Identification of candidate genes for quantitative downy mildew resistance in cucumber – a review

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Abstract

Cucurbit downy mildew (DM), caused by the obligate biotrophic oomycete *Pseudoperonospora cubensis*, is a major foliar disease of cucumber. Cucumber accession PI197088 was previously shown to be one of the most promising donors for DM resistance. The resistance in PI197088 is controlled by multiple quantitative trait loci (QTL), each with a relatively small effect. We developed a cucumber line with a 12 Mb introgression from PI197088 in a susceptible background. This introgression line was partially resistant to DM. Subsequently, we combined fine mapping data with RNAseq and whole genome resequencing to identify candidate genes for the resistance conferred by this introgression. Interestingly, fine-mapping data suggested that the partial resistance is caused by a combination of multiple genetically linked genes, leading to three subQTL, each with a different effect on the disease phenotype. In one of these subQTL we identified several *Receptor Like Kinase* genes (*RLK*). A novel *RLK* gene appeared to be present in resistant genotypes. In susceptible genotypes, including the reference genotype "Chinese Long 9930", this novel gene has a 551 base pair deletion, and was therefore not correctly predicted during the annotation of the cucumber genome. In another subQTL, we found a loss-of-function mutation in the novel susceptibility gene (S-gene) *CsAAP2A*, encoding an amino acid transporter. In the resistant genotype a non-functional allele of this gene is present due to integration of a transposable element in its coding sequence.

Keywords: downy mildew (*Pseudoperonospora cubensis*), cucumber (*Cucumis sativus*), plant-pathogen interactions, PI 197088, QTL mapping, *RLK* genes, *AAP* genes

INTRODUCTION

Cucumber (*Cucumis sativus* L.) is the third most consumed group of vegetables, after onions and tomatoes, with an annual production of over 83 million metric t. Over 75% of all cucumbers produced worldwide are grown in China (FAOSTAT, 2017). Cucumber is grown in glass greenhouses or plastic tunnels as well as in open fields. Several market types of cucumber are recognized, such as cucumbers that are more suited for fresh consumption, cucumbers intended for pickling, and various region-specific types.

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 (DM) and *Phytophthora* fruit rot, but also of great importance are powdery mildew, *Fusarium* wilt, Gummy stem blight, and a variety of viral diseases (Grumet, 2018).

Our project focuses on DM resistance in cucumber. Cucurbit DM is caused by *Pseudoperonospora cubensis* (Berk. & M.A. Curtis) Rostovzev, an oomycete belonging to the order *Peronosporales*, family *Peronosporaceae*. The pathogen was initially discovered in herbarium specimens originating from Cuba in 1868, explaining the name of the species (Berkeley and Curtis, 1868). 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

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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 (Spring et al., 2018).

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 (Jenkins, 1942, 1946). 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 (Epps and Barnes, 1952).

Hereafter, a novel promising source of DM resistance was found in the Indian accession PI 197087, characterized by the development of small brown hypersensitive lesions upon inoculation with extremely sparse sporulation (Barnes and Epps, 1954). Interestingly, the same Indian accession was also used in PM resistance breeding (Barnes and Epps, 1956). 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 (van Vliet and Meijsing, 1974, 1977). 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 (Wang et al., 2019).

Since the year 2004 however, new strains of the pathogen emerged in the USA, which are virulent on plants with *dm-1* (Cohen et al., 2015; Holmes et al., 2015; Thomas et al., 2017a). This is reminiscent of the situation in central and northern Europe two decades earlier, where since the year 1984 all of a sudden strong DM epidemics occur annually on cucumber, presumably due to the emergence of a new, more virulent, strain which has overcome previously existing resistance in the European cucumber genepool (Lebeda and Cohen, 2011). It has been shown, in the USA as well as in Europe, that populations of *P. cubensis* are epidemiologically very diverse, changing rapidly from one year to another with regards to the prevailing pathotype spectra (pathotype being defined as the pathogenicity of an isolate on a range of differential cucurbit host species) (Lebeda et al., 2013; Thomas et al., 2017b).

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 (Holmes et al., 2015; Criswell et al., 2010). The most scrutinous study regarding sources of DM resistance was performed between 2005 and 2009, when 1300 different cultigens from the US. National Germplasm system were scored for DM resistance for multiple years at multiple locations in the USA, Poland and India, using natural infections as well as controlled inoculation experiments (Call et al., 2012). 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 (Call et al., 2012). Several susceptible individuals were observed in F_2 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 (Vandenlangenberg, 2015). 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 (Wang et al., 2018; Li et al., 2018; Yoshioka et al., 2014; Caldwell et al., 2011). 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. (2019), 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.

OVERVIEW OF RESULTS AND DISCUSSION

QTL DM4.1 – Multiple phenotypes, multiple genes?

In our project, 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 (Li et al., 2018; Wang et al., 2018; Caldwell et al., 2011). In order to characterize 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. Subsequently we deployed next generation sequencing methods (RNAseq and whole genome resequencing) in order to identify candidate genes and potential causal mutations.

In our QTL mapping, 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 analyzing 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 (manuscript submitted for publication elsewhere).

After separating the different subQTL, we fine-mapped DM4.1.2 to an interval containing 40 predicted genes, and DM4.1.3 to an interval containing 80 predicted genes. In both QTL, candidate genes were identified through comparative transcriptome and genome analysis of NILs with the subQTL and the susceptible recurrent parent. We found a gene encoding a receptor like kinase with homology to the *Arabidopsis LRK10L2* gene (*CsLRK10L2*) as the most likely candidate for subQTL DM4.1.2. In susceptible genotypes, including the cucumber reference genome ("Chinese Long 9930") a loss-of-function mutation was found (manuscript submitted for publication elsewhere). Whereas the resistance from subQTL DM4.1.2 was dominantly inherited, the resistance of the DM4.1.3 inherited recessively, indicating an impaired susceptibility gene causing this latter subQTL. Indeed, we found a loss-of-function mutation in candidate gene *CsAAP2A*, encoding an amino acid transporter. This mutation is the probable causal mutation leading to a partial loss of susceptibility conferred by subQTL DM4.1.3 (manuscript in preparation).

The predicted causal genes for these two subQTL thus have little in common, one encoding a receptor-like kinase triggering defense 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 defense 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 recognize?**

The *CsLRK10L2* gene which we found as the potential causal gene for QTL DM4.1.2 is part of a cluster consisting two other *RLK*-like genes. One of these predicted genes is rather short and was not expressed based on our data sets, and is therefore potentially a pseudogene, whereas the other gene (*CsLRK10L1*) was abundantly expressed. Interestingly, the first exon of *CsLRK10L2* that encodes the predicted extracellular domain, is 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 much more variable among these *RLK* genes. The kinase domain of CsLRK10L1 had the traditional "RD" motif usually found in most RLKs, however, the kinase domain of CsLRK10L2 was a non-RD kinase. Non-RD kinases are frequently found in a subset of RLKs all involved in defense (Dardick et al., 2012).

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 defense-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* (Feuillet et al., 1997). 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 speculate 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 defense responses. It is therefore tempting to speculate that CsLRK10L2 and related RLKs are involved in OG-mediated defense regulation, although more evidence is needed to support this hypothesis.

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. Whereas this is the first indication that amino acid permeases (*AAP* genes) contribute to susceptibility to oomycete pathogens, a role of *Arabidopsis AAP* genes as susceptibility genes against obligate biotrophic nematode parasites was previously discovered (Elashry et al., 2013; Marella et al., 2013). *AAP* genes were found to be transcriptionally upregulated in syncytia induced by cyst nematodes (Elashry et al., 2013) and giant cells induced by root-knot nematodes (Hammes et al., 2005), causing a drastic increase in amino acid concentrations in these feeding structures (Hofmann et al., 2010). Loss-of-function mutants in several *AAP* genes led to partial resistance toward both types of parasitic nematodes, although this increase in resistance was rather weak, potentially due to redundancy between *AAP* paralogues (Marella et al., 2013; Elashry et al., 2013).

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 such non-related parasites partially depend on similar host genes to obtain nutrients. It was for example shown that the biotrophic pathogen *Hyaloperonospora arabidopsidis,* causing DM on *Arabidopsis*, had 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 (Baxter et al., 2010).

In contrast to nematodes, that 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. 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 (Eves-van den Akker et al., 2014), 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 (Fischer et al., 2002), 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), which could explain 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. A likely explanation for this observation is that this particular *AAP* gene is not primarily leaf-expressed, but is rather expressed in stem tissue. We found that whereas inoculation with *P. cubensis* generally led to an increase in amino acid concentrations (indicating that an infected leaf becomes a sink for amino acids), the amino acid concentration was markedly lower in (inoculated) plants with QTL DM4.1.3, i.e., with the *Csaap2a* mutation, compared to (inoculated) wild-type plants. The loss-of-function mutation in *Csaap2a* thus seems to decrease the flow of amino acids toward the leaves, thereby reducing nutrient supply to the pathogen.

CONCLUSIONS

We introgressed one major QTL from resistant cucumber accession PI 197088 in a susceptible background and 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 causal genes for two of these subQTL. For subQTL DM4.1.2, which had a dominant effect on sporulation, we found a functional *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 *CsLRK10L2* gene has a 551 bp deletion in DM susceptible genotypes compared to resistant genotypes. 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 defense response.

On subQTL DM4.1.3, we identified *Amino Acid Permease 2A* (*CsAAP2A*) as the causal gene. We found that cucumber plants with the loss-of-function allele contained lower levels of amino acids after inoculation compared to WT plants, indicating that the mutation decreases amino acid transport to infected leaves. As such, this gene is a novel *S*-gene for oomycetes.

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