provide them with nutrients. Amino acids, as building block of proteins and precursors for many secondary metabolites, are an important category of nutrients. It should therefore not be surprising that amino acid transporters play important roles in plant-pathogen interactions. This is true for pathogen-encoded amino acid transporters such as the *UfAAT3* gene from the rust fungus *Urmoyces fabae*, which is strongly expressed in haustoria, where it facilitates uptake of amino acids by the pathogen [42]. However, pathogens can also be able to induce host-expressed amino acid transporters, thereby facilitating transport of amino acids towards infected cells, and thus increasing the availability of amino acids for the pathogen. This is best documented for sedentary plant-parasitic nematodes, which induce the formation of highly specialised feeding structures (termed giant cells for root-knot nematodes or syncytia for cyst nematodes) in plant roots from which they acquire nutrients. In roots containing these feeding structures many host-encoded amino acid transporter genes are transcriptionally upregulated [32, 33, 43]. Triggered by these findings two groups studied the interaction of nematodes with *Arabidopsis aap* mutants, and found that reproduction of cyst nematodes

is significantly reduced in *Ataap1*, *Ataap2* and *Ataap6* T-DNA knock-out mutants [33] whereas reproduction of root-knot nematodes is reduced in *Ataap3* and *Ataap6* T-DNA knock-out mutants [34]. Another example of an amino acid transporter which plays an important role in plant-parasite interactions is the *Arabidopsis LHT1* gene, the expression of which is induced by infection with the bacteria *Pseudomonas syringae* as well as the fungi *Erisyphe cichoracearum* (causal agent of powdery mildew) and *Colletotrichum higginsianum* (causal agent of anthracnose). *lht1*-knockout mutants were found to be less susceptible to all three diseases [44].

Several amino acid transporter genes, most notably in the *AAP* and *LHT* families, have now been shown to be susceptibility genes against various pathogens including nematodes [33, 34], bacteria and fungi [44] as well as oomycetes (this research), demonstrating the fact that amino acid transport is important for many unrelated pathogens. In the investigated cases so far, loss-of-function mutations in single amino acid transporter genes were found to confer partial resistance. As plants have a multitude of amino acid transporter genes (e.g. at least 67 genes in *Arabidopsis thaliana* [45]) with partially overlapping substrate specificity and expression profiles, it is to be expected that with regards to their role as S-genes there is functional redundancy between amino acid transporter genes. Therefore, it would be interesting to study the effect of combining loss-of-function alleles of multiple amino acid transporter genes on plant-parasite interactions, which is nowadays feasible thanks to genome editing techniques such as CRISPR-*Cas9* [46].

Downy mildew resistance in cucumber due to *csaap2a* mutations is caused by perturbing pathogen-induced amino acid loading of infected leaves

In plants, one of the major limiting mineral nutrients is nitrogen, as it is required to produce numerous compounds including amino acids, nucleotides as well as several hormones. After inorganic nitrogen (in the form of ammonium or nitrate) is taken up by the roots, it is fixated either directly in the roots or, after transportation of nitrate, in leaves, in an energy-requiring process producing amino acids. Amino acids are the major transport form of organic nitrogen. As plants require several transmembrane transport steps in order for amino acids to flow from source to sink tissues, and additionally different types of amino acids (e.g. basic, neutral and acidic amino acids) require different specificities of transporters, it is not surprising that plants encode a plethora of amino acid transporter genes, divided over ten distinct gene families [47]. One of the most-studied amino acid transporter families is the *Amino Acid Permease* (AAP) family, which generally transport neutral and acidic amino acids, whereas there are also *AAP* genes transporting basic amino acids [37]. Obligate biotrophic pathogens rely on their host to provide them with amino acids, therefore they are dependent on host-encoded

transporters in order to transfer amino acids from source tissues to infected cells. As we study a loss-of-function allele of a cucumber *AAP* gene as a candidate gene contributing to downy mildew resistance, we were triggered to examine the effect of this mutation on amino acid concentrations, in presence as well as in absence of the pathogen. Our results (Figure 4) suggest that the pathogen manipulates the plant to increase amino acid transport towards infected leaves, thereby creating an artificial sink. In contrast, Liu et al. showed that *Arabidopsis* plants inoculated with the powdery mildew causing fungus *Erisyphe cichoracearum* had lower concentrations of several amino acids, especially glutamine, compared to mock-inoculated controls [44]. Apparently *E. cichoracearum* is not able to facilitate amino acid transport towards infected tissue, but rather depletes the existing amino acid pool. Whether this different outcome in the *Arabidopsis-Erisyphe* interaction compared to the cucumber-*Pseudoperonospora* interaction is caused by differences in the pathogen, in the plant host or in both remains to be seen, it might therefore be interesting to study the effect of more pathogen-host combinations on free amino acid concentrations.

Furthermore, our results showed that *CsAAP2A* is involved in amino acid loading of infected leaves, and consequently a loss-of-function mutation leads to decreased amino acid transport, since in inoculated plants the observed increase in amino acids was generally lower in plants with the mutant *csaap2a* allele compared to WT plants (Figure 4). The spectrum of amino acids for which we found significant differences in concentration between both genotypes after inoculation encompassed nine neutral, non-aromatic amino acids, consistent with the previously found specificity of AAP transporters in *Arabidopsis* [45].

An interesting open question is by which mechanism *P. cubensis* is able to increase amino acid transport towards infected leaves. It was shown that several nematode species are able to transcriptionally upregulate *AAP* genes in infected *Arabidopsis* roots [32, 33]. An apparently common mechanism in plant-parasite interactions to influence source-sink relationships is to induce endo-reduplication of host cells, leading to a higher DNA ploidy, which in turn alters the expression of several genes, including many transporter genes [48]. Interestingly, many nutrient sinks (seed endosperm, fruit, trichomes) also undergo endo-reduplication to facilitate increased nutrient transport. Whether downy mildew-causing pathogens are indeed able to induce endo-reduplication is to our knowledge yet unknown.

Whereas this is the first report regarding a role for amino acid transport in plant-downy mildew interactions, other genes involved in amino acid homeostasis have previously been identified to be S-genes for downy mildew. In a forward genetic screen Van Damme

et al. identified several susceptibility genes in *Arabidopsis thaliana* for downy mildew, caused by *Hyaloperonospora arabidopsidis* [30]. One of these genes, *DMR1*, was later found to encode Homoserine Kinase (HSK), a key enzyme in the metabolic pathway converting the amino acid precursor homoserine to threonine, isoleucine and methionine [49]. Missense mutations in *DMR1* did result in elevated levels of homoserine, as was to be expected, but did surprisingly not result in depletion of downstream amino acids, presumably due to a feedback mechanism. It was shown by exogenous homoserine application on wild-type *Arabidopsis* that homoserine either induces defences against *H. arabidopsidis* or has a toxic effect on the pathogen [49]. Later, Stuttmann et al. identified two additional genes involved in amino acid metabolism with effects on downy mildew susceptibility in *Arabidopsis*, i.e. *Aspartate Kinase 2* (*AK2*) and *Dihydrodipicolinate Synthase 2* (*DHDPS2*). Mutant alleles of both genes have increased concentrations of the amino acids lysine, threonine, methionine and isoleucine, leading to resistance [50]. In all three cases (*DMR1, AK2* and *DHDPS2*) it is an overabundance rather than a shortage of certain amino acids causing DM resistance, which is fundamentally different from our findings regarding *CsAAP2A*. Nevertheless, imbalances of amino acid homeostasis seem to have a large effect on host compatibility with DM causing oomycetes.

A CUMULE transposon insertion is responsible for the c*saap2a* loss-of-function allele

The DM4.1.3 allele of the *CsAAP2A* gene contains a Mu-like transposable element (MULE) (Figure 3D). Apart from a *MudrA*-like transposase gene which is characteristic of MULEs [51], the element amplified from *CsAAP2A* also contained an ubiquitin-like protease (*Ulp1*). MULEs containing *Ulp1* sequences were first described in melon (*Cucumis melo*) and are therefore called *Cucumis Mutator*-like element (CUMULE), although CUMULE transposons were also identified in the *Arabidopsis* and rice genomes [35]. Although it is not clear what the benefit of expressing *ULP1* is for transposable elements, it is speculated that ULP1 might downregulate proteins involved in TE control and/or that it is involved in repair of double stranded breaks left after the excision of the transposon [39]. Apart from the two coding parts (*MudrA* and *Ulp1*) of the *CsAAP2*-CUMULE, we also identified a tandem repeat region consisting of nine full and one partial repeat of a 178 bp satellite (Additional Data 6). To our knowledge, such a tandem repeat region is not previously described for Mu-like elements. Whether this tandem repeat has a function for the CUMULE or is merely the result of errors during DNA replication is unknown. The fact that it is located directly upstream of the *Ulp1* gene (Figure 3D) might imply that it is part of the *Ulp1* promoter, and as such have a regulatory role on *Ulp1* expression [52]. Upstream of the tandem repeat region, a short (408 bp) ORF was found to be expressed (Additional Data 5) with no apparent similarity to any known gene. Whether this ORF indeed encodes a protein or is also part of the *Ulp1* promoter remains to be seen.

We identified numerous BLAST hits of elements of the newly identified CUMULE transposon in the cucumber reference genome (Chinese Long 9930 v2 [53]), indicating that this type of transposon is rather common in the cucumber genome, as was to be expected since it has been found before to be present in several *Cucumis* species [35]. The fact that multiple copies of CUMULE transposons, presumably with high sequence homology to one another, are present in the cucumber genome indicates that it is very likely that not all of the reads which we aligned to the newly identified CUMULE insertion in *CsAAP2A* (Additional Data 5) represent transcriptional activity of this particular insertion, but a proportion of this reads will represent other insertions of similar transposons. This also explains the presence of several variable nucleotides ("heterozygous SNPs") in the aligned RNAseq reads (Additional Data 5), which will represent SNPs between various insertions of CUMULE transposons rather than multiple alleles at the same locus of this homozygous inbred line. The fact that even with multiple copies the *MudrA* gene was found to be very lowly expressed (Additional Data 5) indicates that this type of transposons is not very active under the investigated circumstances.

The newly identified *csaap2a* allele was not found in a resequenced core collection of 115 cucumber genotypes [36] (Additional Data 8). This indicates that this *csaap2a* allele is rather rare, which is in agreement with the fact that we identified it in a genotype (NIL DM4.1.3) inheriting its resistance from accession PI 197088, which was only recently identified as one of the most promising sources of DM resistance [15].

Phylogenetic analysis of *AAP* genes

Previously Tegeder and Ward (2012) identified *AAP* homologs in five plant species, containing representatives of non-vascular land plants, non-seed vascular plants, monocots and eudicots, but could not identify *AAP* homologs in algal species, indicating that *AAP* genes evolved in land plants [37]. A phylogenetic tree of *AAP* genes showed separation of *AAP* homologs in four clades, three of which were subdivided in two subclades. In this study, we extended the previously published phylogenetic tree by identifying *AAP* homologs in four cucurbit species with published genomes (cucumber, melon, watermelon and zucchini) as well as tomato.

Whereas we could reproduce the previous findings of Tegeder and Ward (2012) regarding the lower plants *Physcomitrella patens* and *Selaginella moelendorffi*, rice and *Arabidopsis thaliana*, we identified far more *AAP* homologs in barrel clover (*Medicago trunculata*) than previously described (Additional data 10). Previously, 11 *AAP* homologs were described in *M. trunculata*, but in our analysis 28 unique full-length *AAP* proteins were identified. Presumably the cause of this discrepancy is the fact that we identified *AAP* homologs in an improved genome assembly (Mt4.0) which was not yet available

during the previous phylogenetic analysis [54]. The finding that a new, improved, genome assembly leads to the identification of so many additional *AAP* homologs could imply that in other species also more *AAP* homologs exist than are currently identified. As *M. trunculata* is a legume species, which can fixate nitrogen through symbiosis with rhizobia and therefore have high protein contents compared to other species [54], it is tempting to speculate that the expansion of the *AAP* gene family in *M. trunculata* compared to other plant species is associated with the specific needs regarding increased amino acid transport in nitrogen-fixating organisms.

In the resulting ML phylogenetic tree, clustering of *AAP* homologs was largely in line with the previously published phylogeny [37], the main exceptions being that Clade 2A (containing the majority of lower plant *AAP* homologs) was polyphyletic in our analysis, and six of the newly identified *Medicago trunculata AAP* homologs formed an additional subclade (Clade 1B) which was previously not described. Lower than average (five) numbers of *AAP* homologs were identified in the cucurbit species cucumber, melon and watermelon. The zucchini genome contained double the amount of *AAP* homologs compared to the other cucurbit species, in line with the reported whole-genome duplication in *Cucurbita* species compared to other Cucurbitacea [55]. Four out of the five cucumber *AAP* genes, including *CsAAP2A*, belong phylogenetically to subclade 3A. This subclade also includes four *Arabidopsis AAP* genes which are all involved in phloem loading, i.e. transport of amino acids to the phloem, either in source tissues or along the vasculature to import amino acids from the xylem to the phloem [37]. It seems therefore reasonable to speculate that these cucumber genes might also be involved in phloem loading. No cucumber, melon or watermelon homologs were found in Clade 1, even though all other seed plants in the phylogeny had at least one Clade 1 homolog. The function of Clade 1 *AAP* homologs is currently unknown, as none of these genes was functionally characterised. It is interesting however that the studied cucurbit species, with the exception of zucchini, can apparently function without a Clade 1 ortholog, indicating that either there is functional redundancy between Clade 1 *AAP* genes and *AAP* genes in other clades, or Clade 1 *AAP* genes are involved in a process which is not essential for cucurbit biology. The remaining *CsAAP6* gene belongs phylogenetically to subclade 4B, which includes both a gene localized to xylem parenchyma (*AtAAP6*) as well as two genes involved in loading of amino acids in seeds (*AtAAP1* and *AtAAP8*) [37].

Whereas cucurbit *AAP* genes form orthologous groups in the phylogenetic tree (Figure 5), as is to be expected based on their evolutionary relatedness, orthologous relations with *AAP* genes in non-cucurbit species are more difficult to establish, due to subsequent gene duplication events and gene loss in the different evolutionary lineages represented by the different species in the phylogenetic tree. This has consequences

for the possibility to study the effect of mutations in *AAP* genes on host-pathogen interactions in other species, as we suspect there will be some functional redundancy between paralogs within the same (sub)clade. However, our findings indicate that a loss-of-function mutation in *CsAAP2A* causes partial resistance to DM, even though the cucumber genome contains a closely related paralogue, *CsAAP2B.* It would be interesting to study the effect of double *csaap2a/csaap2b* mutations on DM resistance, as this might have an increased effect compared to the single *csaap2a* mutation described in this paper. However, double mutations might also be expected to cause pleiotropic effects on the plants fitness, as is often the case for mutations in *S* genes [25].

Expression analysis of cucumber *AAP* genes

One of the proposed reasons for plants to encode multiple *AAP* genes is that there are many different steps in amino acid transport, including export from source cells, import to phloem cells, xylem-to-phloem transfer along the vasculature, as well as import in sink cells, which should be regulated independently [47]. Therefore, it is interesting to look at the tissue-specific expression profile of cucumber *AAP* genes to learn more about the function of each of the *AAP* genes. *CsAAP2A,* as well as its most closely related paralog *CsAAP2B*, were especially abundantly transcribed in stem tissue, which is consistent with a hypothesis in which *CsAAP2A* and *CsAAP2B* are involved in xylem-to-phloem transport of amino acids along the vasculature, comparable to *Arabidopsis AtAAP2* and *AtAAP4* genes [56]. It was shown that the *Arabidopsis* AtAAP2 transporter is crucial for xylem-to-phloem transport of amino acids, and that an *ataap2* loss-of-function mutant had drastic changes in source-sink translocation of amino acids [57]. It is therefore reasonable to assume that our candidate gene *CsAAP2A* is also important for source-sink transport of amino acids, which is in agreement with our findings regarding amino acid transport to *P. cubensis* infected leaves (Figure 4).

Recently, Burkhardt & Day conducted a transcriptome analysis of PI 197088 in a time course of *P. cubensis* infection, comparing gene expression in resistant PI 197088 with that in susceptible cv. 'Vlaspik'. They found that in both genotypes thousands of genes were differentially expressed in *P. cubensis* inoculated plants compared to mock-inoculated plants. Genes downregulated in PI 197088 at early time points included several genes involved in nutrient transport, indicating that host-driven restriction in nutrient availability to the pathogen plays an important role in slowing down pathogen growth in PI 197088 [58]. We were therefore interested to see the dynamics of *AAP* gene expression in leaves inoculated with *P. cubensis*.

We found a significant reduction of expression of cucumber *AAP* genes in inoculated plants compared to mock treated plants in both the susceptible genotype HS279 and

the partial resistant NIL DM4.1.3 (Figure 6B, Additional data 11). Whether this reduction in gene expression in leaves is biologically relevant for genes *CsAAP2A* and *CsAAP2B* is questionable, as the basal expression of both genes is already very low in leaf tissue (Figure 6A). However, the finding that *AAP* genes in general, and especially *CsAAP3B* and *CsAAP6*, were downregulated upon inoculation with *P. cubensis* is striking. This is in clear contrast with the earlier findings in nematode-inoculated *Arabidopsis* roots, in which *AAP* genes are actively upregulated, presumably by the parasite, in order to increase amino acid availability [33, 34]. A plausible explanation would be that the host plant actively downregulates *AAP* genes upon pathogen sensing, to restrict nutrient flow towards pathogen-infected cells, which would be in agreement with the findings of Burkhardt & Day that host-driven nutrient availability restriction is an important component of cucumber defence against *P. cubensis* [58]. Alternatively, it is also possible that the *AAP* genes which were downregulated in inoculated leaves encode transporters which export amino acids from source leaves towards the phloem, and as such downregulation of those genes would decrease amino acid transport from the leaves, increasing the amino acid concentration in inoculated leaves. Under this second hypothesis, downregulation of these genes would be beneficial to the pathogen, rather than to the host plant. Functional analysis of each of the *AAP* genes (e.g. by making loss-of-function mutations in individual *AAP* genes using CRISPR-*Cas9*) would be needed in order to test whether these genes contribute to susceptibility or rather to resistance against *P. cubensis*.

Quantitative cucumber DM resistance: future prospects

Resistance to DM in the highly resistant cucumber accession PI 197088 was shown to be inherited quantitatively, with up to eleven QTL [17–19]. We have evidence that one of these QTL, DM4.1, consists of several linked subQTL, each contributing for a small part to DM resistance, further exemplifying the highly polygenic nature of PI 197088 DM resistance (**Chapter 4**). In this report we identified a mutation in the *CsAAP2A* gene as potentially causal to subQTL DM4.1.3. As *CsAAP2A* encodes an amino acid transporter gene, and we have shown that plants with DM4.1.3 resistance have generally lower concentrations of amino acids after DM inoculation, we postulate that DM4.1.3-based resistance is due to a limited availability of amino acids to the pathogen. Previously, we identified a receptor-like kinase putatively involved in oligogalacturonan-signalling as a likely candidate gene for another PI 197088-derived subQTL, DM4.1.2 (**Chapter 4**). For another DM resistance locus (*dm-1* from PI 197087) a missense mutation in the *CsSGR* gene, involved in chlorophyll degradation was found to be a likely candidate [13]. There appear to be no functional relations between these three candidate genes for DM resistance, indicating that cucumber resistance to *P. cubensis* is a complex phenomenon dependent on many seemingly unrelated genes and pathways. As recently reviewed

by Corwin and Kliebenstein (2017), quantitative, broad spectrum resistance is not predominantly dependent on signalling of pathogens, such as is the case for qualitative resistance by dominant R genes, but extends far beyond that, and is influenced by many genes involved in diverse pathways leading to for example cell wall strengthening and defence compound synthesis [22]. The polygenic nature of DM resistance in cucumber makes it a suitable study object to further understand the complex mechanisms behind quantitative disease resistance.

Identification of causal genes for quantitative DM resistance will be of great importance for cucumber breeding, as it enables the use of molecular markers closely linked to the causal mutation, thereby decreasing linkage drag. Furthermore the identification of causal genes makes it possible to verify whether DM resistance QTL in other sources of resistance such as PI 605996, PI 330628, PI 197086 and PI 605924 [15] are allelic to the resistance provided by PI 197088 or represent other causal genes which might be combined to obtain higher levels of resistance. Interestingly, PI 330628 (WI7120) was recently shown to have a major QTL on chromosome 4 contributing to DM resistance as well, explaining 20.9-50.7% of the phenotypic variance [59]. Fine-mapping and identification of potential candidate genes of this QTL might improve our understanding of the genetic architecture of cucumber DM resistance, and inform us whether it is possible to combine resistances derived from both genotypes.

METHODS

Plant materials and growing conditions

Four cucumber genotypes were used in experiments for this publication. Plant introduction line PI 197088, highly resistant to DM caused by *Pseudoperonospora cubensis* [15], was originally collected in Assam, India on April 16th 1951 and is maintained by the United States National Plant Germplasm System (NPGS). Breeding line HS279 is a pickling type cucumber, susceptible to DM, with good horticultural characteristics. From a cross between these genotypes, F3BC3S3 mapping populations were developed using HS279 as recurrent parent. Near isogenic lines (NILs) DM4.1 and DM4.1.3 are derived from F3BC3S3 individuals with PI 197088-derived introgressions on chromosome 4 corresponding to the interval Chr4:11,479,953-20,438,834 and Chr4:18,469,868-23,424,119, respectively.

Plants were grown in climate chambers with temperatures of 22 °C (day) and 17 °C (night), with a 16/8h day/night cycle, and a relative humidity of 80%.

P. cubensis inoculum maintenance and disease tests

An isolate of *P. cubensis* obtained from an infected cucumber field in Haelen, the Netherlands, was maintained on fully expanded cucumber leaves, healthy in appearance before inoculation. Detached leaves were kept in closed boxes containing water-soaked paper towels, and inoculated with a spore suspension developed as described below. Boxes containing inoculated cucumber leaves were kept in a climate chamber under 18 °C (day) and 15 °C (night), with a 16/8h day/night cycle for ten days. Heavily infected detached leaves were preserved at -20 °C as inoculum source for <6 months. Spore suspensions were produced by washing spores from frozen infected leaves using tap water, and filtering through cheesecloth. The spore concentration was measured using a haemocytometer, and adjusted to $1x10^4$ spores/ml.

Cucumber plants for *P. cubensis* disease tests were grown in plastic tents, which were closed the day before inoculation to ensure a high relative humidity. Both sides of cucumber leaves were sprayed with spore suspension prepared as described above. After inoculation, plants were left in darkness at 18/15°C (day/night) for 24 hours in closed plastic tents. Starting from seven days post inoculation, yellowing (chlorosis), sporulation and collapsing (necrosis) of leaves were assessed by eye on a 1-9 scale, 9 being fully resistant and 1 being fully susceptible.

Identification and sequencing of CUMULE transposon

DNA was isolated from cucumber leaf samples originating from genotypes PI 197088, HS279, NIL DM4.1 and NIL DM4.1.3 using a CTAB protocol [60]. A PCR was performed on the isolated DNA samples using primers AAP-clon-TE-F and AAP-clon-TE-R as specified in Additional Data 12. In each PCR reaction, 5 µL of template DNA, 2.5 µL of each primer, 1 µL of dNTP, 10 µL of Phusion HF buffer, 28.5 µL of MQ water, and 0.5 µL of Phusion high-fidelity polymerase (Thermo Fisher Scientific, U.S.A.) were mixed. Cycling conditions were: 30 s initial denaturation at 98 °C, followed by 30 cycles of 10 s denaturation at 98 °C, 20 s annealing at 60 °C, and 3 min 30 s extension at 72 °C, followed by a final incubation of 7 min at 72 °C. PCR products were visualized with GelRed staining and analysed by agarose gel electrophoresis. PCR products were cloned using the Zero Blunt TOPO PCR Cloning Kit (Thermo Fisher Scientific, U.S.A.) according to the manufacturer's instructions, and transformed to electrocompetent *Escherichia coli* cells, strain DH5α. Presence of the insert was checked by using primer pairs AAP-clon-TE-F plus AAP-colPCR-R1 and AAP-colPCR-F2 plus AAP-colPCR-R2 (Additional Data 12) on 1 µL of bacterial culture in a 10 µL DreamTaq DNA polymerase (Thermo Fisher Scientific) PCR reaction according to manufacturer's instructions. 12 individual *E. coli* cultures with confirmed AAP-TE inserts were grown in liquid LB culture and plasmids were recovered using the Qiaprep spin miniprep kit (Qiagen, Germany). Plasmids were sent for Sanger sequencing (GATC Biotech, Germany), first using

primers AAP-clon-TE-F and AAP-clon-TE-R, subsequently using primers designed based on the previous sequencing runs. Additional Data 12 gives an overview of the 57 designed sequencing primers. Obtained reads were trimmed and aligned to one another using CLC Genomics Workbench v11. A consensus sequence was built based on majority rule.

For annotation, previously described RNAseq reads of NIL DM4.1.3 were aligned to the consensus TE sequence using TopHat (v2.1.1) [61]. The alignment of RNAseq reads to the TE sequence was manually checked using IGV (v2.3.32) [62]. Alignment of repetitive regions and prediction of ORFs was done using CLC Genomics Workbench v11. BLASTn and BLASTp searches of ORFs and translated ORFs were performed at the NCBI BLAST server.

To check for the occurrence of similar TEs in the cucumber reference genome, BLASTn searches with annotated regions of the TE sequence were performed against the cucumber reference genome, Chinese Long 9930 v2.

To check for occurrence of the CUMULE insertion at the *CsAAP2A* locus in cucumber germplasm, reads of the resequencing project of 115 cucumber accessions by Qi et al. [36] were downloaded from the NCBI short read archive, accession SRA056480. By a simple BASH script, total reads were screened for the presence of 30 bp sequences comprised of:

1.) The last 15 bp of *CsAAP2A* before the TE insertion and the first 15 bp of the TE insertion, in forward (5'-CCAAAAGACATGGGAAATTGACAAAAATAGG-3') or reverse (5'-CCTATTTTTGTCAATTTCCCATGTCTTTTGG-3') orientation;
2.) The last 15 bp of the TE insertion and the first 15 bp of *CsAAP2A* after the TE insertion, in forward (5'-GGCCTATTTTTGTGAATTCCCCAAAAGACAT-3') or reverse (5'-ATGTCTTTTGGGGAATTCACAAAAATAGGCC-3') orientation;
3.) The 30 bp of *CsAAP2A* surrounding the TE insertion site, without TE sequence, in forward (5'- CCCATTCATGATGTCTTTTGGAGTTGTGGAA-3') or reverse (5'-TTCCACAACTCCAAAAGACATCATGAATGGG-3') orientation.

The number of detected reads per accession for each of these six sequences was stored as a tabular file. The genotype of the accessions was determined to be either homozygous TE-allele, homozygous WT-allele or heterozygous.

Amino acid profiling of *P. cubensis* inoculated cucumber plants

For amino acid profiling, 16-day old cucumber plants of genotypes NIL DM4.1.3 and HS279 were inoculated with *P. cubensis* as described above, or a mock treatment con-

sisting of spraying the leaves with tap water instead of spore suspension. Eight days post inoculation, inoculated leafs were sampled and directly frozen in liquid nitrogen. Frozen leaves were ground in liquid nitrogen, and ca. 100-200 mg aliquots were taken for amino acid extraction. Total amino acids were extracted in 750 µL 80% ethanol at 50°C in an ultrasonic bath for 30 minutes. Samples were spun down, the supernatants were collected, a second extraction was performed similarly as described above on the pellet, after which both supernatants per sample were pooled. Solvent was removed by freeze-drying, after which extracts were re-suspended in 100 µL 80% ethanol.

Free amino acids in amino acid extracts were cleaned and derivatized using the EZ:faast GC/FID kit (Phenomenex, U.S.A.). Derivatized samples were injected on an Agilent Technologies 7890A GC system with a FID detector using an Agilent Technologies 7683B series injector. Agilent Chemstation software was used to call peaks in the resulting chromatograms, and to determine the peak area per amino acid.

Phylogenetic analysis

Protein sequences annotated as AAP genes in the *Arabidopsis thaliana* genome were obtained from The Arabidopsis Information Resource (TAIR) on www.arabidopsis.org [accessed on 22-02-2019]. The eight Arabidopsis AAP proteins (AtAAP1-AtAAP8) were used as queries for BLASTp searches against reference genomes of *Medicago trunculata* (Mt4.0v1) [54], *Oryza sativa* (v7_JGI) [63], *Physcomitrella patens* (v3.3) [64], *Selaginella moellendorffii* (v1.0) [65], *Solanum lycopersicum* (ITAG2.40) [66], *Cucumis sativus* (Chinese Long 9930 v2) [53], *Cucumis melo* (cv. DHL92 v3.5.1) [67], *Citrullus lanatus* subsp. *vulgaris* (cv. 97103 v1.0) [68] and *Cucurbita pepo* subsp. *pepo* (Zucchini v4.1) [55]. Identified BLASTp hits (with e-value < 1E-10) were reciprocally used as queries against the *Arabidopsis thaliana* (TAIR10) genome [69] in order to select genes for which the best hit was an *Arabidopsis AAP* gene. The resulting list was manually curated to concatenate short predicted genes with subsequent gene identifiers (as they were most likely gene prediction errors), split a very long predicted gene, and remove remaining short (<300 AA) genes. Protein sequences were aligned to one another using CLC Genomics Workbench v11 using standard parameters. A maximum likelihood phylogeny was determined using CLC Genomics Workbench v11 using standard settings, and 1000 bootstrap replicates.

Expression analysis of *AAP* genes in cucumber using qRT-PCR

For expression analysis, plants of genotypes HS279 and NIL DM4.1.3 were inoculated with *P. cubensis* as described above, or mock treated. At one, three, five and seven days post treatment, leaf samples were taken of three individual plants per genotype/treatment, and immediately frozen in liquid nitrogen. RNA isolation, cDNA synthesis

and qRT-PCR were performed as previously described [31]. For quantification of *AAP* expression, we used the primer sequences as described in Additional Data 12. Primer pairs specific for the cucumber reference gene *TIP41*, as described by Warzybok et al. [70], were used for normalization of expression. Two technical replicates were taken for each sample/gene combination. Ct values per sample were normalised by subtracting the geometric mean of the Ct values for *TIP41*, giving deltaCt, abbreviated as dCt. dCt values were subsequently normalized by subtracting the average dCt value for each gene in mock-inoculated HS279 plants at 1 dpi, giving ddCt. Averages and standard deviations of ddCt values were calculated over three biological replicates. Normality of ddCt distributions was tested using Shapiro-Wilk tests ($P > 0.05$). Differences in ddCt value between time points were analysed with two-way ANOVA tests. Homogeneity of variances were tested using Levene's test. If ANOVA tests showed a significant ($p < 0.05$) treatment x time point interaction effect, one-way ANOVA tests were carried out to test for significance of simple main effects of treatment per time point ($p < 0.05$). All statistical analyses were performed using SPSS v23 software (IBM). Relative transcript abundances were calculated as 2^{-ddCt} for plotting.

AVAILABILITY OF DATA AND MATERIAL

The RNAseq dataset analysed in this manuscript was submitted to NCBI SRA under accession number PRJNA544259 (to be released on publication).

All other data generated or analysed during this study are included in this published article and its additional data files.

AUTHORS' CONTRIBUTIONS

FWKH, FB and WHF developed plant materials and (fine)mapped the DM 4.1.3 locus. HA performed the qRT-PCR experiment. JAB designed, performed and analysed the rest of the experiments, with valuable suggestions by HJS and YB. JAB drafted the manuscript. WHV, RGFV, YB and HJS gave critical feedback on the draft manuscript. All authors read and approved the manuscript.

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ADDITIONAL DATA

All figures and additional datafiles described in this chapter were deposited online, and can be accesed through doi:10.4121/uuid:9b31358d-b8da-449a-8ae8-0fea922bc186

Additional Data 1

Format: XLSX

RNAseq transcript abundance values (in absolute numbers of uniquely mapped fragments, calculated using HTSeq [71]) of 95 genes (when applicable with multiple predicted splice isoforms) in the DM4.1.3 locus in genotypes HS279 and NIL DM4.1.3 (three biological replicates per genotype) three days post inoculation with *Pseudoperonospora cubensis*. Average numbers of fragments per gene, as well as the average fold change between the two genotypes are given. Significance of differential expression (BH-adjusted p value) as determined with R package DEseq2 [72] is given.

Additional Data 2

Format: XLSX

Non-synonymous SNPs between genotypes HS279 and DM4.1.3 in genes within the DM4.1.3 locus, as determined using the SnpEff [73] algorithm. Location of the SNP based on the reference genome (Chinese Long 9930 v2) is given, the allele in both genotypes, the effect (amino acid substitution) on the encoded protein as well as the Gene ID, and the annotation of the gene in which the SNP occurs.

Additional Data 3

Format: PDF

Whole genome sequencing (WGS) data (PE reads, 100 bp) of cucumber genotypes NIL DM4.1 and HS279, as well as RNAseq data (PE reads, 100 bp) of genotype HS279 were manually inspected using Integrative Genomics Viewer software [62], lay-out as described in Figure 2. Exons of the *CsAAP2A* gene (Csa4M573860.1) are indicated above as black boxes. A red box indicates the part of the *CsAAP2A* alignment which is represented in Figure 2 in more detail. The RNAseq data of NIL DM4.1 are not shown, because of the very low transcript abundance for the *CsAAP2A* gene.

Additional Data 4

Format: PDF

Nucleotide sequence alignment of the first 118 bp of the CUMULE transposon sequence identified in the DM4.1.3 allele of the *CsAAP2A* gene with the reverse complement of the last 118 bp of the transposon sequence, indicating a near-perfect terminal inverted repeat.

Additional Data 5

Format: PDF

Alignment of RNAseq data (PE reads, 100 bp) of cucumber genotype NIL DM4.1.3 to the newly identified CUMULE transposon sequence was manually inspected using Integrative Genomics Viewer software [62], graphical representation as described in Figure 2. Reads for which the other mate in the mate pair could not be mapped on the transposon are highlighted with red outlines. Below the alignment, a schematic representation of the annotated CUMULE transposon (as in Figure 3B) is drawn to scale.

Additional Data 6

Format: PDF

Multiple nucleotide sequence alignment of the ten satellites in the tandem repeat region identified in the CUMULE transposon in *CsAAP2A*. A bar graph indicates the percentage conservation between the ten repeats per bp.

Additional Data 7 – BLAST output of CUMULE elements in reference genome

Format: XLSX

A BLASTn search was performed using the 118 bp (5') TIR domain, the 2205 bp predicted *MudrA* gene and the 1599 bp predicted *Ulp1* gene from the CUMULE transposon as queries against the cucumber reference genome (Chinese long 9930 v2). Tabular blast output (standard BLAST output format 6) is given.

Additional Data 8 – In silico screen of presence-absence TE allele in 115 genomes

Format: XLSX

Total reads of 115 recently resequenced cucumber accessions [36] were assayed in silico for the presence of reads indicating the allele of *CsAAP2A* characterised by the insertion of the CUMULE TE insertion, or presence of reads indicative of the wild-type allele. The number of reads indicating presence of either the TE-allele or the WT-allele of *CsAAP2A* are given. Accession codes of the 115 genotypes were obtained from [36].

Additional Data 9 – Alignment of predicted AAP protein sequences with/ without TE insertion

Format: PDF

Multiple amino acid sequence alignment of the predicted sequence of the WT *CsAAP2A* gene with the allele encoded by the TE-allele of the *CsAAP2A* gene identified in the DM4.1.3 locus.

Additional Data 10 – Identified AAP homologs in ten plant species

Format: XLSX

AAP genes identified in the genomes of *Arabidopsis thaliana*, *Medicago trunculata*, *Oryza sativa*, *Physcomitrella patens*, *Selaginella moellendorffii*, *Solanum lycopersicum*, *Cucumis sativus*, *Cucumis melo*, *Citrullus lanatus* subsp. *vulgaris* and *Cucurbita pepo* subsp. *pepo*. Gene name abbreviations as used in Figure 5 are given, plus the gene accession codes of the respective genome annotations, the clade to which the gene belongs according to the phylogenetic analysis described in Figure 5, and the size of the predicted protein (in number of amino acids).

Additional Data 11 – Expression profile of cucumber AAP genes in Pseudoperonospora cubensis inoculated leaves of genotype NIL DM4.1.3

Format: PDF

Relative expression level of cucumber *AAP* genes in leaves of partial DM resistant cucumber genotype NIL DM4.1.3 in response to inoculation with *P.* cubensis or a mock treatment. Relative transcript abundances at 1, 3, 5 and 7 days post inoculation with *P. cubensis* were determined using qRT-PCR. Data were normalized relative to reference gene *TIP41*, and subsequently normalized relative to the average dCt value of mock-treated HS279 plants (Figure 6B) at 1 dpi for each gene. Relative transcript abundances were calculated as 2^{-ddCt}. Each bar shows the relative expression of three biological replicates on a logarithmic scale. Note that the Y-axis for *CsAAP2A* is different compared to the other genes, as the gene was barely detectable by qRT-PCR in this genotype. Error bars indicate standard deviation. Asterisks indicate significant differences between mock treated and inoculated plants (ANOVA, $P < 0.05$).

Additional Data 12 – List of used primers

Format: XLSX

A list of primers used in this manuscript. Names as used in this manuscript are given, plus the nucleotide sequence of the primer, in 5' to 3' orientation.

Chapter 6

General Discussion

In this thesis, the interactions between cucumber and two of its most devastating pathogens are described: powdery mildew (PM), caused by the fungi *Podosphaera xanthii* and *Golovinomyces orontii*, and downy mildew (DM), caused by the oomycete *Pseudoperonospora cubensis*. Whereas the pathogens causing these diseases share similar biotrophic lifestyles and superficially resemble each other, e.g. by their hyphal growth and the formation of intracellular haustoria to take up nutrients, they have different evolutionary histories, leading to a completely different genetic make-up and physiochemical properties, and furthermore they infect different host cell-types. Therefore, to a large extent different genes are involved in resistance against both groups of pathogens. Our special interest was in the identification of susceptibility genes (*S*-genes), mutant alleles of which cause recessively inherited resistance. Resistance based on disabled *S*-genes is thought to be more durable compared to resistance based on dominant, race specific *R*-genes [1]. Moreover, most sources of resistance to these mildews in cucumber are recessively inherited [e.g. 2–4], in contrast to many other pathosystems, and as such a focus on *S*-genes appeared justified.

Our approach was to characterize existing sources of resistance against both diseases, in order to identify causal genes underlying those resistances. By identifying the genes involved in mildew resistance and susceptibility, we aimed at better understanding the complex interactions between cucumber and its pathogens. Furthermore, the identification of causal genes facilitates the development of molecular markers closely linked or even within the resistance gene, limiting linkage drag and the possibility of recombination between markers and resistance.

THE ROLE OF *MLO* GENES AS SUSCEPTIBILITY GENES IN CUCUMBER AND OTHER SPECIES

As *MLO* genes are one of the oldest and best-known examples of susceptibility (S) genes, conferring PM resistance in barley for over seven decades, our first experiments aimed at understanding the role of cucumber *MLO* genes in PM resistance. The most important PM resistance in cucumber is the so-called "hypocotyl resistance", a monogenic, recessively inherited trait which is frequently used in cucumber breeding for several decades without any indications of the pathogen overcoming this resistance [2]. We hypothesized that this resistance might be caused by a loss-of-function allele of the gene *CsaMLO8*, as it has a physical location on the genome consistent with mapped QTL for hypocotyl resistance [5]. Indeed, we found that the hypocotyl resistance is associated with a mutation in the *CsaMLO8* gene. The mutation was caused by the insertion of a transposable element in the coding domain of the gene, causing aberrant splicing of the

mRNA. We showed that heterologous overexpression of the wild-type *CsaMLO8* allele in a tomato *mlo* mutant resulted in full restoration of PM growth of *Oidium neolycopersici*. This proofs that *CsaMLO8* is a functional *S*-gene to PM. The *CsaMLO8* mutant allele with the transposable element, however, failed to restore susceptibility of the tomato *mlo* mutant to *O. neolycopersici*. This shows the causal mutation for the detected hypocotyl resistance **(Chapter 2)**. Subsequently we studied two other, highly related, cucumber *MLO* homologs, *CsaMLO1* and *CsaMLO11*, and found that all three cucumber clade V *MLO* genes were able to restore PM susceptibility in the tomato *mlo* mutant **(Chapter 3)**.

Identification of clade V *MLO* genes in cucumber and other species

After the barley *MLO* gene was cloned, it was found homologues genes could be found in other plant species as well, usually forming a medium-sized family of genes with 10-25 paralogues per plant species. Phylogenetic analysis of *MLO* genes from different species revealed that seven phylogenetic clades could be distinguished [6]. Not all *MLO* genes were found to be *S*-genes. *MLO* genes which are *S*-genes in monocot plant species of the Poaceae family, including the original barley *MLO* gene, all cluster in clade IV. In contrast, all documented *S*-genes in the *MLO* family identified in eudicot plants cluster in clade V [7].

With the cucumber genome sequence publicly available, two groups searched for *MLO* homologs in cucumber. Zhou et al. [8] reported that they identified 14 *MLO*-like genes in the first version of the cucumber genome. Schouten et al. [9] used the updated version of the cucumber genome (v2) and identified 13 *MLO*-like genes, the difference being that in the first version of the genome one *MLO* gene on chromosome 1 was erroneously annotated as two shorter neighbouring genes. Both groups agree that three cucumber *MLO* genes cluster in clade V of the MLO gene family, and can therefore be considered to be candidate *S*-genes. In the nomenclature proposed by Schouten et al. (2014), which we follow, the names of those clade V *MLO* genes are *CsaMLO1*, *CsaMLO8* and *CsaMLO11*.

In this thesis, we did not examine the ten *MLO* homologs in clades other than clade V, as we consider those not as likely *S*-genes, based on the fact that all *MLO S*-genes in other species identified so far cluster in clades V (in eudicot species) and IV (in the Poaceae family). However, one might argue that the focus on clade V genes by us and other groups might turn out to be a self-fulfilling prophecy, as the lack of research on non-clade V (and IV) *MLO* genes might cause us to overlook potential *S*-genes in other clades. Following this line of thought, the *Arabidopsis AtMLO3* gene, which is in clade VI and as such is more closely related to clade V genes than other non-clade V *MLO* genes, was recently characterised [10]. It was shown that *Atmlo3* mutants had characteristics in common with mutants of *AtMLO2* (*S* gene in clade V), including an increase in the

ratio of defence hormones salicylic acid (SA, especially important for defence against biotrophic pathogens) over jasmonic acid (JA, especially important for defence against necrotrophic pathogens), the formation of spontaneous callose depositions and early leaf senescence under stress-free conditions, although all these effects were smaller in amplitude compared to *Atmlo2* mutants. However, *Atmlo3* mutants were not resistant to PM nor to other tested diseases, implying that this gene can indeed not be considered to be an *S*-gene [10]. These results imply that the current focus by most groups on clade V *MLO* genes is probably justified, when trying to identify susceptibility genes. As *AtMLO3* is lowly expressed compared to *AtMLO2*, it would be interesting to test whether the finding that *AtMLO3* is not an *S*-gene is caused by differences in the encoded proteins or by differences in the expression patterns of both genes. As such, one could over-express clade VI *MLO* genes such as *AtMLO3* in PM resistant (clade V) *mlo* mutants, to see whether this would (partially) restore susceptibility. If not, it would be compelling evidence that clade VI *MLO* genes should really not be considered candidate *S*-genes.

Apart from cucumber, the evolution of *MLO* genes was studied in three other cultivated cucurbit species (melon, watermelon and zucchini). It was found that within the phylogenetic tree of *MLO* genes, cucurbit *MLO* genes usually formed orthologous groups, although gene duplications in zucchini often led to multiple zucchini paralogues within these orthologous groups [11]. In clade V, three cucurbit orthologous groups could be identified, which is in agreement with the three clade V *MLO* genes we studied in cucumber. Whereas none of the *MLO* genes in pumpkin/zucchini and watermelon were functionally characterized, a mutant allele of a melon clade V *MLO* gene was found (*CmMLO2*), characterised by a 85 bp deletion, in the PM resistant wild melon accession "C18" [12]. This gene was found to be abundantly expressed in leaf tissue of a susceptible melon cultivar, and upregulated upon inoculation with the PM causing fungus *P. xanthii* [13]. According to the phylogeny of Iovieno et al. this gene, which they call *CmMLO5*, was in the orthologous group also containing *CsaMLO8* [11], which we showed to be the major *S*-gene in cucumber (**Chapter 2**, **Chapter 3**). As such, it is to be expected that the watermelon and pumpkin/zucchini orthologues of these cucumber and melon *MLO* genes also play major roles as *S*-genes for PM.

Csamlo8 mutations in cucumber

In **Chapter 2**, we described how the insertion of a 1449 bp long transposable element (TE) in exon 11 of *CsaMLO8* led to aberrantly spliced mRNA, with two splice isoforms missing either 72 or 174 bp. We demonstrated with an *in silico* approach that this TE-allele of *Csamlo8* allele occurs frequently (27%) in a collection of 115 genotypes representing the cucumber germplasm. Simultaneously, Nie et al. found the same mutant allele of *CsaMLO8* in a PM resistant North China type cucumber inbred line, as the most likely

candidate gene for a PM resistance QTL containing 25 genes [14]. They subsequently characterized this gene, to which they refer in their publication as *CsMLO1*, the results of which confirmed our analysis and added additional information [15].

In addition to characterising the TE-allele of *Csmlo8*, Nie et al. [15] amplified the genomic *CsaMLO8* sequence of 27 additional cucumber inbred lines of diverse ecotype and origin, including well-known sources of PM resistance such as WI 2757 [5] and (a descendant of) PI 197088. Whereas they found the same 1449 bp insertion allele in nine of the additional resistant cucumber lines, including WI 2757, they also identified two additional natural variants in other resistant lines. In four instances they found a T to C point mutation with respect to the susceptible allele at position 1301, leading to erroneous splicing of exon 5. In one case they found a 1 bp frameshift insertion at position 3703, leading to an early stop codon. Whereas most of the susceptible lines (i.e. 11 out of 14) were found to have the wild-type *CsaMLO8* allele, there were some exceptions: one susceptible line, "S94", was found to have the 1449 bp transposable element insertion, and another susceptible line, "9930", which is the genotype of the cucumber reference genome, was found to have the T to C point mutation leading to aberrant splicing of exon 5. Apparently in these two inbred lines the *CsaMLO8* mutation is not sufficient for obtaining full resistance, which might be explained by the fact that hypocotyl resistance is known to be only partially effective, and other genes are required to reach full resistance [2]. It is therefore well possible that a weak, partial resistance in the two susceptible inbred lines with *Csamlo8* mutations was overlooked due to the conditions and scoring mechanism of the disease tests. Alternatively it could be possible that those genotypes have mutated alleles of genes required for *mlo* resistance, like the *ror* genes described in barley [16] and *pen* genes in Arabidopsis [17]. In this light it is interesting that the study which found "S94" to be susceptible to PM used it as a susceptible parent in a QTL mapping experiment, together with genotype "S06", which also has the TE-allele of *CsaMLO8* [15], as the resistant parent. Indeed, in this study PM symptoms were only scored on true leaves, and as such hypocotyl resistance (which supposedly was fixed in this population, given that both parents share the same mutant allele of *Csamlo8*) could have been easily overlooked. As such, QTL mapped in this study (on chromosomes 1, 2 and 5) might represent genes required for *mlo*-based resistance [18].

Interestingly, another susceptible line was found to have a 1451 bp insertion with a sequence very similar (95.6% identical) to the 1449 bp insertion, but at a different location, i.e. in intron 9 instead of in exon 11. This insertion in the intron was found not to alter the coding sequence nor the transcript abundance of *CsaMLO8*, explaining the susceptibility of this genotype. However, this indicates that insertions of this TE did occur frequently in the cucumber genome [15].

We tried to identify lines among the 115 resequenced cucumber genotypes [19] with the other loss-of-function alleles discovered by Nie et al. [15]. For the T to C point mutation at position 1301 described by Nie et al. [15], we should note that as pointed out by the authors, the reference genome "9930" has this mutation. We could identify three other genotypes homozygous for a C at this position, and additionally three heterozygous genotypes (Table 1), confirming the finding by Nie et al. [15] that this allele is less common than the 1449 bp insertion. Interestingly one of the three genotypes homozygous for this allele is "Puerto Rico #6", which is part of the "Puerto Rico" lines also comprising Puerto Rico 37 and Puerto Rico 40, the first reported sources of resistance towards PM in cucumber [20]. We could not identify any lines with the one bp insertion at position 3703 reported by Nie et al. [15], which is remarkable as the genotype in which they identified this mutation, "PI 197088", is a well-known and often used source of resistance against both powdery and downy mildew.

Table 1 - Cucumber genotypes among the 115 lines resequenced by Qi et al. (2013) homozygous or heterozygous for the CsaMLO8 T to C point mutation at position 1301

Individual Code	Zygosity	Accession name	Origin	Ecotype
CG4210	Homozygous	71 Hao Huang Gua	China, Chongqing	East Asian
CG6508	Homozygous	Puerta-Rico #6	Puerto Rico	Eurasian
CG7704	Homozygous	N2/81	Dem. Rep. of the Congo	Eurasian
CG1811	Heterozygous	Qing Dao Qiu Ye Er San	China, Shandong	East Asian
CG1077	Heterozygous	Qing Pi Ba Cha	China, Jilin	East Asian
CG1373	Heterozygous	Ye San Bai	China, Hebei	East Asian

The role of *CsaMLO1* and *CsaMLO11*

After finding that *Csamlo8* mutations are frequently used to confer PM resistance in cucumber, we decided to study the other clade V *MLO* genes in cucumber, *CsaMLO1* and *CsaMLO11* as well. Whereas we found that overexpression of both genes could restore susceptibility in the tomato *mlo* mutant, suggesting both genes can function as *S*-genes, we could not find any evidence for loss-of-function mutations in either gene in cucumber germplasm **(Chapter 3)**. This surprised us, as we would expect that loss-of-functions in these genes, supposedly conferring additional PM resistance, would have been actively selected for. There could be two possible explanations:

- It is possible that mutant alleles of *Csamlo1* and/or *Csamlo11* cause pleiotropic effects, negatively influencing yield due to constitutive defence responses. Pleiotropic effects of *mlo* mutations, such as non-pathogen associated callose depositions and early senescent leaves, are frequently reported, e.g. in barley [21] and in *Arabidopsis* [17]. Indeed PM resistance in cucumber was previously often found to be strongly linked to early leaf chlorosis. Interestingly, Zijlstra et al. [22] wrote over two decades

ago that the pleiotropic chlorosis phenotype linked to PM resistance in cucumber very much resembled the situation of *mlo* resistance in barley.

- Another possibility is that *Csamlo1* and/or *Csamlo11* mutations do not confer PM resistance, and are hence not selected for. In this light it is interesting that overexpression of *CsaMLO11* in tomato had a smaller effect on PM susceptibility compared to *CsaMLO1* and *CsaMLO8*, therefore this gene might be a less-effective *S*-gene **(Chapter 3).** In *Arabidopsis*, one *MLO* gene (*AtMLO2*) plays a major role, and mutations in this gene cause partial resistance. Whereas mutations in two other *MLO* genes (*AtMLO6* and *AtMLO12*) do not cause PM resistance in wild-type *AtMLO2* background, double mutations in two and triple mutations in all three clade V *MLO* genes do enhance PM resistance compared to single *Atmlo2* mutants [17]. It is possible that effects of *Csamlo1* and/or *Csamlo11* mutations are only observable in *Csamlo8* mutant background, decreasing the chance for such mutations to be noticed and selected for, especially since their effect would only be found in homozygous state given the recessive nature of resistance based on disabled *S*-genes.

To further examine the role of *CsaMLO1* and *CsaMLO11*, an interesting line of research could be to induce mutations in these genes, especially in *Csamlo8* mutant background. Technology to induce targeted mutations, especially in the form of CRISPR-*Cas9*, is nowadays frequently used in plant research, and was shown to be effective in cucumber [23, 24], as such this technology could be used to induce *Csamlo1* and/or *Csamlo11* mutations in *Csamlo8* background. However, cucumber is rather recalcitrant to genetic transformation, and as such reported rates of transformation efficiency are usually rather low (ca. 0.1%) [24]. In our hands, experiments aimed at generating cucumber mutants have so far been unsuccessful.

The importance of gene expression patterns

It has often been found that PM causing fungi induce the expression of *MLO* genes, and this PM-mediated induction of *MLO* genes is sometimes regarded as a selection criterion to select promising candidate *S*-genes. However, we could not reproducibly demonstrate PM-mediated induction of the major cucumber *MLO* gene *CsaMLO8* **(Chapter 2, Chapter 3)**. Another group demonstrated upregulation of *CsaMLO8* expression twelve hours post inoculation with the PM causing fungus *P. xanthii*, a time point which we did not take into account in our experiments, whereas they showed, similar to us, that *CsaMLO8* expression was rather constant at earlier and later time points [15]. We found that *CsaMLO1* was significantly upregulated upon *P. xanthii* inoculation **(Chapter 3)**, in line with earlier findings [9].

Whereas PM-mediated *CsaMLO1* upregulation in itself is an interesting finding, we noted that *CsaMLO1* was relatively lowly expressed, even upon PM inoculation, compared to *CsaMLO8*. We found high expression values of *CsaMLO8* in aerial tissues, including leaves **(Chapter 3)**, which was in line with the findings of Nie et al. [15]. *CsaMLO1* and *CsaMLO11* however, were only lowly expressed in aerial tissues, and were much more abundant in roots. We compared these expression patterns with those from the *Arabidopsis* clade V *MLO* genes, and found a rather similar scenario: the gene with a major influence on host-mildew interactions, *AtMLO2*, was abundantly expressed in leaves, whereas the genes with minor effects, *AtMLO6* and *AtMLO12*, were less actively transcribed in leaves. We postulated therefore that expression differences between *MLO* paralogues during absence of the pathogen, rather than transcriptional upregulation following infection, should be considered as a selection criterion to identify major *MLO* genes, as the one-sided focus on upregulation might obscure larger, and therefore more biologically relevant, differences in basal expression between paralogues.

CUCURBIT DOWNY MILDEW RESISTANCE – A COMPLEX TRAIT

Demise of *dm-1* resistance

For almost 50 years, cucumber growers relied on DM resistance conferred by the recessive *dm-1* gene, which was originally identified in the resistant Indian cucumber accession PI 197087 [25]. The fact that this gene conferred broad spectrum resistance for several decades demonstrates the durability that is commonly associated with recessive resistances, conferred by loss-of-function alleles of *S*-genes. It is generally assumed that to overcome non-functional *S*-gene based resistance, a pathogen needs gain-of-function mutations to compensate for the lack of the *S*-gene. In this light it would be interesting to study how *P. cubensis*, the causal agent of cucurbit DM, overcame *dm-1* resistance.

Recently, it was shown that the *dm-1* gene represents an allele of the cucumber *Staygreen* (*CsSGR*) gene, which also provides resistance to the bacterial pathogen *Pseudomonas syringae* pv. *lachrymans*, causing angular leaf spot, and the fungus *Colletotrichum orbicular*, causing anthracnose [26]. SGR proteins catalyse the conversion of chlorophyll *a* into pheophytin *a*, which is the first and therefore rate-limiting step in chlorophyll degradation [27]. Loss-of-function mutants in *SGR* homologs in several plant species are shown to have a "stay-green" or "non-yellowing" phenotype, caused by failure to degrade chlorophyll. Interestingly, the first *sgr* mutant was already studied in 1866, as the recessive "green cotyledon" gene *i* studied by Mendel [28] was later shown to be caused by mutations in the pea *SGR* gene [29]. *Arabidopsis Atsgr* mutants

were shown to have decreased disease symptoms (chlorosis, necrotic area) upon inoculation with either the bacterial pathogen *Pseudomonas syringae* or the necrotrophic fungus *Alternaria brassicola*, demonstrating the role of chlorophyll degradation in disease symptoms. However, bacterial titer of *Atsgr* mutants inoculated with *P. syringae* was not significantly lower, suggesting that *AtSGR* is important for reduced disease symptoms, but does not really reduce pathogen growth [30]. In contrast, the cucumber *dm-1* gene used to confer true resistance prior to 2004, and Wang et al. demonstrated that pathogen growth was arrested on cucumber genotypes with the *dm-1* gene [26], suggesting that the DM pathogen is dependent on *SGR* function for its growth. However, the genotypes used for this experiment had a different genetic background, and the authors can therefore not exclude the existence of other resistance genes contributing to lowered pathogen growth.

After the resurgence of cucurbit DM on cucumber, caused by the decreased effectiveness of the *dm-1* gene, several groups studied (changes in) pathogen population structure. It was generally found that *P. cubensis* isolates collected in the U.S.A. after 2004 represented a more diverse set of pathotypes, and included both mating types (A1 and A2) [31, 32], whereas previously a limited number of pathotypes and only a single mating type (A1) were observed there [33]. Also in other parts of the world, including Europe and Israel [34], shifts in population structures regarding pathotype and mating type were observed. An open question is whether the virulence of current *P. cubensis* isolates on cucumber genotypes with the *dm-1* gene is due to increased overall aggressiveness of these pathogen strains, or whether it depends on specific mutations regarding the dependency of the pathogen on the host *SGR* gene. The fact that genotypes with the *dm-1* gene still have intermediate DM resistance compared to genotypes without this gene might implicate that increased overall aggressiveness might play a major role. It would be interesting to directly compare pathogen strains virulent and avirulent on *dm-1* cucumber, in order to identify genomic differences between such strains.

New sources of DM resistance

Whereas research on sources of DM resistance in cucumber was rather scarce during the second half of the twentieth century, largely due to the success of *dm-1* based resistance, the re-emergence of DM as a serious disease limiting cucumber production led to increased attention to DM resistance in cucumber [31, 35]. The most scrutinous study regarding sources of DM resistance was performed between 2005 and 2009, when 1300 different cultigens from the U.S. National Germplasm system were scored for DM resistance for multiple years at multiple locations in the U.S.A., Poland and India, using natural infections as well as controlled inoculation experiments [36]. Three accessions originating from India and Pakistan, i.e. PI 605996, PI 330628, and PI 197088, were

found to be consistently the most resistant in all tested experiments, indicating that these genotypes have a broad-spectrum resistance. However, several other genotypes with promising levels of DM resistance were found as well [36]. Several susceptible individuals were observed in F2 populations derived from crosses between PI 605996, PI 330628, and PI 197088, suggesting that resistance in each of these three lines is at least partially due to different causal genes [37]. PI 197088 is currently the most studied DM resistant cucumber genotype, and several groups have mapped QTL contributing to DM resistance in this genotype. Major QTL on chromosomes 4 and 5 were found to have the largest and most consistent effect, whereas a large amount of minor QTL were identified in several studies, but were very inconsistent between different mapping studies [38–41]. One of the major QTL which was frequently detected in these studies (DM5.1) had flanking markers consistent with the location of the *dm-1/CsSGR* gene as identified by Wang et al. [26], suggesting that a mutation in this gene occurs in PI 197088 and still confers intermediate resistance, even though this is not the full resistance observed prior to 2004.

QTL DM4.1 – Multiple phenotypes, multiple genes?

In this thesis, we investigated QTL DM4.1 on chromosome 4, which was in several studies found to be one of the major QTL for DM resistance in PI 197088, explaining up to 27% of the variance in resistance [38, 39, 41]. In order to characterise this QTL, we introgressed it in a susceptible background by repeated backcrossing with a susceptible breeding line, in order to reduce the genetic variation in resistance due to other QTL **(Chapter 4)**. The resulting heterozygous backcross inbred line (BIL) was selfed for three generations, in order to develop recombinant populations in which we could fine-map the QTL.

When scoring a population inoculated with *P. cubensis*, several phenotypes can be observed. Usually, the first symptom of DM is the formation of angular chlorotic lesions. Over time, lesions turn necrotic and coalesce. Additionally, a successful infection will lead to sporulation at the abaxial side of the leaf. Due to decreased photosynthetic capabilities of heavily infected plants, plants may become stunted, which can also be scored as a symptom of the disease, especially when an experiment follows an infected population for a longer period of time, although plant growth is obviously a very complex trait, influenced by genes not involved in DM resistance as well. Each of these symptoms is furthermore affected by environmental conditions such as temperature, light and relative humidity. Disease symptoms can be scored individual from each other, or can be combined in one score for a "general impression" of the infected leaf. Furthermore, either the intensity of the symptoms, the percentage of the leaf area showing the symptoms, or a combination of these can be scored. In literature, different groups

often scored different symptoms to quantify the resistance in populations, contributing to different outcomes regarding the amount of QTL and the contribution of each of these QTL to overall resistance, even when studying the same sources of resistance (e.g. [38–40]).

Some groups scored multiple disease symptoms in the same population, making it possible to determine the correlation between these symptoms. For example, Call et al. [42] scored chlorosis and necrosis (as the percentage of symptomatic leaf area), as well as plant stunting, in a collection of cultivars and breeding lines grown in the field under a natural DM epidemic, and found that chlorosis and necrosis were correlated (R^2=0.64), whereas stunting was not correlated (R^2=0.04) to either chlorosis or necrosis. Wang et al. [43], mapped QTL in field-grown F3 populations derived from PI 330628 as source of resistance, measuring both chlorosis and necrosis (as the percentage of symptomatic leaf area), as well as scoring the same plants based on a "general impression". They found that chlorosis and necrosis were significantly correlated, although not with a very high correlation (R^2=0.486), whereas the "general impression" score was as expected significantly correlated to both symptoms (R^2= 0.513 and R^2=0.776 for chlorosis and necrosis, respectively). The generally rather low correlations between different disease symptoms make it advisable to score different disease symptoms, as they might be differently influenced by quantitative resistance genes.

In **Chapter 4**, we quantified three disease symptoms separately from one another: chlorosis, necrosis and sporulation. Each of these three scores were combinations of the intensity of the symptoms (e.g. brighter yellow, more dense sporulation) as well as the symptomatic leaf area. Furthermore we performed our experiments in controlled climate chambers, in order to reduce environmental variation. By mapping the chlorosis, necrosis and sporulation traits separately from one another in our populations, we identified recombinants within the DM4.1 interval that defined three subQTL. Subsequently we confirmed the existence of two of these subQTL by analysing populations segregating for one of the QTL, in absence of the other. SubQTL DM4.1.2 had a major effect on sporulation, without affecting either chlorosis or necrosis, whereas subQTL DM4.1.3 explained the decrease of chlorosis, and furthermore also contributed to a decrease in sporulation, but did not affect necrosis. A third subQTL, DM4.1.1, explaining the decrease in necrosis was found, but we were unable to confirm this subQTL in populations without the other subQTL, potentially because this subQTL is dependent on the presence of the other subQTL.

After separating the different subQTL, we fine-mapped DM4.1.2 to an interval containing 40 predicted genes (**Chapter 4**), and DM4.1.3 to an interval containing 70 predicted

genes (**Chapter 5**). In both QTL, candidate genes were identified through comparative transcriptome and genome analysis of NILs with the subQTL and the susceptible recurrent parent. In **Chapter 4**, we describe finding a gene encoding a receptor like kinase (*CsLRK10L2*) as the most likely candidate for subQTL DM4.1.2, a loss-of-function mutation in which was found in susceptible genotypes, including the cucumber reference genome ("Chinese Long 9930"). In **Chapter 5**, we describe finding a loss-of-function mutation in candidate gene *CsAAP2A*, encoding an amino acid transporter, as the probable causal mutation explaining the resistance conferred by subQTL DM4.1.3.

The predicted causal genes for these two subQTL thus have little in common, one encoding a receptor-like kinase triggering defence responses whereas the other is a susceptibility gene, loss-of-function mutations in which apparently limit nutrient transport to the pathogen. Although more functional studies are necessary to fully comprehend the roles of both genes in DM resistance and susceptibility, our working hypothesis is that the decrease in amino acid transport due to the *Csaap2a* mutation leads to decreased pathogen growth, and thus to a decrease in chlorosis, which is the first disease symptom, as well as a decrease in sporulation later on due to the limitation in nutrient availability. On the other hand, the defence responses triggered by *CsLRK10L2* do apparently not decrease initial pathogen growth, as exemplified by the similar rate of chlorosis of plants with and without subQTL DM4.1.2, but the ability of the pathogen to sporulate is decreased. We do not know why specifically sporulation is affected in plants with subQTL DM4.1.2, as such it would be interesting to histologically characterize plants with and without the QTL, in the stage at which sporangiophores are formed, to see at what specific stage sporulation is prevented. Furthermore it would be very interesting to study cell-specific expression patterns of the *CsLRK10L2* gene, e.g. by *in situ* RT-PCR or single cell RNAseq, to see whether this gene is expressed in specific subsets of cells. It could for instance be possible that the gene is not abundantly expressed in mesophyll cells, in which the pathogen forms haustoria, but is abundantly expressed in for example the stomatal guard cells, through which sporangiophores emerge. Such cellular expression patterns might therefore shed more light on the function of the *CsLRK10L2* gene, and the reasons why it specifically stops sporulation.

CsLRK10L2: Where did it come from, and what does it recognise?

The *CsLRK10L2* gene which we found as the potential causal gene for QTL DM4.1.2 (**Chapter 4**) was part of a cluster, consisting of three related genes, of which one represented a rather short gene which was not expressed in our datasets, and is therefore potentially a pseudogene, whereas the other gene (*CsLRK10L1*) was abundantly expressed. Interestingly, the first exon of *CsLRK10L2*, encoding the predicted extracellular domain, was highly conserved with the first exons of the other genes in the cluster,

whereas the other exons, encoding the predicted transmembrane and intracellular kinase domains, were very different among these *RLK* genes. Whereas the kinase domain of CsLRK10L1 had the traditional "RD" motif usually found in most RLKs, the kinase domain of CsLRK10L2 was a non-RD kinase, which are frequently found in a subset of RLKs all involved in defence [44]. It is known that in RD kinases, the conserved positively charged arginine (R) residue inhibits the catalytic function of the neighbouring aspartate (D) residue, an inhibition which is lifted after autophosphorylation of the nearby "kinase activation loop" of the kinase protein. This mechanism ensures that the kinase is not constitutively active, but only is activated upon signalling its cognate ligand [45]. Phylogenetic evidence suggests that the conserved arginine residue was lost in several kinase subclades containing PAMP-receptors, rather than being lost once in a common progenitor of PAMP-receptors. It is poorly understood why PAMP-receptors appear to preferentially not have the conserved arginine residue, although it could have something to do with decreased possibilities of pathogen effectors interfering with kinase activity of the receptor [44]. Non-RD kinases regularly require a conserved co-receptor, the regulatory *RLK* gene *BAK1*, forming a heterooligomeric complex upon binding the recognized extracellular ligand, although this was primarily shown for kinases with an LRR-ectodomain [46, 47].

Phylogenetic analysis of the kinase-encoding domains revealed that *CsLRK10L2* is part of a clade containing several *Arabidopsis* (non-RD) *RLK* genes involved in disease resistance, with rather variable ectodomains, whereas *CsLRK10L1* belongs to a clade of (RD) *RLKs* with conserved ectodomains. This indicates that it is likely that *CsLRK10L2* arose from a tandem duplication of *CsLRK10L1*, after which recombination led to a domain switch by fusing the first exon to exons encoding a defence-related protein kinase domain. Cucumber paralogues of both *CsLRK10L1* and *CsLRK10L2* belong to three clusters of genes on chromosomes 1 and 5, indicating that these clusters of *CsLRK10L1*/*CsLRK10L2* paralogues likely have arisen through segmental duplication.

Regarding their conserved extracellular domains, CsLRK10L1 and CsLRK10L2 are homologs of *Leaf Rust 10-like RLKs* (*LRK10Ls*). This family of *RLKs* is called after the wheat *LRK10* gene, which confers resistance to leaf rust caused by the fungus *Puccinia recondita* [48]. However, it is yet unknown which signals are perceived by this *RLK* family. As we noticed that the extracellular domains of CsLRK10L1 and CsLRK10L2 contain predicted WAK-associated and oligogalacturonan-binding domains, we speculated that these receptors might be involved in perception of oligogalacturonan (OG). As the breakdown product of the pectin component of the plants cell wall, OG is a potent damage associated molecular pattern (DAMP), triggering defence responses.

OG is perceived by so called *Wall associated kinase* (*WAK*) genes. Previously, five *Arabidopsis WAK* genes were described, occurring in one cluster on the genome, which are highly conserved regarding their extracellular domain [49]. Later, twenty-two additional *WAK-like* (*WAKL*) genes were identified in the *Arabidopsis* genome, based on reiterative BLAST searches using the *WAK1* coding sequence and the WAK1 protein sequence [50]. We identified cucumber and Arabidopsis genes with OG-binding and/or WAK-associated extracellular domains, and found additional to *WAK* and *WAKL* genes a large number of genes which were previously not annotated as *WAKL* genes, including many *LRK10L* genes. However, phylogenetic analysis showed that sequences of *WAK* genes and non-*WAK* genes with WAK-like domains were not closely related. Only one cucumber gene (Csa4M665090) was found in the clade containing the five *Arabidopsis WAK* genes, and none in the clade containing the previously annotated *WAKL* genes, indicating an *Arabidopsis*-specific expansion of this gene family compared to cucumber.

As to our knowledge the only genes for which experimental evidence exists that they indeed perceive pectin/OG are *WAK1* [51] and *WAK2* [52], and sequence similarity of our candidate gene to these *WAK* genes is rather low, we should be careful not to draw too fast conclusions about the nature of the ligands perceived by CsLRK10L2 and related homologs. Whereas it is possible that these LRK10L proteins perceive OG, it is equally possible that they perceive different, perhaps structurally related, ligands. Further functional studies (e.g. by co-immunoprecipitation) are therefore required in order to identify the cognate ligand and therefore elucidate the working mechanism of *CsLRK10L2*. Based on our finding that overexpression of *CsLRK10L2* in *Nicotiana benthamiana* triggered a necrotic response without supplying any ligand or inoculating with any pathogen, we assume that the ligand of this receptor is a molecule normally present in some concentration in the plant apoplast, rather than a pathogen associated molecular pattern or pathogen encoded effector protein.

Roles of *AAP* genes and other nutrient transporters in disease susceptibility

As described above we identified a loss-of-function mutation in the *CsAAP2A* gene as potentially causal for subQTL DM4.1.3 (**Chapter 5**). Whereas this is the first indication that amino acid permeases (*AAP* genes) contribute to susceptibility against oomycete pathogens, a role of *Arabidopsis AAP* genes as susceptibility genes against obligate biotrophic nematode parasites was previously established [53, 54]. *AAP* genes were found to be transcriptionally upregulated in syncytia induced by cyst nematodes [53] and giant cells induced by root-knot nematodes [55], causing a drastic increase in amino acid concentrations in these feeding structures [56]. Loss-of-function mutants in several *AAP* genes led to partial resistance towards both types of parasitic nematodes, although this

increase in resistance was rather weak, potentially due to redundancy between *AAP* paralogues [53, 54].

Whereas nematodes and DM causing oomycetes are obviously not very related, both parasites share an obligate biotrophic lifestyle and both form specialized feeding structures in the plant host. It can therefore be that both parasites partially depend on the same host genes to obtain nutrients and thereby confer susceptibility. It was for example shown that genomes of biotrophic plant pathogens, including the pathogen *Hyaloperonospora arabidopsidis* causing DM on *Arabidopsis*, have lost genes encoding key enzymes for the assimilation and transport of inorganic nitrate and nitrite, thus becoming dependent on the plant host for providing them with organic amino acids as a nitrogen source [57].

In contrast to nematodes, who upregulate *AAP* expression in infected cells, we found that several *AAP* genes were downregulated upon *P. cubensis* inoculation, and our candidate gene *CsAAP2A* was barely detectable at all in leaf tissue, but was primarily expressed in stems (**Chapter 5**). This might reflect the difference in feeding styles between these two groups of parasites: whereas nematodes directly pierce the infected host cell with their needle-like stylets in order to suck up nutrients [58], downy mildew causing oomycetes form a haustorium, invaginating the hosts cellular membrane, and as such nutrients must be secreted from the host cell and subsequently transported from the apoplast over the haustorial membrane. As such, high expression of *AAP* genes, which encode amino acid importers rather than bidirectional transporters [59], in infected host cells might be beneficial for nematodes (as they will increase the amino acid concentration inside the host cell) but disadvantageous for oomycetes (as they will decrease the amino acid concentration in the apoplast), and it should therefore be no surprise that these genes are therefore upregulated by nematodes and downregulated by *P. cubensis*.

Even so, the loss-of-function mutation in *CsAAP2A* contributed to resistance rather than to susceptibility (**Chapter 5**). A likely explanation for this observation is that this particular *AAP* gene is not primarily leaf-expressed, but is rather expressed in stem tissue. Stem expressed *AAP* genes in *Arabidopsis* such as *AtAAP2* and *AtAAP6* have been found to function in xylem-to-phloem transport of amino acids, thereby increasing the flow of amino acids towards sink tissues [60]. Therefore, loss-of-function mutations in stem-expressed *AAP* genes, such as *CsAAP2A*, will likely lead to a decrease in the flow of amino acids towards sink tissues. Indeed, we found that whereas inoculation with *P. cubensis* generally led an increase in amino acid concentrations (indicating that an infected leaf becomes a sink for amino acids), the amino acid concentration was mark-

edly lower in (inoculated) plants with QTL DM4.1.3, i.e. with the *Csaap2a* mutation, compared to (inoculated) wild-type plants (**Chapter 5**).

Apart from amino acids, as a major source of organic nitrogen, obligate biotrophs also require several other nutrients, such as carbohydrates, sulphates and phosphates. The best documented role of nutrient transporters in plant susceptibility is that of the sugar transporters belonging to the *SWEET* family. SWEET proteins are plasma membrane localized uniporters, facilitating import and export of monosaccharides, and can therefore facilitate sugar transport to pathogen-infected tissues, supplying them with a source of carbohydrates [61]. Indeed, the rice genes *OsSWEET11* (*Xa13*) and *OsSWEET13* (*Xa25*) were found to be susceptibility genes for the pathogenic bacterium *Xanthomonas oryzae* pv *oryzae*, which grows in the host apoplast, likely because these *SWEET* genes facilitate sugar export from host cells to the apoplast [61, 62]. *SWEET* genes were found to be upregulated by several pathogens, including both PM causing fungi and DM causing oomycetes, suggesting that they provide susceptibility to these pathogens [61, 63]. Furthermore several biotrophic pathogens, including DM causing oomycetes, have lost key genes for sulphur assimilation [57], and as such it is not surprising that sulphate transporters have been found as (putative) susceptibility genes, being heavily upregulated in poplar (*Populus trichocarpa*) upon inoculation with the rust fungus *Melampsora larici-populina* [64] and providing susceptibility in rice to the bacteria *Xanthomonas oryzae* pv. *oryzicola* [65]. Interestingly, a mutation in a phosphate transporter was recently identified as a potential causal mutation for DM resistance in cucumber, as a resistance QTL on chromosome 5 of resistance accession PI 330628 was fine-mapped to an interval containing only three genes of which one, the phosphate transporter *PHO1*, had a missense SNP and was ca. 60-fold induced upon *P. cubensis* inoculation [66].

MILDEW RESISTANCE IN CUCUMBER – FUTURE PERSPECTIVES

In this thesis we have investigated genes involved in resistance and susceptibility to mildew causing pathogens: the PM causing fungi *Podosphaera xanthii* and *Golovinomyces orontii* and the DM causing oomycete *Pseudoperonospora cubensis*. We studied existing QTL contributing to mildew resistance, to identify candidate genes, either based on homology with known susceptibility genes (*MLO* genes) or based on a combination of fine-mapping with genomic studies (*LRK10L*, *AAP* genes). The results of this work can contribute to breeding mildew resistant cucumber varieties, e.g. by creating molecular markers closely linked to resistance traits, and to screen new potential sources of resistance to verify that they rely on other genes and are not merely allelic variants of currently used resistance sources. However, mildew diseases continue to be a problem

in cucumber breeding, and as such more work is required to identify and characterize other sources of resistance. Here, I will discuss several other approaches that could contribute to obtaining broad and durable mildew resistant cucumbers. Whereas these approaches generally can apply to both PM causing fungi and DM causing oomycetes, our attention is slightly biased towards DM, given the fact that DM resistance is broadly seen as the major priority in cucumber breeding, whereas PM is rather effectively controlled by current sources of resistance.

Characterizing causal genes for other QTL

We have put our focus on two loci contributing to mildew resistance: the recessive *pm-h* gene for "hypocotyl resistance", also known as QTL PM5.2 [5], which is the most important source of (partial) PM resistance for over four decades, and QTL DM4.1, which is one of the major QTL for DM resistance in the highly resistant cucumber line PI 197088. However, neither of these QTL provide full resistance, and as such these QTL should be combined with other sources of resistance. Luckily, such QTL are known for both diseases and are already used in cucumber breeding. It would be helpful to identify causal genes for these other QTL, similar to our work regarding PM5.2 and DM4.1.

For PM, it has been shown that combining *pm-h* with a second genetically linked gene for "leaf resistance", known as *pm-l* or PM5.1, provides full resistance [2, 5, 67]. *pm-l* was previously mapped on chromosome 5 at 57.5 cM, versus 79.3 cM for *pm-h* [67]. In a PM resistance mapping study, a major QTL for leaf resistance, PM5.1, was mapped with peak markers at 54.4, which, based on the marker sequence, corresponds to a physical location of ca. Chr5:17.150.000. However, flanking markers in this study delimited a 2-LOD support interval between 45.5 and 93.1 cM, which is too large to identify potential causal genes [5].

For DM, various studies reported several QTL contributing to resistance, mostly using accession PI 197088 as a source of resistance. Yoshioka et al. [40] reported ten QTL in a cross between PI 197088 and the intermediately resistant cv. Santou, of which one QTL on chromosome 1 (peak at 2 cM) and two QTL on chromosome 5 (peaks at 15 and 96 cM) had major effects. Interestingly, cv. Santou apparently contributed the resistance allele for DM1.1. Wang et al. [38] identified eleven QTL, of which three QTL on chromosome 5 with peaks at 40, 67 and 119 cM had major effects, together with QTL DM4.1 which we studied. Li et al. [39] identified five QTL of which DM4.1 had the largest effect, followed by two QTL on chromosomes 1 (flanking markers at 67 and 90 cM) and 5 (flanking markers at 83 and 98 cM). These results illustrate that especially chromosome 5 and to a lesser extent chromosome 1 are rather abundant in QTL contributing to resistance.

It would be interesting to develop introgression lines in order to fine-map these QTL for PM and DM, ultimately aiming to identify the causal genes.

Forward genetic screens

Apart from characterizing known mildew resistance QTL, other approaches can lead to identification of novel resistances. These other approaches are especially relevant to cucumber breeding, given the rather low genetic diversity of cucumber and the difficulties in introgressing traits from wild relatives (discussed below). One of these approaches is to identify mildew resistant mutants in forward genetic screens, also known as mutation breeding. Mutation breeding involves treating plants with physical (e.g. x-rays), chemical (e.g. ethyl methane sulphonate, EMS) or biological (e.g. *Agrobacterium tumefaciens* mediated T-DNA insertions) mutagenic agents. Mutagenized seeds will contain numerous mutations, usually in heterozygous state. As mutations usually lead to loss-of-function alleles, which are recessive, phenotyping in order to find interesting mutant families is usually performed in M2 populations which segregate for the induced mutations [68, 69].

Mutation breeding can be successfully applied to find new sources of resistance. Due to the induction of loss-of-function alleles, resistances identified through mutation breeding are usually susceptibility genes. The *mlo* gene in barley was for example originally discovered in a mutation breeding experiment involving X-ray treatment of barley, leading to 12000 primary mutants, which were selfed to produce M2 populations. Three of these populations segregated for resistance to PM caused by the fungus *Blumeria graminis* f. sp. *hordei* [70]. Mutation breeding has subsequently led to lines with resistance to numerous pathogens in many crop plants [69]. Due to its small size, short generation time and prolific seed production, the model plant *Arabidopsis thaliana* is highly suited for forward genetic screens. Several mutant screens for pathogen resistance have been performed in *Arabidopsis*, which have for example led to the identification of the *PMR* genes [71–73] and *DMR* genes [74] as *S*-genes for PM and DM, respectively.

Forward genetic screens can be an interesting strategy to identify novel sources of mildew resistance in cucumber. Mutant cucumber populations have been developed by several groups by treating cucumber seed with EMS, leading to many mutant phenotypes in shape, size and colour of fruits, flowers, leaves and tendrils [75–77]. In two cases, the causal mutated genes were identified, i.e. *Csycf54* for light green coloration in fruits and leaves [78] and *Cscyp85a1* for extreme dwarf growth due to lack of internode elongation [79]. To our knowledge, it has not yet been successfully attempted to use mutation breeding to obtain disease resistance in cucumber. In order to avoid

overlooking novel *S*-genes with relatively small partial effects, it can be preferable to perform mutagenesis experiments in a highly susceptible background, as was done in the *DMR* screen, which used a hyper-susceptible *eds1* mutant background [74]. Subsequently, newly identified mutant alleles can be introgressed in elite lines. Generally, large numbers of M2 mutants are required in order to find disease resistant mutants: 20 mutants with multiple alleles of the six *PMR* genes were identified in a population of ca. 26.000 M2 plants, and nine mutants with alleles of the six *DMR* genes were identified in a population of ca. 100.000 M2 plants, belonging to 3.600 M1 families. The relative amount of resistant mutants that can be found is of course dependent on the mutation frequency, and thus on the mutagenizing treatment (e.g. the concentration of EMS, the duration of the treatment).

Previously, identification of the causal genes underlying a new mutant allele usually required outcrossing a homozygous mutant plant with another genotype, in order to create an F2 population in which the mutant phenotype can be mapped, as pre-existing molecular markers are usually not polymorphic in a segregating mutant population derived from one background genotype. However, due to the advances in next-generation sequencing technologies, it is nowadays feasible to directly sequence mutant plants in order to score the (mutation-induced) polymorphisms as molecular markers. When sequencing bulks of resistant and susceptible plants in a segregating mutant population (i.e. bulk segregant analysis, BSA), most induced mutations will have an allele frequency of on average 50% in both bulks, whereas mutations genetically linked to the causal mutation will have higher frequencies, obviously ranging up to 100% for the causal mutation and mutations closely linked to it. Therefore, a BSA sequencing approach can readily identify candidate causal mutations, thereby greatly accelerating forward genetic pipelines [80–82].

Characterization of cucumber *S*-gene orthologues

Whereas forward genetic screens can potentially lead to the identification of novel *S*-genes, as discussed in the previous paragraph, such screens have already been performed in other species, most notably in the model organism *Arabidopsis thaliana*. As there are already many *S*-genes known against various diseases, it can be assumed a likely possibility that a large proportion of *S*-genes identified in a forward genetic screen will later turn out to be cucumber homologues of known *S*-genes in other species. Because a forward genetic screen requires a lot of work, involving the development and screening of thousands of mutant families, it could be a faster approach to perform reverse genetic screens, based on the identification of cucumber orthologues of known *S*-genes.

A review by van Schie and Takken provided a comprehensive overview of more than 180 *S*-genes identified in various plant species (including over 110 genes identified in *Arabidopsis*), although this list contains some redundancy due to the inclusion of multiple orthologues of the same gene family in different species (e.g. 10 *MLO* genes, 11 *eIF4E* genes) [1]. As *S*-genes are often found to be conserved between different plant species, cucumber homologues of these genes can readily be identified by means of the BLAST algorithm, followed by phylogenetic analysis to distinguish cucumber orthologues from less related homologs. To illustrate this, we identified cucumber orthologues from 26 *S*-genes reviewed by van Schie and Takken [1], implicated as *S*-genes conferring susceptibility to DM in *Arabidopsis*. For 25 of these 26 genes, cucumber orthologues could be identified. For one gene, *IOS1*, which encodes an LRR-RLK conferring susceptibility to *Hyaloperonospora arabidopsidis* [83], no clear cucumber orthologue could be identified, due to numerous *Arabidopsis*-specific gene duplications in this gene family. Figure 1 gives an overview of the genomic positions of the cucumber orthologues of known DM *S*-genes.

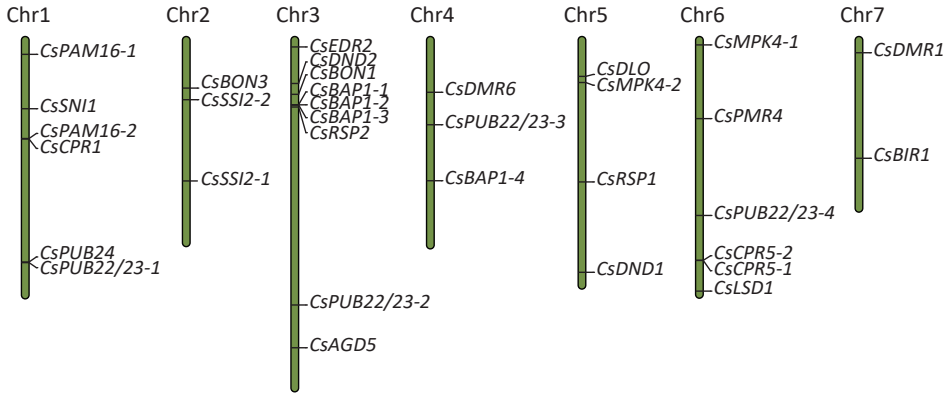

Figure 1 - Cucumber orthologues of DM S-genes reviewed by van Schie and Takken [1] as identified by BLAST followed by phylogenetic analysis. Names are based on *Arabidopsis* gene symbols, with added numbers identifying cucumber paralogues in cases of species-specific gene duplications.

Whether a cucumber orthologue of an *S*-gene identified in another species also contributes to susceptibility against pathogens in cucumber should be verified by reverse genetic approaches. One approach is to screen (e.g. EMS mutagenized) mutant libraries for loss-of-function mutations in the gene of interest, and subsequently test identified mutants for resistance, an approach known as TILLING (targeted induced local lesions in genomes). As a proof of principle, this approach was successfully used in cucumber to obtain mutants in the sex determination gene *CsACS2* [84].

Another possibility is to make use of several techniques relying on transformation, such as targeted knock-downs (by RNAi) or knock-outs (e.g. using CRISPR-*Cas9*) of the gene of interest in cucumber to induce resistance, or complementation in a mutant genotype of another species by heterologous overexpression of the cucumber gene of interest, to see whether this restores susceptibility. RNAi was successfully used in cucumber to study several genes involved in development, such as sex determination gene *CsGAMYB1* [85], wax biosynthesis gene *CsCER1* [86] and the gibberellin receptor gene *CsGID1a* [87]. CRISPR-*Cas9* was used to induce targeted knock-out mutants in the cucumber orthologue of *S*-gene *eIF4E*, resulting in a broad recessive resistance against three different viral diseases [23]. Complementation studies with cucumber *S*-gene orthologues were performed for example by us regarding cucumber *MLO* genes in tomato *slmlo1* (**Chapters 2 and 3**), and by Nie et al. [15] in *Arabidopsis Atmlo2/Atmlo12*.

Genomic and genetic studies of cucumber mildew pathogens

In this thesis we studied the cucumber side of cucumber-mildew interactions. However, arguably of equal importance in these interactions are the genes of the pathogens and the processes in which they are involved. Studying pathogen-encoded genes might shed more light on which processes are crucial in the establishment of a compatible cucumber-mildew interaction, and as such might reveal new ways in which we can manipulate this interaction in order to obtain resistance. Identification of important pathogen encoded effector proteins might lead to the discovery of novel sources of resistance, as the targets of those effector proteins could be products of *S*-genes. Furthermore, receptors recognizing such effectors might be identified or developed, representing novel *R*-genes.

A hurdle in the study of mildew genomics is the fact that these biotrophic pathogens cannot be cultured, and as such it is rather difficult to isolate pathogen DNA or RNA without isolating large amounts of host-derived DNA/RNA. However, several significant steps have been made to study the genomes and transcriptomes of DM and PM species.

A draft genome of *P. cubensis*, the causal agent of cucumber DM, was assembled in 2011, based on DNA isolated from purified sporangia. However, this draft genome represented only 12.1 Mb, divided over 42.799 contigs, whereas the genome size of *P. cubensis* was predicted to be ca. 88 Mb [88]. A second *P. cubensis* genome was subsequently assembled based on deeper sequencing data of sporangia of another isolate, which represented 64.4 Mb divided over 35.546 contigs. Gene annotation led to the identification of 23.522 predicted genes [89].

Transcriptomic studies based on RNA isolated from both purified sporangia as well as a time series of (susceptible) cucumber leaves inoculated with *P. cubensis* gave insight in the genes required for several stadia in infection. Whereas a large majority (88 to 97%) of RNAseq reads from the time series mapped to the cucumber genome rather than the pathogen genome, deep sequencing gave enough power to detect expression of 7821 pathogen genes in at least one time-point [90]. Among the most abundantly transcribed genes were 271 genes predicted to encode secreted effectors. Effectors of oomycetes frequently contain a conserved N-terminal domain containing the amino acid sequence RxLR, in which x can represent any amino acid, flanked by a high frequency of D/E residues. This so-called RxLR motif is not required for effector activities, but rather acts as a signal to translocate the effector protein to the host cell, and as such RXLR effectors generally have a function within the host cell [91]. Interestingly, expressed predicted effector genes in *P. cubensis* encoded 271 proteins with RxLR-like motifs, but the first arginine (R) residue was found to be not at all conserved, but could also be substituted for ten other residues with similar frequencies [90]. Whether or not these xxLR-effectors also facilitate host translocation, or result in apoplastic effectors, is not yet studied. Several expressed *P. cubensis* effectors also belonged to a second class of oomycete effectors, the so-called Crinklers (CRNs), containing another translocation motif, LxLFLAK. Furthermore, a high proportion of expressed genes, especially at later time points, were found to encode secreted hydrolytic enzymes such as proteinases, lipases and carbohydrate active enzymes (CAzys), which probably have a function in breaking down plant structures in order to obtain nutrients [90].

One *P. cubensis* RxLR effector gene, *PscRXLR1*, was found to be abundantly expressed during early stages of infection, and was subsequently characterised. Interestingly, the PscRXLR1 protein was found to localize to the plant plasma membrane, even though the predicted protein sequence lacked a predicted transmembrane domain. Heterologous overexpression of this gene in *N. benthamiana* resulted in chlorosis, followed by a necrotic response, indicating that this effector can play a role in disease symptom development of cucurbit DM [89].

One aspect of which is not yet extensively studied are the genetics of *P. cubensis*. Whereas the pathogen mostly relies on asexual reproduction, sexual reproduction between strains of opposing mating types (A1 and A2) can occur, which leads to recombination. It is generally assumed that recombination events have led to more frequent outbreaks of novel pathogen types with altered host ranges and pesticide resistances over the last two decades [92]. It has been shown to be possible to efficiently induce oospore formation by inoculation of detached leaves with *P. cubensis* strains of opposing mating types, leading to on average 600 oospores per cm^2 of infected leaf area. A

major hurdle in these experiments is that oospores are dormant structures, which do not efficiently infect new susceptible hosts under laboratory conditions [93]. However, it has been shown that oospore dormancy can be broken by storing decayed plant material containing oospores for several months in a refrigerator, leading to disease on ca. 46% of inoculated plants [94].

It would for example be highly interesting to try to identify genes controlling host specificity, by crossing *P. cubensis* isolates with different pathotypes, and mapping the host specificity in resulting progeny. Identification of pathogen encoded virulence genes might subsequently lead to the identification of interacting partners in cucurbit host species, which would shine new light on the reasons for which cucumber appears to be universally susceptible to *P. cubensis* strains, in contrast to other cucurbit species.

In addition to the DM pathogen *P. cubensis*, some work has already been performed to study the genomes of both fungal species which can cause PM on cucumber, although this work was not performed on pathogens growing on cucumber itself, but on different hosts, as both species have a rather broad host range. A low-quality draft genome of an *G. orontii* isolate growing on *Arabidopsis* was published, representing 65 Mb of the estimated 160 Mb genome, divided over 61.834 contigs. Interestingly, this genome size is very large compared to non-PM ascomycetes, whereas similar genome sizes were observed in two other PM species [95].

Furthermore a *G. orontii* transcriptome was sequenced by specifically isolating haustoria from inoculated *Arabidopsis* leaves, following by RNA extraction . In this manner, 85% of sequencing reads corresponded to the *G. orontii* draft genome. Genes involved in protein synthesis were highly abundant in the *G. orontii* transcriptome, whereas nutrient transporter transcript abundance was surprisingly scarce, given the fact that the haustorium is thought to be primarily a feeding structure. Furthermore, 70 predicted effector genes were identified, of which 19 were among the top 50 of most abundantly expressed *G. orontii* genes. [96]. A (*de novo*) transcriptome of *P. xanthii* was also sequenced, by isolating mycelium from heavily infected zucchini leaves as well as by washing of conidiospores, allowing the assembly of 37.241 contigs. Similar to the *G. orontii* transcriptome, genes involved in protein synthesis and modification were most abundantly expressed. 137 putative secreted effectors were identified among the *P. xanthii* transcripts [97]. It would be interesting to see whether the identified transcriptomic trends are similar in both species when inoculated on cucumber, or whether there are genes which are specifically upregulated when infecting a cucumber host.

Interestingly, it was shown that *P. xanthii* can be transformed by incubating conidiospores in an *Agrobacterium tumefaciens* suspension, followed by inoculation on detached cotyledons. By washing the cotyledons with selective antibiotics, transformed *P. xanthii* colonies can be selected, as exemplified by fluorescence of *GFP*-transformed strains. However, stable transformation could not be observed, for inoculation of fresh cotyledons with spores derived from transformant colonies led to loss of fluorescence within two generations, unless selective antibiotics were applied every generation [98]. The fact that *P. xanthii* can be transformed opens up possibilities of studying effector genes of this pathogen. It would be interesting to test whether a similar approach could also work to transform the DM pathogen *P. cubensis*, although its endophytic lifestyle might prove to be an additional hurdle compared to the epiphytic *P. xanthii*, regarding the antibiotic resistance selection.

Transferring resistances from other cucurbits

In many crop species, a rich source of desirable traits, such as disease resistances, is represented by wild relatives of the crop species, accessions of which are frequently maintained by gene banks. Plant breeders can often introgress such traits by recurrent backcrossing to cultivated crops. Over 50 different wild *Cucumis* species have been identified, which could potentially be a large reservoir of interesting novel traits for cucumber breeding [99]. However, introgression breeding with wild relatives of cucumber is prevented by the fact that cucumber has a different chromosome number (2N = 14) compared to other *Cucumis* species (2N = 24).

Through embryo rescue, viable F1 hybrids between cucumber and its most closely related wild relative, *C. hystrix*, were obtained, which indeed had a chromosome count of 2N = 19, and were therefore both male and female sterile [100]. Later, through tissue-culture induced chromosome doubling, amphidiploid *C. hystrix* x *sativus* hybrids were obtained with a chromosome count of 2N = 4X = 38 [101]. After this amphidiploid was shown to be fertile, it was described as a new synthetic species, *C.* x *hytivus* [102]. Backcrossing of *C.* x *hytivus* to the diploid *C. sativus* parent aided by embryo rescue resulted as expected in an allotriploid [103]. Interestingly, treatment of allotriploid shoots with colchicine, which is normally used to induce chromosome doubling, did not lead to allohexaploids as was expected, but instead led with low frequency to "monosomic alien addition lines" (MAALs), with a chromosome count of 15, i.e. the complete *C. sativus* genome supplemented with a single haploid *C. hystrix* chromosome [104]. Furthermore, selfing of the (less fertile) allotriploid for several generation was found to lead to introgression lines, some of which had a chromosome count normal for cucumber (2N = 14) but with some morphological characteristics derived from *C. hystrix*, putatively due to natural translocations from *C. hystrix* chromosomal fragments in *C. sativus* chromosomes. Some

of these introgression lines were found to have intermediate resistances against DM and *Fusarium* wilt, traits likely inherited from the *C. hystrix* progenitor [105, 106].

Whereas these results indicate that it is possible to introgress traits from the most closely related wild *Cucumis* species to cucumber, this is an extremely time- and labour consuming process. Furthermore, it is unsure whether such an approach will also work for other, less related *Cucumis* species. Experiments in the 1980s at the Institute for Horticultural Plant Breeding aiming at producing hybrids between wild cucumber (*C. sativus* var. *hardwickii* and *C. sativus* var. *sikkimensis*) with several distantly related African *Cucumis* species showed that pollen tube growth was arrested, unless pollen of the wild relatives was mixed with "mentor pollen", i.e. pollen of the female parent which is rendered "genetically dead" by gamma-irradiation. However, even after successful pollination, embryo development was arrested in these wide crosses [107].

An attractive alternative to introgression breeding with wild *Cucumis* species would be to make use of genetic modification, by isolating genes involved in resistance in wild *Cucumis* species and transforming them to cultivated cucumber. This concept is known as cisgenesis, to distinguish it from transgenesis, which makes use of non-crossable species [108]. By cisgenesis, the process of introducing a novel gene in cucumber would be greatly enhanced compared to introgression breeding, whereas linkage drag of unwanted traits from the wild species is avoided. Obviously, in order for a cisgenic approach to work, the causal genes for resistance should first be identified in the wild species, e.g. by crossing resistant and susceptible accessions of the same species followed by (fine)mapping.

CONCLUSION

We have identified several genes involved in interactions between cucumber and mildew causing pathogen species. Regarding PM, it is hopeful that the frequently used "hypocotyl resistance" trait appears to be caused by a *Csamlo8* loss of function mutation. As *mlo* resistance is very durable in other species (barley, pea), it seems reasonable to assume that this resistance in cucumber will not soon be broken. It would be interesting to further characterize the effects of loss-of-function mutations in the other cucumber clade V *MLO* genes, to see whether these could increase the PM resistance conferred by *Csamlo8*. Furthermore, other sources of PM resistance which are currently used, most importantly the "leaf resistance" gene on chromosome 5, should be characterized in order to draw a conclusion on the durability of current PM resistance in cucumber.

Regarding DM, we identified (likely) causal genes for one of the major QTL from resistance source PI 197088. The results of this work can be readily used to develop molecular markers in order to use these genes in breeding programs, resulting in (partial) resistant cultivars. However, the effects of QTL DM4.1 on its own are not large enough to provide an adequate level of DM resistance, and as such more work is needed to find and characterize other sources of resistance. We have discussed several options to identify novel candidate genes for DM resistance in cucumber, including the introgression and fine-mapping of other QTL from PI 197088 and other resistant accessions, forward and reverse genetic screens, a more thorough study on the pathogen side of cucumber-mildew interactions, and approaches on transferring resistances from other cucurbit species.

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Summary

Two of the most limiting diseases in cucumber production are downy mildew (DM), caused by the oomycete *Pseudoperonospora cubensis*, and powdery mildew (PM), caused by the fungi *Podosphaera xanthii* and *Golovinomyces orontii*. Whereas the pathogens causing DM and PM are not related, they share a similar obligate biotrophic lifestyle.

In my PhD thesis we aimed at identifying genes involved in susceptibility and resistance to these diseases, in order to better understand the interactions between cucumber and mildew pathogens, and contribute to breeding disease resistant cucumbers. One focus point in my research was the concept of susceptibility (*S*) genes. Loss-of-function of *S*-genes lead to resistance, which is more durable than classical race-specific resistance (*R*) genes. A second pillar of my PhD research was to identify causal genes for QTL contributing to disease resistance, which are frequently used in cucumber breeding. Regarding PM, the most important source of resistance is "hypocotyl resistance", which causes partial resistance characterized by PM-free hypocotyls and stems, and can lead to full resistance when combined with other sources of resistance. One of the most famous examples of an *S* gene is *MLO*, loss-of-function alleles of which provide durable PM resistance in barley already for several decades. After the cloning of the causal gene it was found that *MLO* genes occur in all sequenced plant genomes to date, and that in several plant species *MLO* homologues in phylogenetic clades IV and V were found to be *S* genes for PM. In the first two chapters of my thesis I investigated *MLO* genes in cucumber, leading to the identification and functional characterization of a loss-of-function allele of the clade V *CsaMLO8* gene as causal for hypocotyl resistance in cucumber (**Chapter 2**). Furthermore, we functionally characterized the other clade V *MLO* genes in cucumber, *CsaMLO1* and *CsaMLO11*, and found that both are functional *S*-genes too, although they are rather weakly expressed in leaves, presumably leading to a minor role regarding PM susceptibility (**Chapter 3**).

In the second half of my thesis the focus is on identification of genes involved in DM resistance. Currently, the most often used source of DM resistance in cucumber is the Indian semi-wild accession PI 197088, the resistance in which inherits as a polygenic trait. We introgressed one major QTL from this resistant accession in a uniform susceptible background, in order to fine-map it. We found that this QTL consists of several subQTL, each explaining a different aspect of the resistance conferred by the full QTL. Through a combination of transcriptomics and whole genome sequencing, we identified likely candidate genes for two of these subQTL. For subQTL DM4.1.2, which had a dominant effect on sporulation, we found a novel *RLK* gene (*CsLRK10L2*) which was strongly upregulated by the pathogen. This gene has homology to *LRK10* genes, which were originally found as candidate genes for leaf rust resistance in wheat. This *LRK10-like* gene had a 551 bp deletion in DM susceptible genotypes compared to resistant

genotypes. The cucumber reference genome also shows this deletion. The presence of a predicted oligogalacturonan-binding domain in the CsLRK10L2 protein suggested that this receptor might be involved in sensing cell-wall damage, triggering a defence response (**Chapter 4**). For subQTL DM4.1.3, which had an effect on both chlorosis and sporulation, we identified a mutation in the *Amino Acid Permease 2A* (*CsAAP2A*) gene, encoding a transporter for amino acids. Homologs of this gene were previously found to be *S*-genes for several obligate parasitic nematode species, as they heavily rely on their host to provide them with amino acid nutrients. We found that cucumber plants with the loss-of-function allele of *CsAAP2A* contained lower levels of amino acids after inoculation compared to WT plants, indicating that the mutation might decrease amino acid transport to infected leaves. As such, this gene is a novel *S*-gene for oomycetes (**Chapter 5**).

Combined, the results described in this thesis increase our knowledge about the interactions between cucumber and two of its most notorious pathogens, and will facilitate cucumber resistance breeding.

Samenvatting

Twee van de grootste beperkingen in de productie van komkommers zijn de ziekten valse meeldauw (Engels: "downy mildew"), veroorzaakt door de oömyceet *Pseudoperonospora cubensis*, en echte meeldauw (Engels: "powdery mildew"), veroorzaakt door de schimmels *Podosphaera xanthii* en *Golovinomyces orontii.* Hoewel de pathogenen die valse en echte meeldauw veroorzaken niet nauw verwant zijn aan elkaar delen beide groepen een biotrofe levenswijze, hetgeen betekent dat ze afhankelijk zijn van parasitisme op een levende waardplant.

Het doel van mijn proefschrift was om genen te identificeren die betrokken zijn bij vatbaarheid en resistentie tegen deze ziekten. Door de interacties tussen komkommer en meeldauwpathogenen beter te begrijpen, kunnen we bijdragen aan de veredeling van ziekteresistente komkommers. Een focuspunt van mijn onderzoek was het concept van zogenaamde "vatbaarheidsgenen" (Engels: "Susceptibility genes", vaak afgekort tot S-genen). Het verlies van deze S-genen leid tot een resistentie die duurzamer is dan die van klassieke ras-specifieke resistentiegenen (R-genen).

Een tweede pijler van mijn onderzoek was het identificeren van de causale genen voor kwantitatieve resistentie (Engels: "Quantitative trait loci", afgekort tot QTL), die vaak gebruikt worden in komkommerveredeling. Wat betreft echte meeldauw is de belangrijkste bron van resistentie de zogenaamde "hypocotyl-resistentie", een kwantitatieve resistentie die gekenmerkt wordt door meeldauw-vrije hypocotylen (het deel van de stengel onder de zaadlob) en stengels. Gecombineerd met andere vormen van resistentie kan hypocotyl-resistentie leiden tot volledige resistentie.

Het bekendste voorbeeld van een vatbaarheidsgen is het *MLO*-gen (afkorting voor "Meeldauw Locus O"), waarvan mutant-allelen in gerst al decennialang gebruikt worden voor duurzame meeldauwresistentie. Nadat het causale gen gekloneerd was, bleek dat *MLO*-genen voorkomen in alle plantsoorten waarvan het genoom bekend is, en dat in diverse plantsoorten *MLO*-genen (specifiek die genen in de fylogenetische groepen of klades IV en V) vatbaarheidsgenen zijn voor echte meeldauw. In de eerste twee experimentele hoofdstukken van mijn proefschrift onderzocht ik *MLO*-genen in komkommer, wat leidde tot de identificatie en de functionele karakterisatie van een mutant-allel van het klade V gen *CsaMLO8* als het causale gen voor hypocotyl-resistentie (**Hoofdstuk 2**). Verder hebben we ook de twee andere klade V *MLO* genen in komkommer (*CsaMLO1* en *CsaMLO11*) functioneel gekarakteriseerd. Beide genen zijn functionele vatbaarheidsgenen, alhoewel ze maar zwak tot expressie komen in bladeren, wat waarschijnlijk zorgt voor een beperkte rol wat betreft meeldauw-vatbaarheid (**Hoofdstuk 3**).

In de tweede helft van mijn proefschrift ligt de focus op de identificatie van genen betrokken bij resistentie tegen valse meeldauw. Momenteel is de meest gebruikte bron van valse meeldauw-resistentie het genotype PI 197088, een semi-wilde komkommerlijn uit India. Resistentie van deze bron overerft als een polygene eigenschap. Wij hebben een belangrijk QTL uit deze resistentielijn ingekruist in een meeldauw-vatbare achtergrondlijn, met als doel dit QTL te fijn-karteren. We ontdekten dat dit QTL uit meerdere sub-QTL bestaat, waarvan elk een ander aspect van de resistentie verklaart. Door middel van een combinatie van transcriptoom-analyse en genoom-sequentiebepaling hebben we de meest waarschijnlijke kandidaatgenen voor twee van deze subQTL geïdentificeerd.

Voor het dominant overervende sub-QTL "DM4.1.2", dat leidde tot vermindering van sporulatie van het pathogeen, vonden wij een nieuw receptor-kinase gen (*CsLRK10L2*) als kandidaat, een gen dat sterk geïnduceerd werd door de pathogeen. Dit gen is verwant aan *LRK10* genen, die oorspronkelijk ontdekt zijn als kandidaat-genen voor resistentie tegen de ziekte bruine roest in tarwe. Ons *LRK10*-achtige gen had een deletie van 551 baseparen in vatbare komkommer genotypen, in vergelijking met resistente genotypen. In het komkommer referentie-genoom is deze deletie ook aanwezig. De aanwezigheid van een voorspeld "oligogalacturonan-bindend domein" in het eiwit dat gecodeerd wordt door *CsLRK10L2* duidt erop dat deze receptor betrokken kan zijn bij het registreren van schade aan de celwand, wat leidt tot een afweerrespons (**Hoofdstuk 4**).

Voor het recessief overervende sub-QTL "DM4.1.3", dat leidde tot vermindering van chlorose en sporulatie, vonden wij als kandidaat een mutatie in het gen *CsAAP2A*, dat codeert voor een transport-eiwit voor aminozuren. Homologen van dit gen zijn in het verleden ontdekt als vatbaarheidsgenen voor verscheidene parasitaire nematode-soorten, omdat die sterk afhankelijk zijn van hun gastheer voor de toevoer van nutriënten zoals aminozuren. We ontdekten dat, na inoculatie met de pathogeen, komkommerplanten met een mutant-allel van *CsAAP2A* een lagere concentratie aan aminozuren bevatten dan wild-type planten, wat een aanwijzing is dat deze mutatie leidt tot een verminderd aminozuur-transport naar geïnfecteerde bladeren. Hiermee is dit gen een nieuw vatbaarheidsgen voor oömyceten (**Hoofdstuk 5**).

De resultaten in dit proefschrift vergroten onze kennis omtrent de interacties tussen komkommer en twee van haar meest beruchte pathogenen, hetgeen bij zal dragen aan de resistentieveredeling van komkommer.

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About the author

Jeroen Anne Berg was born in Groningen, on October 28th, 1988. Throughout his childhood he moved several times with his parents. First to Bakkeveen in Friesland, where he learned not only the standard primary school subjects, but the Frysian language as well. After moving to Buitenpost for a short interlude, they ended up in Groningen were he started his secondary education. He went to the "Willem Lodewijk Gymnasium", just like his father had done before him. Whereas Jeroen usually liked school, he liked food sometimes even more. One example of this is that he wanted to drop ancient Greek in the fourth grade, but resumed it after he found out that they had 'pepernoten' during the lessons. Furthermore his interest in (eating) hot peppers started: A girl at his school told that her father required aspiring sons in-law to prove their worth by eating three very spicy peppers (*Capsicum chinense* cv. 'Madame Jeannette'). Obviously, Jeroen had to eat them, just for the sake of it. In 2007 he obtained his Gymnasium diploma and moved to Wageningen in order to study Biotechnology.

In Wageningen he met a lot of new people, especially at his youth organisation, J.V. Unitas. In the first year of the study Biotechnology almost all courses were shared with the study Molecular Life Science. As many of his new friends studied Molecular life Science he decided to switch studies after two months. However, one and a half year later, Jeroen found out that Molecular Life Science was for him too much focussed on chemistry, and did not contain enough 'life'. Therefore Jeroen switched to study Biology, as third time's a charm. He followed all the courses about plants, animals, and ecology and decided he would perform his BSc thesis in the lab of Entomology. His BSc thesis was entitled: "The role of *PKG* expression on the mobility of parasitoid wasp larvae". Next to his study Jeroen was very active in many committees of J.V. Unitas, such as the cooking and theatre committees. During one of the trips with Unitas friends they visited Leipzig, where Jeroen (of course) had to eat the hottest curry sausage in the world a.k.a. "Mutter der Schmerzen".

After finishing his Bachelor degree in 2011 he stopped studying for a year and became a fulltime board member at J.V. Unitas, where he functioned as treasurer. He learned many additional teamwork skills during this year, and he had time to think about which Master programme he would start afterwards. During his Bachelor degree his interest in plants had grown more and more, although molecular biology was also still very interesting. Therefore, Jeroen started his Master's degree in Plant Biotechnology in 2012. With this decision all his interests: biotechnology, molecular biology and plant biology,

came together. It turned out that this MSc program was a very good decision, as it laid the fundament for his PhD thesis and his potential future career in plant breeding.

During his Master, with a specialization in Molecular Plant Breeding and Pathology, he performed his MSc thesis at the laboratory of Phytopathology, which was entitled: "The role of R1/AVR1 subcellular localization and the Exocyst complex in *Phytophthora*-host interactions". Afterwards Jeroen performed an internship at the potato breeding company Solynta in Wageningen about "Fine-mapping the *Self-Locus Inhibitor* (*Sli*) gene in diploid potato". Both research projects were awarded high grades and in 2014 Jeroen obtained his MSc degree *cum laude.*

In 2014 Jeroen started his PhD project at Plant Breeding about "Identification of genes for Mildew Susceptibility in Cucumber" under the supervision of prof. Yuling Bai, dr Henk J. Schouten and prof. Richard G.F. Visser. It was a very interesting project that made him enthusiastic about Cucurbitaceae and especially Cucumbers, also leading to a sharp increase in his cucumber consumption. The project was a nice collaboration between Plant Breeding and the breeding company Nunhems, nowadays part of BASF. Apart from hard work in the lab, in the greenhouse or behind the computer, Jeroen enjoyed to present his work at many international conferences. A summer school in Hungary, a conference in Warsaw, a conference in China, which he combined with a holiday with colleagues in order to climb the Yellow Mountains and enjoy the Chinese cuisine. Furthermore he went to a conference in California, which of course had to be combined with a holiday again, this time together with his supervisor Henk.

During these years Jeroen did not only develop himself as a researcher in order to become an independent scientist, but also as a person to become a family man. In 2017 he became father of our wonderful son Waldemar and during the last month of writing his thesis he became father of our beautiful daughter Hasse. He and his wife held a competition who would be first to deliver, she (the baby) or he (his thesis). You hold the result of his delivery in your hands and hopefully you enjoyed reading it!

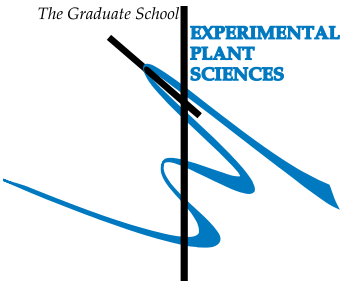
Written by Marloes van Splunter-Berg

Education certificate

EDUCATION STATEMENT

Education Statement of the Graduate School

Experimental Plant Sciences



Issued to: **Jeroen A. Berg**
Date: **11 October 2019**
Group: **Plant Breeding**
University: **Wageningen University & Research**

1) Start-Up Phase	*date*
► **First presentation of your project**	
Identifying mildew S-genes in cucumber	12 May 2015
Subtotal Start-Up Phase	*1.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD Get2gether 2015	29-30 January 2015
EPS PhD Get2gether 2016	28-29 January 2016
EPS PhD Get2gether 2017	9-10 February 2017
► **EPS theme symposia**	
EPS theme 2 symposium & Willie Commelin Scholten Day 2015	20 February 2015
EPS theme 2 symposium & Willie Commelin Scholten Day 2017	23 January 2017
EPS theme 2 symposium & Willie Commelin Scholten Day 2018	24 January 2018
EPS theme 4 symposium 2015	15 December 2015
EPS theme 4 symposium 2016	16 December 2016
► **Lunteren Days and other national platforms**	
NWO Lunteren day 2015	13-14 April 2015
NWO Lunteren day 2016	11 April 2016
NWO Lunteren day 2017	10-11 April 2017
NWO Lunteren day 2018	9-10 April 2018
Najaarssymposium Nederlandse Vereniging voor Plantenbiotechnologie en Weefselkweek (NVPW) 2015	11 December 2015
Najaarssymposium Nederlandse Vereniging voor Plantenbiotechnologie en Weefselkweek (NVPW) 2017	8 December 2017
TKI-U Network Day 2016	12 April 2016
► **Seminars (series), workshops and symposia**	
Minisymposium Phytopathology	24 November 2014

2nd WageningenPhDSymposium"Connecting Ideas, Combining Forces"	6 May 2015
Keygene symposium "Applications and Challenges of Oxford Nanopore Sequencing in the Life Science Industry"	14 April 2016
Symposium and Inaugural lecture prof. Guido van der Ackerveken	9 May 2017
KNPV Oomyceten Meeting	28 June 2017
Plant Breeding Research Day 2014	30 September 2014
Plant Breeding Research Day 2015	29 September 2015
EPS Flying Seminar Prof.dr. Yves van de Peer 'The evolutionary significance of gene and genome duplications'	3 February 2015
Plant Science Seminar "Into the Battle between Plants and Viruses, but what about EVEs?"	12 May 2015
EPS Flying Seminar Gero Steinberg "Long-distance endosome trafficking drives fungal effector production during plant infection"	5 June 2015
EPS seminar "Plant intracellular immunity: evolutionary and molecular underpinnings"	21 January 2016
Public lecture "Rewriting our genes? Crispr-Cas systems as tools for genome editing"	30 September 2016
WEES Seminar Prof. dr. Berenike Maier "Bacterial Gene Transfer"	24 November 2016
EPS Seminar Dr. Birgit Kemmerling "The Arabidopsis BIR family - negative regulators of BAK1 receptor complexes and more"	25 November 2016
WEES Seminar Dr. Mart Krupovic "Natural history of viral capsids"	22 February 2017
B-Wise Seminar, Justin van der Hooft and Victor Carrion "Using sequence information to predict protein function"	9 January 2018
► **International symposia and congresses**	
"2nd International Academic Conference for Graduate Students", Nanjing Agricultural University, China	27-30 October 2015
"XIth Eucarpia meeting on Genetics and Breeding of Cucurbitaceae (2016)", Warsaw, Poland	24-28 July 2016
"Cucurbitaceae 2018", UC Davis, USA	12-15 November 2018
► **Presentations**	
Talk: 2nd International Academic Conference for Graduate Students, Nanjing Agricultural University	28 October 2015
Talk: Najaarscongres NVPW 2015	11 December 2015
Talk: TKI-U Network day	12 April 2016
Talk: XIth Eucarpia meeting on Genetics and Breeding of Cucurbitaceae (2016)	27 July 2016
Poster: Cucurbitaceae 2018	13 November 2018
► **Excursions**	
EPS Company visit Tomatoworld	14 October 2016

	Subtotal Scientific Exposure	*17.8 credits**
3) In-Depth Studies		*date*
►	**Advanced scientific courses & workshops**	
	2015 Powdery Mildew Summer School, Eger, Hungary	3-7 August 2015
	Bioinformatics – A User's Approach, Wageningen	24-28 August 2015
	The Power of RNAseq, Wageningen	10-12 February 2016
	Course Linux/BASH scripting, Wageningen	18-20 April 2016
	Course Python for bioinformatics, Wageningen	17-19 May 2016
	Data Analysis and Visualizations in R, Wageningen	11-12 May 2017
	Advanced course Phylogenetics: principles & methods, Wageningen	23-26 April 2018
►	**Journal club**	
	PhD Discussion club Plant Breeding (monthly)	2017-2018
	Subtotal In-Depth Studies	*8.1 credits**
4) Personal Development		*date*
►	**General skill training courses**	
	Project and Time Management	6 October - 24 November 2015
	Data Management Planning	23 May 2016
	Reviewing a Scientific Paper	23 March 2017
	Career Perspectives	14 September - 12 October 2017
	Subtotal Personal Development	*3.6 credits**
	TOTAL NUMBER OF CREDIT POINTS*	**31.0**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS with a minimum total of 30 ECTS credits.
** A credit represents a normative study load of 28 hours of study.*

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