AACAAGGATATAA
TATCTCACTTTGT
TCTGCGCCTTGTG
TATTGCCGAACGG
AGGACTTGAGAA
AATGGTTTCTTTC
TATGGAAACTATG
CTATTCCGACACC
GTTTTCTTTCTGA
AAGTCCGAATCT
CAGAAGCAGGTA
TTTGCGGAGCTT
GGTCTCTCATACA
AAGCAATCAGTA
ATGCAGAATTTGA
GGCTGTAAGGGA
CAAATTGGTTCA
AGTTGCTCGGTT
GATTGGTCAGCCT
GCACCAAGTAGT

FORGING RESILIENCE FROM FRAGILITY

Insights on loss of susceptibility in Cucurbitaceae

Lampros Siskos

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ATGGAACCCTATC
TTCCTCAGAGAA
AATC CGTCTC
GAC CGACT
ACC CAC
TCTACCGC CTGC
TCCTCCTC TGAA
GTCAGGG GAA
GATT GCTTT
ATGCC AGATCG
TCTTCGAGTTCTT
AAA TTCT
GATC CTC
GAGGAAA AAT
CTGAAGAAATGG

Propositions

1. *DNA primase* is the most robust genetic asset for resistance breeding against geminiviruses in any crop.
   (this thesis)

2. The GSVK motif of PELOTA holds potential for universal plant resistance against geminiviruses.
   (this thesis)

3. Novel technological advances catalyse a next agricultural revolution.

4. Mimicry of subjective experience is the *ipso facto* limitation of AI in producing art.

5. Contemporary economies are fundamentally unable to face climate change.

6. Genuine meaning emerges only by embracing the totality of human existence.

Propositions belonging to the thesis, entitled

Forging resilience from fragility: Insights on loss of susceptibility in Cucurbitaceae

Lampros Siskos

Wageningen, 24 November 2023

Forging resilience from fragility

Insights on loss of susceptibility in Cucurbitaceae

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Forging resilience from fragility

Insights on loss of susceptibility in Cucurbitaceae

Lampros Siskos

Thesis

Submitted in fulfillment of the requirements for the
degree of doctor at Wageningen University
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Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
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οἳ πρῶτα μὲν βλέποντες ἔβλεπον μάτην,
κλύοντες οὐκ ἤκουον, ἀλλ᾽ ὀνειράτων
ἀλίγκιοι μορφῆσι τὸν μακρὸν βίον
ἔφυρον εἰκῇ πάντα, κοὔτε πλινθυφεῖς
δόμους προσείλους ᾖσαν, οὐ ξυλουργίαν,
κατώρυχες δ᾽ ἔναιον ὥστ᾽ ἀήσυροι
μύρμηκες ἄντρων ἐν μυχοῖς ἀνηλίοις.
ἦν δ᾽ οὐδὲν αὐτοῖς οὔτε χείματος τέκμαρ
οὔτ᾽ ἀνθεμώδους ἦρος οὔτε καρπίμου
θέρους βέβαιον, ἀλλ᾽ ἄτερ γνώμης τὸ πᾶν
ἔπρασσον, ἔστε δή σφιν ἀντολὰς ἐγὼ
ἄστρων ἔδειξα τάς τε δυσκρίτους δύσεις.

Aeschylus – Prometheus bound (447-458)

Chapter 1

General introduction

Prometheus unbound: Harnessing the full natural potential

Nature is a dynamic operating system where energy is constantly recycled and transformed into different forms. This concept is known as the conservation of energy or the first law of thermodynamics. Living organisms, being part of this larger system, obey the same rules, for example, sunlight is converted into chemical energy through photosynthesis in plants, which is then consumed by animals and converted into mechanical energy for movement or heat energy for maintaining body temperature. A key quality feature distinguishing humans from the rest of the living organisms is the ability to actively direct and manipulate the transformations of energy and matter contrary to being passive receivers or objects to these changes. That particular key element, stemming from intellect and higher cognitive abilities, worked in favour of shaping this world in accordance not only to our survival needs but to our well-being, a term innate and relative to humans.

In that context, neolithic hunter-gatherers around 11,000 years ago begun to cultivate the land, domesticate plants and animals and settle in societies, an event that changed dramatically human civilization, known as first agricultural revolution (Skoglund et al., 2012). The creation of a food surplus, the population growth and the shift from nomadic lifestyle to organized societies, were only a few of the impacts of this revolution that paved the way for who we are today (Larsen et al., 1995). Agriculture became a trademark human activity that nourished great civilizations with material and immaterial heritage. During the 18th and 19th century, with the advancements in agricultural knowledge and technology (Allen, 2008) and the changes in land ownership patterns (Landes, 1969), a second agricultural revolution sprouted also known as British Agricultural Revolution. It mainly included the first transformation of animal breeding into a scientific practice (adoption of inbreeding for stabilizing desired genetic traits in cattle, and livestock culling) which resulted in the development of new more productive livestock breeds (Doherty et al., 2023). Other significant impacts of the British Agricultural Revolution included the adoption of crop rotation, mechanization of agriculture and the use of synthetic fertilizers (Zanden et al., 1991). The dire warnings of an impending future marked by scarcity and hunger, initially articulated by Thomas Malthus in relation to population growth outpacing food production, have gradually receded into distant echoes. This transformation can be attributed to remarkable advancements in agricultural productivity and the increasingly efficient utilization of resources. The further scientification of agriculture that followed mid- 1900, primarily with the hybrid crop breeding and genetic modification as well as the use of chemical pesticides, were only some of the few, yet some of the most significant means to secure food production for an increasing worldwide population.

Cutting-edge technological advances are appearing as the first quarter of the 21st century approaches to its end. The introduction of precise genome editing tools such as CRISPR/Cas in combination with the relatively recent progress on machine learning, artificial intelligence and nanobiotechnology catalyze the conditions for a new agricultural revolution that seems to be approaching. Agricultural process has been post-industrialized and is largely expelling outdated tactics of the past. While the Green Revolution significantly increased agricultural productivity (Evenson & Gollin, 2003), it also had negative environmental impacts, such as excessive fertilizer use, water pollution, and soil degradation (John & Babu, 2021). Additionally, energy use in form of fossil fuel had to increase 50 times (Gianpetro & Pimentel, 1993), something that is not acceptable to today's standards taking into account predictions regarding the limited availability of non-renewable energy sources and the impact of C02 emissions on climate change. Crop production is transforming into a fine-tuned exploitation of large datasets, using real-time monitoring and advanced analytics to make informed decisions for optimization of farming practices, also known as precision agriculture.

There is a significant decrease in time concerning agricultural processes, as the field is post- industrialized to meet the ever-increasing market needs. In these terms agriculture currently functions out of the scope of natural times compared to the conventional agriculture of previous centuries. It is striking for example, that hundreds of years were required for wild plants to turn to landraces through domestication, for later breeding and the creation of cultivars we know today. Natural genetic variation has always been the fundamental element of plant domestication. Natural mutations, products of a lengthy evolutionary process, were selected by humans for their favorable occurring traits. In modern times, advances in genetic engineering techniques such as CRISPR-Cas, have made it possible to induce specific heritable mutations in the genome of crops with precision and efficiency (Liu et al., 2021). These techniques allow scientists to directly edit the genetic material of an organism, introducing or modifying specific traits in a much shorter time frame compared to traditional breeding methods. Moreover, artificial intelligence and machine learning algorithms could play a role in predicting the effects of specific mutations on certain traits (Jamal et al., 2020; van Dijk et al., 2020; Rai, 2022). By analyzing large datasets of genomic and phenotypic information we can identify patterns and correlations that can inform researchers about the potential outcomes of genetic modifications. Additionally, *de novo* domestication using precise genome editing is a new way to introduce or modify specific genes in wild or semi-domesticated species in order to enhance, or modify desired traits (Razzaq et al., 2021). For instance, it could be used to confer disease resistance, improve yield, enhance nutritional content, or make other beneficial modifications. Therefore, utilizing contemporary genome editing techniques a process such as domestication that required

hundreds of years, can now be done in a few plant generations or less (Zsögön et al., 2018). Last but not least, the impressive recent advances of nanobiotechnology applications in plants, such as made to order synthetic plant immune receptors that improve plant immunity and confer the plants resilient against specific diseases, cannot be overlooked (Kourelis et al., 2023). With these innovations and forthcoming advancements, our aim as plant scientists is to ensure sustainable food production that meets the nutritional needs of the predicted global population of more than 10 billion people by the year 2100 (United Nations, 2022).

Cucurbits over the centuries: origin and domestication

Among several vegetables crops that have been domesticated throughout the course of time cucurbits are a significant source of food worldwide possessing a wide range of edible parts, including fruits, leaves, flowers, and seeds. Cucurbit plants exhibit a considerable morphological diversity in their fruits which are nutrient-rich and serve as essential components of a healthy diet. The fruits are characterized by their low-calorie content, making them a favorable choice for individuals seeking for a balanced nutrition. Moreover, they are low in fat while at the same time being rich in vitamins such as vitamin C and A (Manchali et al., 2021), minerals (including potassium and magnesium), and dietary fibers (Fulgoni et al., 2022). Their consumption supports overall health and it is a part of a well-balanced diet.

Cucurbits include plants like cucumbers, melons, squashes, and pumpkins which have originated in different regions around the world. Cucumis sativus (cucumber) is believed to have originated from India, and specifically from the foothills of the Himalayans (Sebastian et al., 2010). Genetic data suggest that cucumbers were first domesticated from the wild Indian C. sativus var. hardwickii (Qi et al., 2013) and it was firstly cultivated in the wider region Southeast Asia (Chomiki et al., 2020). Cucumis melo (melon) has undergone separate domestication events in both Asia and Africa. However, it is noteworthy that the Chinese melon, dating back to 3.000 B.C., stands out as the oldest known domesticated variety within this species (Endl et al., 2018). Citrullus lanatus (watermelon) is believed to have originated from Africa (Paris, 2015). Ancient Egyptian tomb paintings from around ~4450 BC depict watermelons, indicating their early presence in the region (Renner et al., 2019) (Figure 2).

Finally, Cucurbita genus which includes several squash and gourd species, is believed to have originated in the Americas while archaeological data suggest that squash and gourds were cultivated by indigenous civilizations in Mesoamerica, including parts of present-day Mexico and Central America (Chomicki et al., 2020; Pickersgill, 2016).

Figure 1. Morphological diversity of cucurbit fruits. (Grumet et al., 2021).

Cucurbita species were introduced to other parts of the world including Europe after the discovery of the Americas by the Europeans.

Nowadays, cucurbits are cultivated all over the world as their adaptability to different climates and growing conditions has led to their widespread cultivation in both temperate and tropical regions. China is the largest producer of cucurbits globally and cultivation includes a wide range of cucurbit crops, including watermelons, melons, pumpkins, and several types of gourds. In 2020, China reached an 80% share of the worldwide cucumber production and a 59% share of the watermelon production (FAOSTAT, 2020). The main European cucurbit production is centered throughout Mediterranean region, with Spain, Italy, France, and Greece being the most notable producer countries.

Major cucurbit diseases affecting yield, quality and thus the economy

Cucurbits are susceptible to various diseases that can significantly impact both the yield and economic viability of the production. Understanding the main diseases affecting cucurbit crops and their economic implications is crucial for farmers, researchers, and industry in developing effective disease management strategies.

Figure 2. Watermelon depicted on the walls of ancient Egyptian pharaonic tomb, 4450 BC. (Renner et al., 2019)

Fungal & Oomycete pathogens

Powdery mildew (PM), caused mainly by the fungi *Podosphaera xanthii* is one of the most common and economically significant diseases in cucurbit crops. It appears as a white, powdery coating on the leaves, stems, and fruits, leading to reduced photosynthesis and plant vigor. Infected plants exhibit stunted growth and diminished fruit quality, resulting in reduced market value and yield losses of up to 30-50% (Zhang et al., 2021; Lebeda & Sedláková, 2010).

Downy mildew (DM), caused by the oomycete pathogen *Pseudoperonospora cubensis*, is a devastating disease affecting cucurbit crops worldwide (Cui et al., 2022). It primarily affects leaves, causing yellow lesions on the upper surface and greyish-purple spore masses on the undersides. Downy mildew can lead to defoliation, reduced fruit set, and premature senescence, resulting in yield losses ranging from 30% to complete loss of crop production. The impact of downy mildew on the economy is substantial, causing significant economic losses that directly impact farmers' income and have broader implications on the overall supply and availability of cucurbit crops in the market (Savory et al., 2010).

Fusarium wilt, caused by the soilborne fungus *Fusarium oxysporum* f. sp. *niveum*, poses a significant threat to cucurbit crops, such as watermelons and other melons. This devastating pathogen infects the vascular system, impairing water and nutrient uptake, leading to wilting, stunted growth, and plant death. Fusarium wilt has a profound economic impact, causing substantial yield losses and reduced marketable fruit production (Zhang et al., 2015).

Bacterial pathogens

Bacterial wilt caused by *Erwinia tracheiphila* affects cucurbit crops, particularly cucumbers and melons in the USA whereas it is uncommon in the rest of the world (Shapiro et al., 2018). The bacteria invade the vascular system transmitted by cucumber beetles leading to wilting, stunting, and eventually plant death. Affected fruits may become unmarketable, leading to financial losses for growers. There is a limited availability of commercially resistant cultivars in cucurbits and most measures rely on controlling the insect vectors (Rojas et al., 2013).

Viral pathogens

Tomato leaf curl New Delhi virus (ToLCNDV) is one of the most significant viral diseases affecting cucurbit crops, especially in Europe. ToLCNDV has primarily infected tomato and tobacco plants, but since the previous decade it also causes severe epidemics in melon crops across the Mediterranean (Fortes et al., 2016). ToLCNDV is transmitted by the whitefly vector *Bemisia tabaci.* Leaf curling, mosaic, yellowing, stunting, and reduced overall vigor are the main symptoms of the virus in melon. Infected melon fruits may also exhibit deformities, affecting their marketability and quality (Siskos et al., 2022).

Cucumber mosaic virus (CMV) is one of the most widespread and economically significant viral pathogens affecting cucurbits. It infects a wide range of cucurbit crops, including cucumbers, melons, and squash. CMV causes mosaic symptoms, leaf deformation, stunting, and reduced fruit quality. The economic impact includes reduced yields due to poor fruit set, smaller-sized fruits, and decreased market value (Jacquemond, 2012)

Watermelon mosaic virus (WMV) is an aphid borne pathogen affecting mainly watermelon crops. It causes mosaic symptoms, severe leaf deformation, and reduced fruit quality. WMV can lead to significant yield losses, negatively impacting the marketability and economic value of watermelon fruits (Abdalla et al., 2021).

Current challenges in European cucurbit cultivation

Cucurbit cultivation in Europe currently faces significant challenges primarily attributed to the prevalence of PM, DM, and the relatively new viral pathogen ToLCNDV. Especially in the cucurbit greenhouses of Southeast Spain which is one of the main European centers of cucurbit production, the signs and symptoms of the aforementioned diseases are quite common (Figure 3). These pathogens pose a substantial threat to crop productivity, and effective control measures are required. Controlling ToLCNDV especially, presents a particularly arduous task due to its viral nature, and it is mainly limited on controlling whitefly vector populations. To address these pressing concerns, the development of resistant varieties stands out as a necessary approach. The generation of resistant cultivars holds immense potential for mitigating the impact of these pathogens on cucurbit crops. Notably, the urgent need to combat PM, DM, and ToLCNDV underscores the significance of focusing breeding efforts on incorporating resistance traits specific to these pathogens.

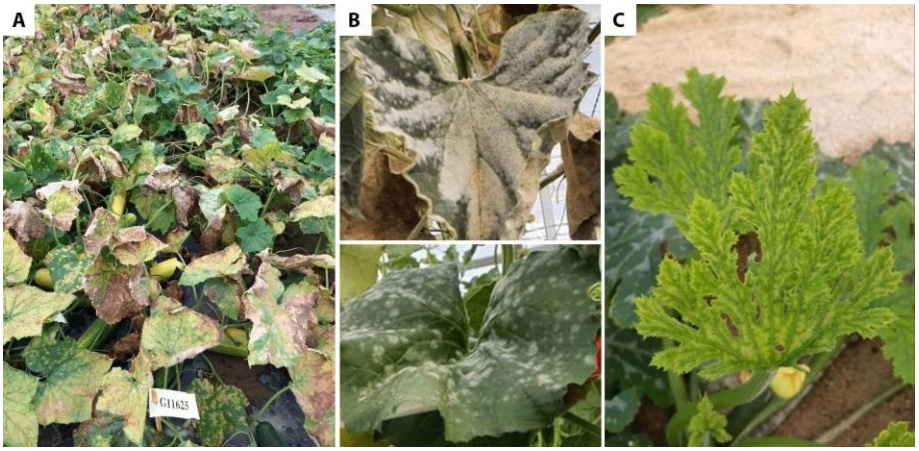

Figure 3. Common phenotypic representation of diseased cucurbit fields in Almeria, Spain, in 2021. **A)** Open field cucumber infected with downy mildew (picture by Daniel Belon Dona, BASF vegetables, Spain), **B)** Greenhouse melon infected with powdery mildew (picture by Jose Riado, SAKATA vegetables, Spain) **C)** Greenhouse zucchini exhibiting ToLCNDV symptoms (picture by author, zucchini greenhouse, Spain).

Understanding the fundamentals of plant immunity

Plant pathogens have detrimental effects on the economy and contributed social and cultural changes in the past. A notable example is the Irish potato famine caused by late blight disease during the years 1845 to 1852. The famine had devastating consequences, resulting in the death and/or emigration of millions of people and leaving a lasting impact on Irish society and culture (Yoshida et al., 2013). Plants, like all living organisms, face

constant challenges from a range of potential threats, including pathogenic organisms and environmental stresses. As a response, plants have evolved sophisticated mechanisms to defend themselves. Understanding these mechanisms is pivotal in the development of sustainable crop production strategies that promote resilience, reduce reliance on chemical interventions and sustain the economy.

Plant immunity can be broadly classified into two main levels. The first level considers the basal immunity, which represents the first line of defense in plants (Hou et al., 2011). It involves basic defense mechanisms that provide a general level of resistance against a broad range of potential pathogens. The key components in basal immunity are pattern recognition receptors (PRRs) which are proteins present on the plant cell surface (Macho et al., 2014). PRRs recognize specific molecular patterns associated with pathogens, known as pathogen-associated molecular patterns (PAMPs). Upon PRR-PAMP recognition, a cascade of defense responses is triggered, including the production of antimicrobial compounds, strengthening of the cell wall, and activation of defense-related genes resulting to PAMP triggered immunity (PTI) (Macho et al., 2014; Bigeard et al., 2015)

Adapted plant pathogens employ a strategy of secreting a large array of effector proteins, which effectively suppress the response of basal immunity in several ways. As a second level of immunity, plants subsequently produce resistance (R) proteins that possess the capability to directly or indirectly recognize the pathogen effectors and trigger a secondary immune response known as effector-triggered immunity (ETI). This interaction known as "gene-for-gene interaction," underlies the functional association between plant resistance proteins and pathogen effectors (Selin et al., 2016; Rajamuthiah & Mylonakis, 2014; Gassmann et al., 2012; Nguyen et al., 2021).

Following recognition of specific molecules derived from the pathogen, such as pathogen-associated molecular patterns (PAMPs) or effector molecules, a vital defense mechanism employed by plants to combat pathogen invasion is activated, known as hypersensitive response (HR) (Balin-Kurti, 2019). The accumulation of defense signaling molecules such as salicylic acid (SA), jasmonic acid (JA) and especially reactive oxygen species (ROS), in the infected cells ultimately leads to programmed cell death (PCD), which forms a physical barrier restricting the pathogen's spread (Coll et al., 2011). Thus, HR is a direct and localized cell death reaction that limits the spread of invading biotrophic pathogens and enhances overall plant resistance. The activation of this specific defense mechanism plays a major role in the plant's ability to defend against invading pathogens. Eventually, HR triggers systemic acquired resistance (SAR), a long-lasting defense response that offers systemic protection of the plant against future pathogen attacks (Conrath et al., 2006).

Introgression of resistance (R) genes is a popular plant breeding approach that involves the introgression of specific genes from a donor plant into a recipient plant to enhance its resistance against specific pathogens. The introgressed R genes code for resistance proteins that recognize specific pathogen effectors as described above and trigger defense responses to combat pathogen attacks. By introducing R genes, plants gain the ability to detect and respond effectively to the presence of the corresponding pathogen strains. The type of resistance that results from R gene introgression is also known as dominant resistance since the resistance trait is expressed when the dominant gene is present. However, the durability of R gene-mediated resistance can be a challenge. Pathogens have the potential to evolve and overcome the recognition by R genes through the production of novel effectors or mutations in existing effectors or downregulation of the effectors. This can lead to the breakdown of resistance over time, requiring the continuous identification and incorporation of new R genes or combinations of multiple R genes to maintain effectiveness (de Ronde et al., 2014).

Comprehending the mechanisms of plant susceptibility

Plant susceptibility genes play a significant role in both levels of plant immunity. They are genetic components innate to plants that are hijacked upon infection by the pathogen effectors manipulating plant defense mechanisms and creating a hospitable environment in order to establish a successful infection (van Schie & Takken, 2014). Evolutionary these genes are not there primarily to support pathogens, but they are responsible for important physiological and developmental functions. Mutations in susceptibility genes, whether they occur through natural (Lapidot et al., 2015) or artificial means (Zaidi et al., 2018) such as genome editing, can often lead to a loss of susceptibility and an increase in plant resilience against pathogens. The loss of susceptibility that is occurring due to non-functional S-genes is also known as recessive resistance. These mutations can result in altered or disrupted functions of the susceptibility genes, leading to enhanced resistance or tolerance to pathogens. S-genes were categorized by van Schie & Takken, 2014, according to their functional role in susceptibility in:

a. **Genes for basic compatibility**: Genes modulating cuticle and cell wall properties (Uppalapati et al., 2012; Hansjakob et al., 2011), controlling stomatal movement (Liu et al., 2009), and altering membrane dynamics (Buschges et al., 1997).

b. **Immune suppressors**: S-genes that function as immune suppressors play a crucial role in dampening the plant's defense responses, allowing pathogens to evade detection and establish infection. These genes include regulators of hormonal defense signaling, such as SA/JA signaling pathway regulators (Zhang et al., 2013; von Saint et al., 2011), which are key players in plant immune

responses. Additionally, these genes include PTI suppressors (Trujillo et al., 2008; Ellis et al., 2002), which target components of the plant's first line of defense mechanisms, and ETI suppressors, which interfere with the plant's recognition and response to specific pathogen effectors (Choi et al., 2018). By manipulating these immune signaling pathways and defense mechanisms, pathogens can effectively undermine the plant's ability to mount an effective immune response, providing them with a favorable environment for infection and proliferation.

c. **Genes that support pathogen nutrition**: S-genes that function as nutrient providers are critical in supporting the needs and growth of the invading pathogen within the host plant. These genes include various components such as sugar transporters, enzymes involved in metabolite biosynthesis, and viral replication machinery. Sugar transporters facilitate the uptake of sugars from the host plant, serving as a nutrient source for the pathogen (Huai et al., 2022). Genes involved in metabolite biosynthesis contribute to the production of essential compounds that nourish the pathogen (Xian et al., 2020). In the case of viral pathogens, genes involved in viral replication are crucial for the successful proliferation and spread of the virus within the host plant (Siskos et al., 2023). By exploiting these S-genes, pathogens can acquire the necessary resources and energy to thrive within the host, enhancing their ability to cause disease.

Shifting immunity towards loss of susceptibility

Advances in genomics and molecular biology techniques have enabled researchers to identify and characterize susceptibility genes in various plant species. This knowledge can be leveraged to engineer plants with enhanced disease resistance by modifying susceptibility genes through CRISPR/Cas genome editing or through the detection of natural mutations of S-genes in any crop germplasm. The integration of disrupted susceptibility genes into crop plants through breeding is commonly referred to as recessive resistance breeding (Hashimoto et al., 2016). Recessive resistance is characterized by the need for two copies (in a diploid) of the resistance allele (homozygous) for effective protection and is considered more durable than dominant resistance. In dominant resistance the pathogen can exert selective pressure against its own factors (effectors) that make it recognizable by the plant’s R proteins. On the other hand, recessive resistance often targets essential steps in the pathogen's life cycle or interactions with the host plant. Disrupting these critical interactions makes it more challenging for pathogens to overcome the resistance without compromising their own fitness. This poses a higher barrier for the pathogen to evolve mechanisms to bypass the disrupted susceptibility genes, making recessive resistance more durable.

Over the years, extensive breeding efforts have been undertaken to enhance disease resistance in cucurbit crops, particularly against the key diseases affecting European cucurbit cultivation, PM, DM, and ToLCNDV (Cui et al., 2022, Siskos et al., 2022). These endeavors have yielded several disease-resistant plant varieties that are currently available in the market. However, there remains an imperative to develop novel cultivars with enhanced durability and broad- spectrum resistance to address the evolving market demands. Notably, ToLCNDV poses a recent threat to cucurbit crops in Europe, and the number of resistant varieties is currently limited. Moreover, the continuous emergence of resistance-breaking strains, exacerbated by the high selective pressure exerted by pathogens in intensive agriculture, renders R-gene stacking through introgression breeding a short-term solution. Consequently, the industry has shifted its focus toward breeding for recessive resistance by incorporating susceptibility genes with loss- of-function characteristics into commercial varieties. Given these circumstances, a top research priority requires investigating the hidden susceptibility loci within the cucurbit genomes associated with the aforementioned diseases. Eventually, either by disruption of these loci through CRISPR/Cas genome editing or by identifying already existing naturally disrupted susceptibility loci existing in cucurbits germplasm we focus on generating durable and broad- spectrum resistance against these important diseases. By achieving this, we aim to address the industry's needs and secure the future of cucurbit crop production.

Scope of this thesis

The objective of this thesis was to investigate recessive resistance in cucurbit crops for the three most economically important diseases, PM, DM and ToLCNDV. Our aim was to examine the potential of mutations in candidate susceptibility loci in cucumber and melon for conferring resistance to the aforementioned pathogens. Three approaches were utilized in order to investigate recessive resistance in these crops.

The first approach involved diving into the melon germplasm to detect naturally occurring high- impact mutations within candidate susceptibility gene homologues. These candidate genes were selected based on their similarity to known S-genes in other plant species or their involvement in established recessive resistance Quantitative Trait Loci (QTLs) in cucumber and melon.

The second approach involved the complementation of candidate S-genes from cucurbits in tomato mutant resistant backgrounds for examination of any potential restoration of susceptibility.

The third approach was the optimization of CRISPR/Cas9 genome editing in cucumber and melon in order to knock-out candidate S-genes.

In **Chapters 2 and 3**, we present a systematic overview of the three current major cucurbit diseases, PM, DM and ToLCNDV as well as all known resistance loci in melon germplasm against these diseases. Eventually, we propose strategies for breeding melon cultivars with durable and broad-spectrum resistance to these pathogens.

In Chapter 4, we have identified the *DNA primase large subunit* (*PriL*) as a susceptibility gene that participates within a known recessive QTL associated with resistance to ToLCNDV in melon. Through genomic analysis conducted on CGN melon germplasm, this study identified specific mutations in the coding sequence of *PriL* that consistently associate with highly resistant phenotypes to the virus across multiple melon accessions. Additionally, by applying a reverse genetics approach in *Nicotiana benthamiana* we have shown that reduced expression of the native *PriL* results in limited viral proliferation of ToLCNDV and two more geminiviruses in the plant. These findings altogether emphasize a conserved function of this gene in geminiviral replication. Eventually we present a model explaining the role of *PriL* during initiation of geminiviral DNA replication.

In **Chapter 5**, by complementation of melon *PELOTA* in a ty-5 background tomato we show a restoration of susceptibility for TYLCV both in viral titers and symptoms, revealing the conserved functional role of this gene in TYLCV susceptibility independent of the host plant. At the same time considering the gene's reported association with susceptibility to multiple geminiviruses, we confidently suggest it as a noteworthy candidate S-gene for ToLCNDV, a significant melon pathogen.

In **Chapter 6**, Utilizing re-sequencing data from 100 melon genomes we performed allele- mining on known S-genes homologues for powdery mildew and downy mildew. Through our analysis, we successfully identified accessions harboring loss-of-function mutations in a melon homolog of the well-known S gene, *TCP14*, known for its role in DM susceptibility in arabidopsis. Additionally, we discovered a knock-out mutation in a Clade V *MLO* gene, which has been previously linked to susceptibility to PM in cucumber, which was able to confer partial resistance to PM in melon.

In **chapter 7**, we present our work on CRISPR/Cas9 genomic editing of candidate S-genes of cucumber and melon through stable *Agrobacterium* mediated transformation. We successfully managed to induce mutations in melon's *AGD5* gene, which is a candidate susceptibility factor for DM. Despite obtaining transgenic calli which

harbored the CRISPR/Cas9 mutations, due to the recalcitrant nature of these plants in shoot regeneration it was not possible to produce full transgenic plants for further evaluation of the resistance. Optimization steps in cucumber and melon transformation and regeneration are proposed.

Finally, in **Chapter 8** I present a comprehensive overview of our major findings, along with a detailed discussion on how to effectively apply the insights gained from our research to foster the development of a robust and disease-resilient cucurbit production.

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Chapter 2

Breeding melon (Cucumis melo) with resistance to powdery mildew and downy mildew

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Abstract

Melon (*Cucumis melo* L.) production is often restricted by a plethora of pests and diseases, including powdery mildew and downy mildew caused respectively by the fungal species *Podosphaera xanthii*/ *Golovinomyces orontii* and oomycete species *Pseudoperonospora cubensis*. Many efforts have been directed on identification of resistant sources by screening (wild) melon germplasm. In the current review, we summarized such efforts from various publications of the last 50 plus years. Resistance to powdery mildew has been identified in 239 melon accessions and downy mildew resistance in 452 accessions of both *C. melo* and the wild relative species *C. figarei*. Among the resistance sources, *C. melo* var. *cantalupensis* accessions PMR 45, PMR 5, PMR 6, and WMR 29 as well as *C. melo* var. *momordica* accessions PI 124111, PI 124112, and PI 414723 have been considered as the most valuable germplasm because multiple resistance genes have been identified from these accessions and are widely used in melon resistance breeding. Further genetic mapping in a number of resistant sources has enabled identification of 25 dominant genes, two recessive genes and seven QTLs conferring powdery mildew resistance, as well as eight dominant genes and 11 QTLs for downy mildew resistances. Based on the reported sequences of associated markers, we anchored physically (many of) these genes and QTLs to chromosomes of the melon cv. DHL92 genome. In addition to presenting a comprehensive overview on powdery mildew and downy mildew resistance in (wild) melon germplasm, we suggest strategies aiming at breeding melon with durable and broad-spectrum resistance to pathogens and pests.

Introduction

Melon (*Cucumis melo* L.) belonging to the *Cucurbitaceae* family is a worldwide economic important horticultural crop. It is highly appreciated for its edible fruits that can be consumed either immature (not sweet) or in most cases, mature (high sugar content and thus sweet) (Pitrat, 2008, 2016; Grumet et al., 2021). Melon displays a great genetic diversity, which is exhibited in many aspects, such as fruit size, shape, color, ripening behavior (i.e., climacteric and a non-climacteric type), peel texture (smooth-skinned to netted), and sex determination (i.e., monoecious and andromonoecious) (Garcia-Mas et al., 2012; Zhao et al., 2019).

Based on phenotypic variations on flower (i.e., sex expression, sepals, and hypanthium and ovary hairs) and fruit traits (i.e., size, shape, exocarp, mesocarp, placentas and seeds), melon has been classified into 19 botanical groups including feral and (non-) cultivated types. They are *Cucumis melo* var. *conomon*, *makuwa*, *chinensis*, *momordica*, *acidulus*, *cantalupensis*, *inodorus*, *ibericus*, *cassaba*, *tibish*, *chandalak*, *ameri*, *flexuosus*, *chate*, *dudaim*, *chito*, *agrestis*, *indicus* and *kachri* (Pitrat, 2016; Pitrat et al., 2000). Among them, *C. melo* var. *chito* is not domesticated and thus represents a feral type. *C. melo* var. *agrestis* is domesticated, but not cultivated (Pitrat, 2016). Melon cultivars belonging to the *cantalupensis* (known as cantaloups and galia), *inodorus* (honeydew), and *ibericus* (Piel de Sapo) groups are economically the most important and most consumed.

Melon cultivation in open fields is confined to the tropical and subtropical countries with warm temperature. In less favourable climates during cool seasons, melon is also widely grown in greenhouses (Burger et al., 2010). These different cultivation practices are challenged by a plethora of pests and diseases caused by different pathogens. Some of the common pests and diseases in melon production are listed in Table 1. In this review, emphasis has been placed on two of them, i.e., powdery mildew and downy mildew, with the aim to summarize the results of the last 50 plus years on identification of resistant melon accessions and their use in introgression breeding.

Table 1. Some common pests and diseases of melon (*Cucumis melo*)

Class	Disease name	Pathogen	Country	Reference
Bacterium	Bacterial fruit blotch	*Acidovorax avenae* subsp. *citrulli*	Worldwide	Bahar 2009
	Internal fruit rot	*Pantoea ananatis*	Japan	Kido 2008
Fungus	Anthracnose	*Colletotrichum orbiculare; C. lagenarium*	Worldwide	Kuan et al., 2011; Damm et al., 2013
	Powdery mildew	***Leveillula Taurica, Golovinomyces orontii, Podosphaera xanthii***	**Worldwide**	Cohen et al. 2004; Kuzuya et al. 2006
	Fusarium wilt	*Fusarium oxysporum* f. sp. *melonis*	Worldwide	Wechter et al., 1995
	Monosporascus root rot	*Monosporascus cannonballus, M. eutypoides*	Worldwide	Castro et al., 2019
Nematode	Root-knot nematode	*Meloidogyne* spp.	Worldwide	Guan et al., 2014
Oomycete	**Downy mildew**	***Pseudoperonospora cubensis***	**Worldwide**	Lebeda and Cohen, 2011
Virus	Cucurbit yellow stunting disorder	cucurbit yellow stunting disorder virus	Worldwide	Marco & Aranda, 2005
	Cucumber green mottle mosaic	cucumber green mottle mosaic virus	Worldwide	Dombrovsky et al. 2017
	Leaf curl	tomato leaf curl New Delhi virus	India, South Asia Mediterranean Basin, Europe, North America	Fortes et al., 2016; Romay et al., 2019
Pest	Melon-cotton aphid	*Aphis gossypii* (Homoptera: Aphididae)	Worldwide	Capinera, 2000
	Leafminer	*Liriomyza trifolii* (Diptera: Agromyzidae)	Worldwide	Dogimont et al., 1999
	Melon fruit fly	*Bactrocera cucurbitae*	Worldwide	Dhillon et al., 2005
	Cucumber beetles	*Diabrotica* spp., *Acalymna vittatum*	North America	Sasu et al., 2010; Weber, 2018
	Red beetle pumpkin	*Aulacophora foveicollis* (Coleoptera: Chrysomelidae)	South Asia	Rashid et al., 2014

Powdery mildew

Powdery mildew is one of the most important plant diseases worldwide and commonly occurring in different cucurbit crops. The disease is caused by biotrophic fungi (Erysiphales) that are able to infect leaves, stems, flowers and fruits, significantly downgrading both the quality and quantity of fruits (Křístková et al., 2009). Three species are reported to affect cucurbits namely, Leveillula taurica, Golovinomyces orontii and Podosphaera xanthii. The most common one in cucurbits is P. xanthii, whereas L. taurica is of minor economic importance (Glawe, 2008; Lebeda et al., 2016). The first report of powdery mildew in melon dated back to the 1920s in California, the USA with P. xanthii (Erysiphae cichoracearum) (Jahn et al., 2002) being the causal agent. Nowadays, the disease is reported over the world (Table 2). Relatively high temperatures and humidity seem to be optimal for P. xanthii. On the other hand, *G. orontii* requires lower temperatures and is flourishing in colder climates and more Northern latitudes (Jagger, 1926; Jahn et al., 2002). *G. orontii* was predominant powdery mildew in cucurbits until 1958. The shift of predominance between the two powdery mildew species could be a true evolution of the pathogen but could also be attributed to misidentification of the pathogen throughout the world (Cornell University, https://www.vegetables.cornell.edu/pest- management/disease-factsheets/cucurbit-powdery-mildew/; Jagger, 1926; Jahn et al., 2002).

Table 2 Powdery mildew species infecting cucurbits

Species	Occurrence in Cucurbits	Favored conditions	Reference
Leveillula taurica	Mexico, Libya, Russia, USA, Greece, Bulgaria, Japan, Kenya, Lebanon, Romania, Senegal, Morocco	Wide range of environmental conditions. Usually, warm and dry conditions for cucurbits	El-Ammari, 1983; Palti, 1988; Forster, 1989; Sokolov and Sokolova, 2010; Beltrán-Peña et al., 2018
Golovinomyces orontii	Italy, Czech Republic, Iran, Hungary, Belarus, Bulgaria, Germany	Low temperatures, cold climates	Pirondi et al., 2016
Podosphaera xanthii	China, India, Argentina, Israel, South Korea, Japan, Mexico, Russia, Thailand, USA, Italy, Myanmar, Spain, Egypt, Iraq, Libya, France, Greece, Czech Republic	Relatively high temperatures and humidity	Jagger, 1926; Palti, 1988; del Pino et al., 2002; Delhey et al., 2003; Liang et al., 2007; Voytyuk et al., 2007; Rajamuthiah and Mylonakis, 2014; Li et al., 2019; Xu et al., 2020

Disease cycle and symptoms

Powdery mildew infection initiates when an ascospore (sexual spore), or more often, a conidium (asexual spore) lands on plant surfaces (e.g. leaves, stems, cotyledons) (Fig. 1). After germination of the conidium, a germ tube is formed and elongates into a swollen hyphal structure called appressorium. Appressoria provide turgor pressure to the penetration pegs for the fungus to breach the host epidermal cell. After entering the plant cells, *P. xanthii* forms haustoria. In addition to the uptake of nutrients, haustoria play a key role for the delivery of molecules (e.g effectors) to maintain a biotrophic relationship with the host. From growing hyphae, conidiophores are formed, which carry new airborne conidia ready for the next round infection. The hypha develops on top of plant surfaces (leaves, stems, cotyledons) producing white powdery spot formations (Fig. 1), resulting in a significant inhibition of photosynthetic ability of the plant (Jagger, 1926). The less frequent sexual disease cycle appears with merging of compatible mating hyphae and the production of chasmothecia.

Podosphaera xanthii races

Research groups from various countries have identified 21 *P. xanthii* races on melon (Table 3). The first three race, Race 1, 2 and 3, were identified in the USA prior to the 1980s (Jagger and Scott, 1938; Thomas, 1978). Since 1984, 18 new races have emerged in different regions, including three more races in the USA (McCreight and Coffey, 2011), five in France (Bertrand and Pitrat, 1989), three in the Czech Republic (Lebeda et al., 2004) and seven in Asia (Hosoya et al., 2000; Liu et al., 2010a; Hong et al., 2018). In Fig. 2 (upper panel), the timeline of emerging new races and their spreading is presented.

Yield loss and control methods

Powdery mildew can lead to significant yield losses under conditions favorable to the melon pathogen (http://ipm.illinois.edu/diseases/series900/rpd925/). The main control measure remains the application of fungicides. However, resistance of *P. xanthii* to various systemic fungicides has been frequently reported since 1967, when the first *P. xanthii* race resistant to benomyl was identified (Schroeder and Provvidenti, 1969). Today, Sterol demethylation inhibitor fungicides (SDMI) are used effectively against powdery mildew in cucurbits. The main advantage for the use of this class is that it requires polygenic changes of the pathogen to become resistant (Pérez-García et al., 2009). Further, several biological control agents are used against powdery mildew in cucurbits, including *Bacillus* bacteria (*e. g. B. subtilis, B. amyloliquefaciens, B. pumilus*), epiphytic fungi (e.g. *Pseudozymaaphidis)* and other mycoparasitic microorganisms (e.g. *Gliocladium catenulatum)* (Gafni et al., 2015; Ni and Punja, 2021). Apart from application of chemical plant protection products and biological control agents, plant breeding has made great efforts towards producing resistant varieties.

Genetic resources of powdery mildew resistance

With various screening and assessment methods (Table S1), *C. melo* germplasm collections including traditional/modern cultivars, breeding lines, and landraces as well as wild relatives have been screened for powdery mildew resistance (Table S2). In total, 239 accessions were considered powdery mildew resistant to different races (Table 3). In addition, we summarized 568 susceptible accessions reported in 16 studies (Table S3), with the aim to avoid redundant screenings in future studies.

It is worth mentioning that accessions PMR 5, PMR 6, and WMR 29 belonging to *C. melo* var. *cantalupensis* as well as accessions PI 124111, PI 124112, and PI 414723 corresponding to *C. melo* var. *momordica* showed resistance to multiple races (Fig. 3) and have been explored in resistance breeding (Fig. 2, lower panel). PMR 45 carrying the dominant gene *Pm-1* (Table S4) was the first powdery mildew resistant variety released 11 years after the official report of race 1 (Bohn and Davis, 1964; Harwood and Markarian, 1968; Kenigsbuch, 1992). On year after introduction of the powdery mildew resistant variety, the resistance in PMR 45 was broken by the next emerging race, race 2. A few years later, variety PMR 5, resistant to both races 1 and 2, was released (Epinat et al., 1992). This resistant cultivar carries several resistance genes (*Pm- 1*, *-2*, and *-D*, Table S4) and provided good protection against *P. xanthii* for more than three decades until a new race 3 was identified in the USA in 1976 (McCreight and Coffey, 2011) and in India in 1986 (Kaur et al., 1986). Melon accession PI 414723 (with resistance loci *Pm- 7*, *-x*, *-x1,5*, and *-x3*, Table S4) originating from India, was identified in 1986 as an effective source to combat race 3 (Fazza et al., 2013). One decade later in 1996, races 4 and 5 were first observed in France (Bertrand, 2002). Immediately afterwards in 1997, cultivars PI 414723 and PMR 5 were tested to be resistant to both races, while Edisto 47 (with resistance loci *Pm- Edisto47-1*, *-2*, Table S4) was effective to race 4 (del Pino et al., 2002; Rabelo et al., 2017). In 2000, races N1, N2, N3, and N4 were reported in Japan (Hosoya et al., 1999), and the first variety AR 5 (with resistance QTLs CMBR111 and CMBR8-CMBR120, Table S4) showed resistance to race N1 was released in 2008 (Fukino et al., 2008). Later, race N1 resistant variety TGR 1551 (with resistance QTL, Pm-R, Table S4), was released in 2011 (Yuste-Lisbona et al., 2011). Race S and accession PI 313970 being resistant to this race, were reported simultaneously in 2003 (McCreight and Coffey, 2011). Different from other resistance sources, one recessive gene, designated *pm-s*, was identified in accession PI 313970 that delivers resistance to *P. xanthii* race S (McCreight and Coffey, 2011). Recently in 2018, races KN 1 and KN 2 were both first reported in South Korea, and their resistant accession PI 124112 (carrying several *Pm*-genes including *Pm-4*, *-5*, *-V.1*, and *-XII.*1, Table 4) and variety MR1 (with a QTL, *Bpm12.1*, Table S4) derived from PI 124111 also have been selected in the same year (Fig. 2) (Hong et al., 2018).

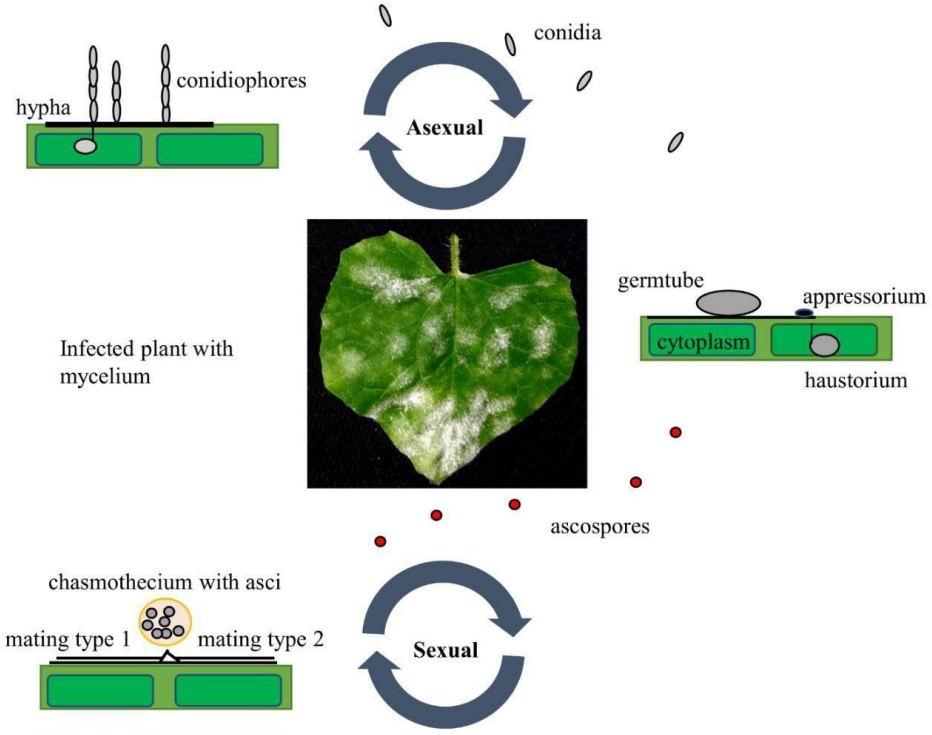

Figure 1. Sexual and asexual infection cycles of *Podosphaera xanthii*. **Asexual**: Conidia from infected leaves land on plant tissue. An appressorium is formed at the end of an elongated germ tube. A haustorium is subsequently formed in plant cell. Finally, conidiophores are formed on the invading hyphae. **Sexual:** The sexual disease cycle occurs less often and appears when two compatible mating hyphae merge to produce spherical structures called chasmothecia. Chasmothecia produce asci with ascospores which are dispersed to start an infection similar to conidia.

So far, 25 dominant resistant loci, two recessive loci/genes and seven QTLs have been identified from a number of resistance sources (Table S4 and S5; Fig. 4). The dominant loci are mostly clustered on chromosomes 2 and 5, indicating a potential allelic relationship of the ones in the same cluster. Four QTLs namely QTL1 (Fukino et al., 2008) on chromosome 2 and qCmPMR-12, QTL2, BPm12.1 (Fukino et al., 2008; Li et al., 2017; Cao et al., 2021) on chromosome 12 co-localize with the genomic positions of several resistance loci (Fig. 4). Next to dominant resistance genes and QTLs, recessively inherited resistance genes have also been identified in melon wild species *C. chate* accession C18 and *C. melo* var. *acidulus* accession PI 313970 (Hong et al., 2015; McCreight and Coffey, 2011). A loss-of-function mutant of a *MLO* (*mildew resistance locus O*) homolog was responsible for powdery mildew resistance to *P. xanthii* race 1 in accession C18 (Hong et al., 2015). In accession PI 313970, one recessive gene,

designated *pm-s*, was identified as conditioning resistance to *P. xanthii* race S (Fig. 3) (McCreight and Coffey, 2011). Whether *pm-s* encodes a loss-of-function mutant of the *MLO* allele remains to be investigated.

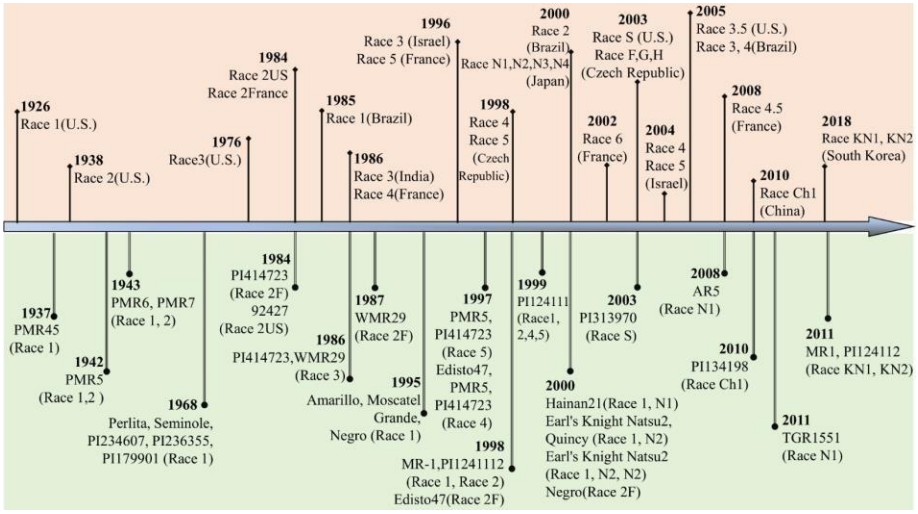

Figure 2. *Podosphaera xanthii* races emerging year and resistance breeding history of melon **Upper panel:** the timeline showing the years when new races were first reported and the reported races appeared in new areas. **Lower panel:** the timeline showing the years when resistant melon lines were introduced into cultivation and the resistance spectrum.

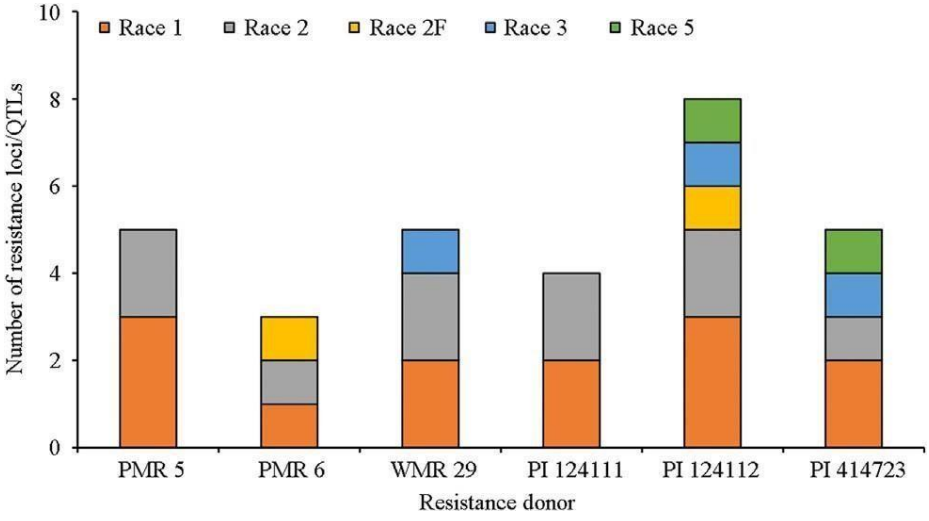

Figure 3. Number of resistance loci/QTLs identified in *C. melo* var. *cantalupensis* (PMR 5, PMR 6, and WMR 29) or *C. melo* var. *Momordica* (PI 124111, PI 124112, and PI 414723) accessions with broad-spectrum resistance to different *P. xanthii* races

Table 3. Previously identified sources of *Cucumis* species resistant to *Podosphaera xanthii* races

	***Podosphaera xanthii* races [b]**																			
Cucumis groups/species [a]	**0**	**1**	**2**	**2F**	**2U.S.**	**3**	**3.5**	**4**	**5**	**6**	**Ch1**	**G**	**H**	**KN1**	**KN2**	**N1**	**N2**	**N3**	**N4**	**S**	**Not known**
C. melo		42	8	5	3			14	1		1					2	3				5
C. melo* var. *acidulus		2		1	1	1	2		1											1	6
C. melo* var. *agrestis		1	1						1												
C. melo* var. *ameri																					**2**
C. melo* var.*cantaloupensis	**7**	**7**		**6**	**2**	**1**	**1**	**3**	**2**	**2**			**1**		**2**	**6**	**3**	**3**	**3**		**6**
C. melo* var. *ibericus		**1**																			
C. melo* var. *inodorus		**3**																			**1**
C. melo* var. *kachri																					**1**
C. melo* var. *makuwa																					**2**
C. melo* var. *momordica	**4**	**9**		**4**	**3**	**9**	**5**	**4**	**9**	**2**		**3**		**2**	**2**						**13**
C. africanus		**1**	**1**																		
C. anguria		**1**	**1**																		
C. prophetarum		**1**	**1**																		
C. zeyheri		**1**																			

Note: [a] *C. melo* denotes taxonomy groups that were not shown in the corresponding reports. Detailed information of resistance source per group is provided in Table S2.
[b] *P. xanthii* races not mentioned in the original publication were categorized as not known.

Resistant *C. melo* accessions to *P. xanthii* races were summarized from the following publications: Floris and Alvarez, 1996; Pan and More, 1996; Hosoya et al., 2000; McCreight, 2003, 2006; Wu et al., 2004, 2008; Alvarez et al., 2005; Fukino et al., 2008; Liu et al., 2010b; McCreight and Coffey, 2011; Sales Júnior et al., 2011; Wang et al., 2011; Yuste-Lisbona et al., 2011; Guan et al., 2014; Nunes et al., 2015; Li et al., 2017; Rabelo et al., 2017; Hong et al., 2018; Manchali et al., 2019; Thakur et al., 2019.

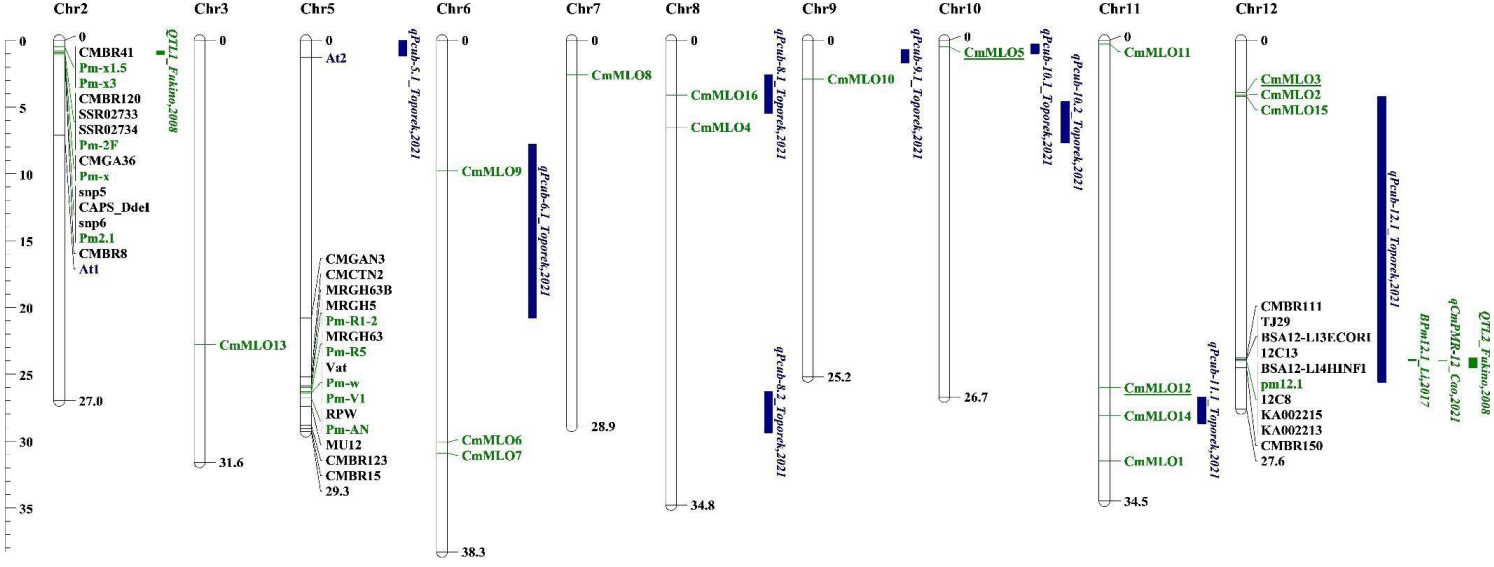

Figure 4. Physical positions at the Megabase scale of *MLO*-like genes, previously described resistance genes, QTLs, and resistance-linked molecular markers (black) to powdery mildew disease (green), and downy mildew disease (blue) on the chromosomes of *Cucumis melo* according to the *C. melo* L. cv. DHL92 genome sequence database (CuGenDB, http://cucurbitgenomics.org/). QTLs/genes were mapped by retrieving the reported sequences of associated markers andprimer pairs (Table S5). *In-silico* mapping was performed using BLASTN. QTL (Italic) for resistance are displayed as bars and the original names given by the authors first describing them are maintained. The *MLO*-like genes that clustered in a neighborhood joining tree together in Clade V are underlined.

Podosphaera xanthii effectors

The dominant resistance for powdery mildew identified so far is shown to be short-lived since it can be easily broken by new emerging races (Fig. 2). The emergence of new resistance breaking strains is an evolution of the pathogen at the molecular level. Pathogens including *P. xanthii* secrete a large array of effector molecules that manipulate the host mechanisms for a successful disease establishment. For example, the pathogen effectors intervene with the PAMP-triggered immunity (PTI) to suppress host defense. PAMPs are pathogen-associated molecular patterns including various types of pathogen-derived molecules from nucleic acids to proteins, lipids and carbohydrates. Upon perception of PAMPs, plant patter recognition receptors (PRRs) trigger rapid PTI responses. It is possible that many QTLs identified in melon for powdery mildew resistance could encode PRRs. Mostly likely, the dominant genes may encode resistance proteins that recognize specific powdery mildew effectors leading to effector- triggered immunity (ETI) (Pedersen et al., 2012; Rajamuthiah and Mylonakis, 2014; Toruño et al., 2016). Traditional breeding is focusing on introgression of resistance genes from wild relatives in commercial varieties. However, due to evolutionary changes the pathogen effector often loses the ability to be recognized by a specific resistance protein leading to compromised resistance.

Research on *P. xanthii* effectors is limited due to the obligate biotrophic lifestyle of the pathogen. Knockout of genes that lead to loss of virulence also make the pathogen non-viable. Only recently, studies on the epiphytic transcriptome of *P. xanthii* have been published, aiming to identify candidate effector proteins. Authors identified 137 secreted proteins with 53 being candidate effector proteins as they showed similarity to previously identified fungal pathogen effectors and included the N-terminal conserved motif Y/F/WxC known to be present in many candidate effector proteins of other powdery mildew species (Vela-Corcía et al., 2016). Host induced gene silencing (HIGS) was performed by the same group later on and revealed that six of these candidates were required for powdery mildew pathogenesis (Martínez-Cruz et al., 2018). PHEC27213 is one of these six candidates, and was a highly expressed haustorial candidate effector, belonging to lytic polysaccharide monooxygenases (LPMOs) that catalyze chitin oligosaccharides. Chitin is a fungal component and chitin oligosaccharides are PAMPs acting as important elicitors to trigger PTI. PHEC27213 is able to suppress this chitin-associated PTI to promote *P. xanthii* virulence (Polonio et al., 2021).

Downy mildew

Downy Mildew is one of the main foliar diseases in cucurbit crops affecting over 40 cucurbit species including melon (Palti and Cohen, 1980). The disease is caused by the obligate biotrophic oomycete *Pseudoperonospora cubensis*. In the 19th century, the pathogen was originally classified into the *Peronospora* genus. The change in taxonomy occurred after the observation that the *Pseudoperonospora* species had the ability to produce zoospores unlike the ones belonging to the *Peronospora* genus (Savory et al., 2011).

The disease outbreak is favored by high relative humidity and temperatures of 15-20 ºC when germination of infective spores is optimal. *P. cubensis* has a wide host range and poses serious problems in warmer regions where melon cultivation takes place all year and the hosts are always present for the perpetuation of the inoculum (Savory et al., 2011).

Disease cycle and symptoms

Downy mildew infection initiates when zoospores are released from germinated sporangia (asexual spores) on a susceptible host under wet conditions (Fig. 5). Sporangia can be dispersed by travelling with wind currents over long distances or by rain splash and infected agricultural equipment. Interestingly, Cohen et al. (2014) reported that *P. cubensis* may be transmitted by seeds since the pathogen was detected in ovaries, fruit seed cavities and seed embryos of various cucurbit crops. Encysted zoospores produce the penetration hypha that enters the leaf tissue through stomata and forms intercellular hyphae with haustoria. Infected leaves exhibit chlorotic and angular lesions on the adaxial side that are limited by the veins. As disease progresses, chlorotic lesions become necrotic and eventually necrosis expands in the whole leaf (Savory et al., 2011). The sexual cycle which includes the production of oospores is extremely rare due to the cultivation length of those crops.

P. cubensis genetic variability and effectors

Depending on the ability to cause disease in different sets of cucurbit species, *P. cubensis* has been divided into 10 pathotypes (Table 4). The pathotypes have been firstly officially identified by Thomas (1987), who summarized the pathotypes of *P. cubensis* by testing isolates that were collected from Japan, Israel and the USA on 13 representative Cucurbitaceae host species from seven genera including *Benincasa*, *Citrullus*, *Cucumis*, *Cucurbita*, *Lagenaria*, *Luffa*, and *Mormordica* (Table 4). Based on their differential reactions, five distinct pathotypes of *P. cubensis* were identified, including pathotypes 1 and 2 from Japan, 3 from Israel, as well as 4 and 5 from the USA. It is worth mentioning that all five pathotypes were able to infect cucumber (*C. sativus*) and muskmelon (*C. melo* var. *reticulatus*). Pathotype 1 was not able to

infect other hosts than these two aforementioned hosts, whereas pathotypes 2-5 had expanded their host ranges including *C. melo* var. *conomon* (pathotypes 2-5), *C. melo* var. *acidulus* (pathotypes 3- 5), *Citrullus lanatus* (pathotype 4-5) and *Cucurbita maxima* (pathotype 5) (Thomas, 1987). A similar approach followed by Cohen et al. (2003) in Israel revealed a sixth pathotype which has a broader range of cucurbit hosts in comparison to pathotype 3 (Cohen et al., 2003). In 2012, Cohen (2015) collected isolates and described it as pathotype 7 in China. Pathotype 8 and pathotype 9 were found in Vietnam and Russia, respectively in 2013 (Thomas et al., 2017), and pathotype 10 was observed in India in 2014 (Cohen et al., 2015).

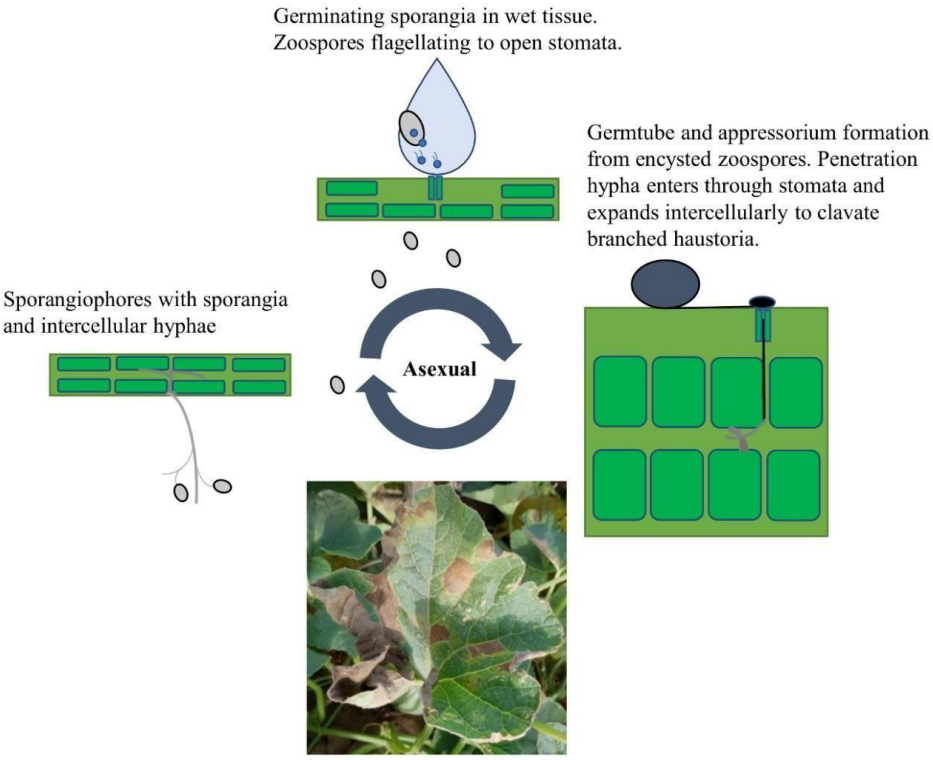

Figure 5. Typical *Pseudoperonospora cubensis* disease cycle Sporangia are germinating under wet conditions on a susceptible host tissue. Zoospores are formed which flagellate into the stomata. After germtube and appressorium formation penetration hyphae expands intercellularly and clavate branch haustoria. Finally, sporangiophores are formed on the elongated hyphae.

Experimental evidence suggests that there are more pathotypes existing in Europe. Lebeda et al. (2004) used a differential set of cucurbits including 12 representatives from six genera, *Benincasa*, *Citrullus*, *Cucumis*, *Cucurbita*, *Lagenaria* and *Luffa* to identify pathogenic variability of 22 *P. cubensis* isolates collected from four European countries (Czech Republic, Spain, France, and the Netherlands). These 22 isolates which differed from pathotypes 1 to 5 described by Thomas et al. (1987) were further categorized into 13 pathotypes. Later during 2003 to 2010, according to the classification system of Lebeda et al. (2004) by including more cucurbit species and genotypes, the amount of recognized pathotypes rapidly extended to nowadays 67. As many of the pathotypes were rather rare, the 10 pathotypes of *P. cubensis* presented in Table 4 are considered the major ones (Cohen et al., 2015).

Oomycetes release effectors in cytoplasm where they interact with effector targets encoded by plant genes to promote disease by suppressing PTI and/or ETI. Cytoplasmic effectors of oomycetes are two types: a, RXLR types which are restricted to *Peronosporales* and b, Crinkler (CRN) types which are present in all oomycetes. RXLR effectors are comprised of a signal peptide in their N terminal followed by a conserved RXLR-(d)EER motif which is responsible for translocation of the protein into the host cell. Most CRN effectors instead have a LXLFLAK motif, which plays the same role as RXLR motif. The domain downstream of the RXLR and LXLFLAK domain are responsible for the interactions of the effectors with host cell proteins (Mestre et al., 2016; Morgan and Kamoun, 2007). Most RXLR effectors exhibit sequence divergence between downy mildew species as a result of different adaptative strategies required for different hosts (Ai et al., 2020). Nevertheless, conserved RXLR effectors have been reported. HaRxL23 and PsAvh73 effectors from *Hyaloperonospora arabidopsidis* and *Phytophthora sojae* respectively are two conserved effectors that could suppress PTI in *Nicotiana benthamiana* and *Arabidopsis thaliana* and ETI in soybean (Deb et al., 2018). Conservation between effectors could mean that they might share similar effector targets in different hosts. One example is that RxLR24 effectors from *P. brassicae* and *P. infestans* were found to interact with the same host factor inhibiting the secretion of antimicrobial compounds in *Arabidopsis thaliana* (Tomczynska et al., 2018).

P. cubensis secretes at least 32 identified RXLR effectors through haustoria into the cytoplasm of host cells to promote its virulence (Tian et al., 2011). Apart from RXLR, prevalent in *P. cubensis* are the QXLR effectors (Tian et al., 2011). Moreover, Savory et al. (2012) have shown that alternative splicing of non-effector genes from the Drug/Metabolite Transporter (DMT) superfamily in *P. cubensis* can make them fully functional effector proteins. These results indicate that the pathogen is in an evolutionary process of improving its molecular machinery in view of the arms race

with the plant. Further research on the host mechanism of the previously identified effectors would be a good step towards understanding *P. cubensis* - host interactions and breeding for durable resistance.

Yield loss and control methods

The downy mildew outbreak in 1984 and its further expansion the years after in Central Europe made the disease for the first time a very serious economic threat for cucumber production in Europe (Olczak-Woltman et al., 2011). Although similar large-scale downy mildew epidemics on European melon have not been recorded so far, the problem is still lurking in the dark since the pathogen destroys plants and downgrades the quality of the produced fruits. Besides breeding for downy mildew-resistant cucurbit cultivars, chemical protection is one of the most common ways of controlling the disease. Fungicides used for downy mildew can be fully systemic (Phenylamides, Carbamates), partially systemic (Cinnamic acids, Complex III respiration inhibitors) and non-systemic (Dinitroanilines, Inorganic copper fungicides) (Lebeda and Cohen, 2011). As expected, downy mildew resistant to many systemic fungicides appeared all over the world soon after the introduction of fungicides. A classic example is the report in 1979 on *P. cubensis* isolates resistant to the systemic fungicide Metalaxyl in Israeli greenhouses (Reuveni, 1980). After that incidence, metalaxyl-resistant downy mildew isolates have been reported in many other countries like Greece, Italy, Australia and Czech Republic (Urban and Lebeda, 2006). Strobilurin fungicide resistant downy mildew isolates are another typical example. Following the introduction and expansion of these isolates in Japan, they became one of the most common fungicide-resistant strains for downy mildew (Ishii et al., 2001). On the other hand, contact fungicides which are contrary to the specialized single site inhibitor fungicides targeting the pathogen at multiple sites, are less likely to generate resistant populations. However, the efficiency of contact fungicides is generally less and they should be used in combination with systemic ones. An integrated management program that includes multiple product applications and preventative measures such as minimization of the exposure of leaves to wet conditions can further reduce the risks and lead to better downy mildew control.

Genetic resources of resistance

In order to identify resistant sources, worldwide efforts have been carried out to screen *C. melo* accessions and their wild relatives using several screening and assessment methods (Table 5 and Table S6) (Epinat and Pitrat, 1989; Pan and More, 1996; Angelov and Krasteva, 2000; More et al., 2002; Lebeda et al., 2007; dos Santos et al., 2009; Shashikumar et al., 2010; Goswami et al., 2011).

Table 4. Ten major pathotypes of *Pseudoperonospora cubensis*

Pathotype	Country (year)	Plant Species	Reference
1	C1, Japan (1987)	*C. sativus*, *C. melo var. reticulatus*	Thomas, 1987
2	C2, Japan (1987)	*C. sativus*, *C. melo var. reticulatus*, *C. melo var. conomon*	Thomas, 1987
3	M1, Japan; M2, Japan (1987) 83, 85, Israel (1987)	*C. sativus*, *C. melo var. reticulatus*, *C. melo var. conomon*, *C. melo var. acidulus*	Thomas, 1987
4	C, the USA (1987)	*C. sativus*, *C. melo var. reticulatus*, *C. melo var. conomon*, *C. melo var. acidulus*, *Citrullus lanatus*	Thomas, 1987
5	T, the USA (1987)	*C. sativus*, *C. melo var. reticulatus*, *C. melo var. conomon*, *C. melo var. acidulus*, *C. lanatus*, *Cucurbita maxima*	Thomas, 1987
6	Unpublished isolates, Israel (1987)	*C. sativus*, *C. melo var. reticulatus*, *C. melo var. conomon*, *C. melo var. acidulus*, *C. maxima*, *Cucurbita moschata*, *Cucurbita pepo subsp*, *pepo*, *Lagenariavulgaris*	Cohen et al., 2003
7	Harbin 10P, China (1987)	*C. sativus*, *C. melo var. reticulatus*, *C. melo var. conomon*, *C. melo var. acidulus*, *C. maxima*, *C. moschata*, *C. pepo subsp*, *pepo*, *L. vulgaris*, *Luffa cylindrica*, *Luffa accutangula*	Cohen et al., 2003
8	Unpublished isolates, Vietnam	*C. sativus*, *C. melo var. reticulatus*, *C. melo var. conomon*, *C. melo var. acidulus*, *L. vulgaris*, *L. cylindrica*, *L. accutangula*, *Benincasa hispida*, *Mormodicabalsamica*	Cohen et al., 2003
9	Unpublished isolates, Russia	*C. sativus*, *C. melo var. reticulatus*, *C. melo var. conomon*, *C. melo var. acidulus*, *C. lanatus*, *C. maxima*, *C. moschata*, *C. pepo subsp. pepo*, *L. vulgaris*, *L.cylindrica*	Cohen et al., 2003
10	Luf-2, Ash-1, India	*C. sativus*, *C. melo var. reticulatus*, *C. melo var. conomon*, *C. melo var. acidulus*, *C. lanatus*, *C. maxima*, *C. moschata*, *C. pepo subsp. pepo*, *L. vulgaris*, *L.cylindrica*, *L. acutangula*, *Luffa aegyptica*, *M. balsamica*, *B. hispida*, *Trichosanthes cucumerina*, *Humulus lupulus*	Cohen et al., 2003

Table 5. Previously identified sources of *Cucumis* species resistant to *Pseudoperonospora cubensis* pathotypes

***Cucumis* groups/species** [a]	***Pseudoperonospora cubensis* pathotypes**[b]		
	3	**4**	**Not known**
C. melo	**1**	**142**	**154**
C. melo var. *acidulus*			**7**
C. melo var. *agrestis*			**1**
C. melo var. *ameri*			**3**
C. melo var. *cantaloupensis*	**1**		**99**
C. melo var. *chandalak*			**5**
C. melo var. *chate*			**2**
C. melo var. *conomom*			**3**
C. melo var. *indicus*			**2**
C. melo var. *inodorus*			**1**
C. melo var. *kachri*			**1**
C. melo var. *momordica*	**3**	**3**	**24**
C. figarei			**1**

Note: [a] *C. melo* denotes taxonomy groups that were not shown in the corresponding reports. Detailed information of resistance source is provided in Table S7. [b] *P. cubensis* pathotypes not mentioned in the original publication were categorized as not known. Resistant *C. melo* accessions to *P. cubensis* pathotypes were summarized from thefollowing literatures: Sambandam et al., 1979; Swamy et al. 1981; Epinat et al., 1989; Thomas and Jourdain, 1992;Dhiman et al., 1993; Dutta et al., 1993; Somkuwar and More, 1993; Epinat and Pitrat, 1994; Pan and More, 1996;Lebeda, 1999; More, 1999; Thomas, 1999; Angelov and Krasteva, 2000; More et al., 2002; Wu et al., 2002; Lebeda et al., 2007; Wu et al., 2008; Shashikumar et al., 2010; Goswami et al., 2011; Malik, 2012; Albuquerque et al., 2015; Manchali et al., 2019; Thakur et al., 2019.

In total, 452 resistant accessions were identified in *C. melo* species as well as the wild relative species *C. figarei* (Table 5 and Table S7). For the majority of the resistant sources, the corresponding pathotypes were not reported. Various resistant accessions were effective against pathotype 3 (5 accessions) and 4 (145 accessions). In addition, we summarized susceptible accessions from 13 studies (Table S8). Among these accessions, three of them were susceptible to pathotype 3, nine to pathotype 4, and 110 accessions susceptible to un-reported races.

Resistance loci and QTLs against *P. cubensis* have been identified in a small number of accessions, including PI 124111, PI 124111F, PI 124112, PI 414723, and 5-4-2-1 (Thomas et al., 1988; Epinat and Pitrat, 1989; Angelov and Krasteva, 2000; Perchepied et al., 2005; Lebeda et al., 2011; Savory et al., 2011) (Table 6). Resistance in accession PI 124111 and its derived F_7 progeny PI 124111F (*C. melo* var. *momordica*) was based on two complementary incomplete dominant genes *Pc-1* and *Pc-2* (Cohen et al.,1987; Thomas, 1988, More et al., 2002). Resistance in PI 124111F to six *P. cubensis* pathotypes

has been attributed to two glyoxylate aminotransferase-encoding genes *At1* (located on chromosome 2) and *At2* (located on chromosome 5) (Fig. 4), which are mainly expressed in leaf and seed (Taler et al., 2004). Their abundant expression was required for complete resistance to *P. cubensis* in PI 124111F (Taler et al., 2004; Savory et al., 2011).

Resistance in PI 124112 was controlled by two complementary incomplete dominant loci *Pc- 4* and *Pc-5* (Kenigsbuch, 1992). In addition, several QTLs for pathotype 3 have been detected in a recombinant inbred line population derived from PI 124112 (Perchepied et al., 2005). Partial resistance to pathotype 3 in PI 414723 was conditioned by *Pc-3* (Kenigsbuch, 1989). In the segregating F_2 and backcross generations derived from the resistant parental line 5-4-2-1 and susceptible parental line K 15-6, a dominant type of inheritance for downy mildew resistance was conferred by *Pc-5* in 5-4-2-1 combined with a modifier *M-Pc-5* in K 15-6 that is expressed only in the presence of *Pc-5* in 5-4-2-1 (Angelov and Krasteva, 2000) (Table 6).

Table 6. Resistance loci/genes and QTLs for downy mildew resistance

Genes/QTLsa	Donor	Pathotype	Inheritance mode	Reference
Pc-1	PI 124111, PI 124111F		Two complementary incompletely dominant	Cohen and Eyal (1987); Thomas (1988)
Pc-2				
Pc-3	PI 414723	3	Partial resistance	Epinat and Pitrat (1989)
Pc-4	PI 124112		Incompletely dominant	Kenigsbuch (1992)
Pc-5			Pc-5 is dominant in presence of M-Pc-5, recessive in the absence of M-Pc-5	Angelov and Krasteva (2000)
M-Pc-5	5-4-2-1			
pcXII.1, pcIV.1, pcVI.1, pcVI.2, pcVIII.1, pcII.1, pcXI.2, pcXI.1, pc16.1, pc24.1, pc32.1	PI 124112	3	Partial resistance	Perchepied et al. (2005)
At1	PI 124111F			Savory et al. (2011); Taler et al. (2004)
At2	PI 124111F			

Note: [a] Resistance genes are in italics.

Introgression breeding for powdery mildew and downy mildew resistance

Genetic resources PI 124111, PI 124111F, PI 124112, and PI 414723 have been considered as the most valuable germplasm and are widely used resistant sources in melon breeding programs for both powdery mildew and downy mildew. PI 124111 originates from India and it was identified at the Horticultural Field Station in the USA,

somewhere in the period from 1938 to 1948 (Thomas, 1988). Various lines and cultivars have been developed using PI 124111 as resistance donor including line MR-1 (derived from 90319, an inbred line of PI 124111) and PI 124111F (Lebeda et al., 2011) (Fig. 6). PI 414723 has a distinct breeding pedigree which was originally derived from PI 371795 (McCreight et al., 1992).

Variety Phoot (*C. melo* var. *momordica*) has been a core source of downy mildew resistance. For example, at least two lines were derived from Phoot including 55-2 and 55-1 (Fig. 6) (Sambandam et al., 1979; Somkuwar and More, 1993). Lines 55-2 and 55-1 derived from Phoot showed higher downy mildew resistance than those of accessions PI 124111F and PI 124112 (More et al., 2002). VRM 31-1-2 (F_{7-9} generation of the cross between Phoot and Monoecious 4) were resistant to both downy mildew and cucumber green mottle mosaic virus (CGMMV) (Fig. 6). Unlike PI 124111F, VRM 31-1-2 had higher yield and acceptable commercial quality (Pan and More, 1996). Other resistance resources have been identified in India, including cultivars Budama 1, Budama 2, Budama 3, EX-1, Phoontee Goomuk, Annamalai, Nakka Dosa Kaya and FM 1. However, information on their pedigree is not available (Sambandam et al., 1979).

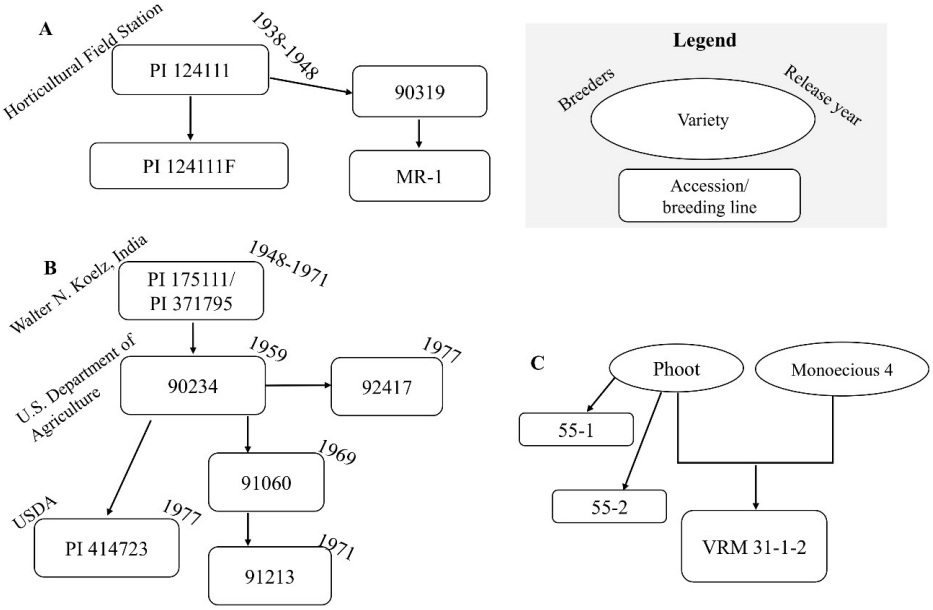

Figure 6. Pedigree trees of resistant lines and cultivars **A**. PI 124111 and its derived breeding lines; **B.**PI 371795 is the parental accession of PI 414723; **C.** Variety Phoot and its derivatives. Top right indicates how to interpret Fig A through C

Novel resistance breeding strategy by loss of function of susceptibility genes

Although resistance can be obtained by introgression of major resistance genes (*R* genes) from wild species into elite varieties, this has rarely been durable due to the evolutionary arms race with the ever-evolving pathogens. Durability could be improved by stacking more *R* genes. Hence, other sources/forms of resistance are highly needed. An alternative approach is to explore recessive resistance conferred by impaired susceptibility genes (*S*-genes) in the host (Pavan et al., 2010). All plant genes required for pathogen survival and proliferation as well as supporting compatibility can be considered *S* genes (Van Schie and Takken, 2014).

Multiple examples of *S* genes have been reported over the last years for downy mildew and powdery mildew in several crops (Table 7). For downy mildew, well-known examples are the *DMR1* and *DMR6* genes, encoding a homoserine kinase protein and a (2OG)-Fe(II) oxygenase, respectively (Van Damme et al., 2008; van Schie and Takken, 2014). Additional to downy mildew, silencing of the two genes led to resistance to a number of different pathogens in both tomato and potato (Huibers et al., 2013; Sun et al., 2016, 2022). For powdery mildew, *S* genes have been identified in various crops and with many different functions (Table 7). The use of impaired *MLO* genes in plant breeding for powdery mildew resistance is considered a very successful example of the *S* gene concept. Each plant species harbors a certain number of *MLO* homologues classified into seven phylogenetic clades. Members of clade IV in monocots and V in dicots are described as susceptibility factors toward pathogens causing the powdery mildew disease (Kusch and Panstruga, 2017). In several plant species, such as *Arabidopsis* (Consonni et al., 2006), wheat (Wang et al., 2014), tomato (Bai et al., 2008), pepper (Zheng et al., 2013), pea (Pavan et al., 2011), apple (Pessina et al., 2016), rice (Elliott et al., 2002), and rose (Kaufmann et al., 2012), recessively inherited powdery mildew resistance was found to be caused by naturally occurring mutations in *MLO* homologs of clade IV or V. In cucumber, three Clade V *MLO* genes namely *CsaMLO1*, *CsaMLO8*, and *CsaMLO11* were proven to play a major role in susceptibility to powdery mildew (Berg et al., 2015, 2017). Following this approach, the search for *MLO*-like proteins in the melon genome available at the Melonomics melon genomic database (https://melonomics.net) yielded 16 putative members of the MLO family (Iovieno et al., 2015). These *MLO*-like genes are scattered among eight chromosomes. Chromosomes 6 (*CmMLO6*, *CmMLO7*, and *CmMLO9*), 8 (*CmMLO4* and *CmMLO16*), 11 (*CmMLO1*, *CmMLO11*, *CmMLO12*, and *CmMLO14*), and 12 (*CmMLO2*, *CmMLO3*, and *CmMLO15*) appear to contain more than one *MLO*-like genes (Fig. 4). While the other four chromosomes including 3 (*CmMLO13*), 7 (*CmMLO8*), 9 (*CmMLO10*), and 10 (*CmMLO2*) contain one *MLO*-like gene. Three putative MLO proteins (*CmMLO3*,

CmMLO5, and *CmMLO12*) from melon were positioned in clade V and these underlying genes are considered as candidate genes for susceptibility to powdery mildew (Fig. 4).

S genes are shown to be conserved among species. This feature provides the potential to identify melon orthologs of the known *S* genes and consequently to perform allele-mining in crops when sequences and/or transcriptomics data are available. During the last decade, the genome of diverse accessions that represent the full spectrum of melon diversity has been re- sequenced, together with multiple wild species (Sanseverino et al., 2015; Zhao et al., 2019; Demirci et al., 2021). Analyzing the re-sequenced genomes enables to identify allelic variation (of known *S* genes) occurring in melon genetic pool and to advance breeding for melon crop improvement.

Conclusion

Here, we have described two important diseases, powdery mildew and downy mildew, in melon production. Host resistance is generally the most favorable control method when considering environmental, economic and social reasons. To achieve durable and broad-spectrum resistance to powdery and downy mildews in melon, tools that facilitate disease evaluation need to be further developed. For example, methods for high-throughput and accurate artificial inoculation as well as disease scoring in order to unambiguously measure the resistance level. In view of resistance-breaking and lack of good dominant resistance sources to these two diseases, we recommended to utilize impaired susceptibility genes as an alternative strategy for melon resistance breeding. We expect that a strategic combination of dominant R genes/QTLs with mutant alleles of S genes will aid to the sustainability of disease resistance in melon.

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Supplementary material

Supplementary material associated with this article can be found, in the online version.

Table 7. Previously reported susceptibility genes for downy mildew and powdery mildew in several crops

Disease	*S*-gene	Function	Crop	Reference
Downy mildew	DMR1	Homoserine kinase: amino-acid pathways and metabolite biosynthesis	Arabidopsis thaliana	Van Schie and Takken, 2014
	DMR6	(2OG)-Fe(II) oxygenase, participates in salicylic acid homeostasis	A. thaliana	Van Damme et al., 2008
	AGD5	GTPase-activating protein at the *trans*-Golgi network	A. thaliana	Schmidt et al., 2014
	MKP1	Mitogen-Activated Protein Kinase Phosphatase 1, regulates the production ofreactive oxygen species during immune responses	A. thaliana	Escudero et al., 2019
	TCP14	Leaf-specific expressed transcription factor	C. sativus	Zheng et al., 2019
	NUP133	Nup133 nucleoporin family protein	A. thaliana	Ried et al., 2019
	AAP	Amino-acid permease gene, transport of amino-acid in the cell	*A. thaliana*, *C. sativus*	Berg et al., 2020
	STAYGREEN	Magnesium dechelatase, key regulator in chlorophyll degradation	C. sativus	Wang et al., 2019
Powdery Mildew	PMR4	Callose synthase, salicylic acid signaling	Solanum lycopersicum A. thaliana	Santillan et al., 2020 Nishimura et al., 2003
	PMR5	Unknown function	A. thaliana	Vogel et al., 2004
	PMR6	Pectate lyase, affects pectin accumulation in cell wall	A. thaliana	Vogel et al., 2002
	EDR4	Negative regulator of defense, by regulating relocation of *EDR1*	A. thaliana	Wu et al., 2015
	MLO	Membrane proteins with transmembrane domains involved in a variety of physiological processes. negative regulation of vesicle-associated and actin- dependent defense pathways	*Hordeum vulgare*, *A. thaliana*, *S. lycopersicum*, *Pisumsativum*, *Fragaria vesca*,*Capsicum annuum*, *Triticum aestivum*, *C. sativus*, *Rosa hybrida*, *Nicotiana tabacum*, *C.melo*, *Vitis vinifera*, Malus domestica	Jørgensen et al., 1992; Consonmiet al., 2006; Bai et al., 2008; Humphry et al., 2011; Cheng et al., 2013; Jiwan et al., 2013; Zheng et al., 2013; Wang et al., 2014; Berg et al., 2015; Qiu et al., 2015; Fujimura et al., 2016; Pessina et al., 2016a, 2016b

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Chapter 3

A new challenge in melon resistance breeding: the ToLCNDV case

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Abstract

Tomato leaf curl New Delhi virus (ToLCNDV) is a whitefly transmitted plant virus that is affecting European melon cultivation for over a decade. Since its first introduction in the Mediterranean basin the virus has been associated with significant economic losses including lower yields and cracked non-marketable fruits in Spain and other key cucurbits production areas. Since there is no chemical application against viral pathogens the focus is geared towards resistance breeding. Various QTLs associated with ToLCNDV resistance have been reported over the recent years in melon and other cucurbits. In the current review we summarize the latest advances in melon breeding for ToLCNDV resistance and present all relevant loci known so far in cucurbits. As a way forward in the future we propose an alternative to traditional resistance gene introgression breeding by exploiting the knowledge on genes that confer susceptibility to the virus in melon and other cucurbits.

Introduction

Melon (*Cucumis melo* L.), also known as muskmelon, is an important dicotyledonous annual fruit crop (Jeffrey 1980; Pitrat 2008, 2016). It belongs to the Cucurbitaceae family, which also includes many economic important crops, such as cucumber (Cucumis sativus), watermelon (*Citrullus lanatus*), zucchini (*Cucurbita pepo*), pumpkin (*Cucurbita moschata*) and bitter melon (*Momordica charantia*). The global melon production was 28.5 million tons in 2020 (www.fao.org/faostat), with China being the leading producer (13.8 million tons), followed by Turkey (1.7 million tons), India (1.3 million tons) and Kazakhstan (1.2 million tons). Melon cultivation is favored by high temperatures ideally between 21 °C and 35 °C under long days and high light intensities, conditions which lead to high sugar accumulation (Pitrat 2008). It is cultivated for the consumption of fruits that are harvested either immature (not sweet) or most often mature (high sugar content and thus sweet) (Pitrat 2008, 2016).

Compared with wild species, cultivated melons have increased their sizes of fruits with thicker flesh, larger leaves and seeds, and loss of bitterness during the domestication (Pitrat 2016; Zhao et al. 2019). Although East Africa was regarded as the place of origin, recent data suggests that melon and cucumber may originate from India and Australia (Sebastian et al. 2010; Garcia- Mas et al. 2012; Chomicki et al. 2020). Cultivated melon grown today can be traced back to two wild lineages and is believed to have been first domesticated in Asia as early as 4 thousand years ago. A second area of domestication is in Africa (Chomicki et al. 2020). The Asian lineage (C. melo subsp. melo) gave rise to all the commercially important cultivars and their market types including the most widely cultivated and consumed ‘Galia,’ ‘Cantaloupe’, and ‘Honeydew’ melons (Chomicki et al. 2020), while the wild African lineage (*C. melo* subsp. *meloides*) gave rise to the ‘Tibish’ and ‘Fadasi’ melons, landraces grown in the Sudanian region (Endl et al. 2018).

Melon’s genome sequence of a doubled-haploid line (DHL92), derived from the cross between PI161375 (kachri type) and Piel de sapo T111 (inodorus type), was obtained, assembled and released in 2012. This assembled genome sequence is 375-Mb in size with 27,427 predicted protein-coding genes (Garcia-Mas et al. 2012). The number of predicted disease resistance genes has been estimated as 396 or 459 depending on different studies (Garcia-Mas et al. 2012; Qin et al. 2021), and 81 or 60 of them encode the nucleotide-binding site and leucine-rich repeat (NLR) type of proteins, while, the rest codes for receptor-like kinases (205 or 359) or receptor like proteins (110 or 40) (Table 1). In cucumber (C. sativus var. sativus) and its wild species (*C. hystrix*), 57 and 77 annotated NLR genes have been identified, respectively (Table 1) (Qin et al. 2021).

In watermelon (*C. lanatus* subsp. *vulgaris*) and its semiwild and wild species (subsp. *Mucosospermus* and subsp. *lanatus*), 44 and 6 (semiwild and wild species) annotated NLR genes have been identified (Table 1) (Guo et al. 2013). The numbers of NLR genes identified in cucurbit crops are relatively low when compared with Arabidopsis (149), tomato (260), grape (302), poplar (398), and rice (600) (Andolfo et al. 2014; Casacuberta et al. 2016; Tuskan et al. 2006; Wang et al. 2008; Meyers et al. 2003).

The published melon reference genome assembly has still 40-Mb of unassigned sequences primarily due to the high percentage of repetitive sequences and 50-Mb of sequencing gaps. Different approaches have been undertaken to improve the melon genome assembly and annotation in recent years (Castanera et al. 2020; Ruggieri et al. 2018). By incorporating the available melon RNA-Seq data collections, a new annotation has been released consisting of 29,980 protein-coding genes roughly 1,500 more than the original annotation.

Apart from melon, the genomes of other important cultivated cucurbits have become available over the last 20 years. The first breakthrough in cucurbits research occurred in 2009 with the sequencing of cucumber genome Chinese long inbred line 9930 (Huang et al. 2009). Due to its high agricultural and research interest newer versions of the cucumber genome came out in 2012 (Yang et al. 2012) namely of Gy14, an inbred line with excellent horticultural traits in North America. A more recent 2019 Chinese Long version followed (Li et al. 2019). The first genome of watermelon occurring from 20 watermelon resequencing data became publicly available in 2013 (Guo et al. 2013). The genome of Charleston Gray watermelon, which is a popular American cultivar resistant to Fusarium and anthracnose disease, was published in 2019. Finally, *C. moschata* and *Cucurbtia maxima* genomes became available in 2017 (Sun et al. 2017), followed by C. pepo genome in 2018 (Montero-Pau et al. 2018). Due to the collinearity of the aforementioned genomes mapping of resistance in one cucurbit crop could be very fruitful for another one.

Melon, cucumber, and watermelon which are major crop species in the Cucurbitaceae family, but distant from Cucurbita species, have different chromosome numbers (melon, 2n=2x=24; cucumber, 2n=2x=14; watermelon, 2n=2x=22). The current assembly of cucumber and watermelon genomes span 243.5 Mb containing 26,682 predicted genes and 353.5 Mb containing 23,400 predicted protein-coding genes, respectively (Guo et al. 2013; Huang et al. 2009). These genome sequences offer many opportunities to understand the genome structure/size and cucurbit genome evolution (Garcia-Mas et al. 2012; Guo et al. 2013). There is high synteny among these genomes (Huang et al. 2009; Li et al. 2011). In addition to several intra- and inter-chromosomal rearrangements, there are likely ancestral fusions and fission of melon chromosome pairs in cucumber

and watermelon (Garcia-Mas et al. 2012; Guo et al. 2013). The increased size of the melon/watermelon genome compared with cucumber is suggested to be partly caused by the accumulation of transposable elements (Garcia-Mas et al. 2012; Guo et al. 2013). Regarding the more distant cucurbits, *C. maxima* and *C. moschata* and *C. pepo* have genome sizes of 271, 270 and 263 Mb respectively. Interestingly, the genomes of *C. pepo*, *C. maxima* and *C. moschata* are the result of a genome duplication that occurred 30–40 million years ago which is not observed in the sequenced genera of Cucumis and Citrullus (Montero-Pau et al., 2018).

Within melon, genetic variability at the whole genome level has been analyzed regarding single nucleotide polymorphisms (SNPs), structural variation (SV) and transposon insertion polymorphisms (Demirci et al. 2020; Sanseverino et al. 2015). This variability was found to be greatly reduced among elite varieties, likely due to selection during breeding. In the elite cultivars, some chromosomal regions particularly associated with agronomic traits such as fruit ripening and stress response showing a high differentiation indicates these are the candidate regions under strong selection (Demirci et al. 2020; Sanseverino et al. 2015).

Because cultivated melon is highly susceptible to various diseases, knowing the progenitors and (closest) wild relatives of melon is important for the introgression of resistance genes. Furthermore, through genome-wide association studies (GWAS), genes or quantitative trait loci (QTLs) that underlie complex traits related to domestication such as fruit mass and quality have been uncovered which provides valuable resources for melon breeding (Zhao et al. 2019).

Table 1. Number of resistance genes in *Arabidopsis* and the three *Cucurbitaceae* species and their (semi) wild species.

Resistance gene [a]	***Arabidopsis*** [b]	**Cucumber** [c]		**Watermelon** [d]	**Melon** [e]
		Cucumis sativus	*Cucumis hystrix*	*Citrullus lanatus*	*Cucumis melo*
NBS-LRR	149	57	77	44	60* - 81
RLP	57	49	55	35	40* - 110
RLK	239	436	347	162	205- 359*

[a] NBS-LRR, nucleotide-binding site-leucine-rich repeat; RLK, receptor-like kinase; RLP, receptor-like protein.

[b] Number of resistance genes in *Arabidopsis* (Wang et al., 2008; Meyers et al., 2003)

[c] Cucumber and wild species, *Cucumis sativus* and *C. hystrix* (Huang et al., 2009; Qin et al., 2021*; Wan et al., 2013);

[d] watermelon, semiwild, and wild species *Citrullus lanatus* subsp. *vulgaris*, subsp. *mucosospermus*, and subsp. *lanatus* (Guo et al., 2013)

[e] Numbers of resistance genes in melon, *Cucumis melo* are taken from Qin et al., 2021.

[f] Numbers are taken from Garcia-Mas et al., 2012.

Tomato leaf curl New Delhi virus

Melon is affected by many important diseases caused not only by fungi and oomycetes but also by viruses. ToLCNDV is a relatively newly emerging virus in the European continent threatening cucurbit crops (Fortes et al., 2016). The virus is prevalent in India where it infects mainly solanaceous species and causes significant economic losses in tomato cultivation (Moriones et al. 2017). The virus, however, has been expanding its genetic diversity, infecting new hosts and occurring in new territories. In 2012, it was first detected in the Mediterranean basin affecting zucchini crops in Spain (Juarez et al. 2014), and since then ToLCNDV is a major problem for open field and greenhouse grown cucurbits. Sequence analysis of the strain that was spreading in the Mediterranean territory showed a new isolate named ToLCNDV-ES which originated from recombination of ToLCNDV strains. ToLCNDV-ES is better adapted to cucurbits than its Indian relative and it poses a danger for European cucurbit production (Fortes et al. 2016). ToLCNDV is a whitefly transmitted bipartite Geminivirus. Its highly flexible genomic organization is thought to be a contributing factor to its wide distribution in hosts (43 dicotyledonous species so far) and areas around the world (Pakistan, India, Bangladesh, Iran, Sri Lanka, Malaysia, Taiwan, Thailand, Indonesia, Tunisia, Spain, Italy, Morocco, Algeria, Portugal, Estonia, Greece) (Zaidi et al. 2017; Bragard et al. 2020). The virus belongs to Geminiviridae (genus: Begomovirus) a family of circular single stranded DNA viruses and has two genome components DNA-A and DNA-B.

DNA-A codes for a replication enhancer protein, a transcriptional activator protein, a coat protein AV1), an AV2 protein involved in viral movement and AC4 protein. DNA-B encodes a movement protein and a nuclear shuttle protein (Zaidi et al. 2017) (Table 2). Finally, the viral components can easily interact with beta satellites often in mixed infections enhancing the viral pathogenesis and virulence (Sivalingam et al. 2012). Disease cycle and symptoms ToLCNDV, like other begomoviruses is transmitted through viruliferous whiteflies in the field/greenhouse which feed on the host plant. The whitefly acquires virus particles from the host plant's phloem upon herbivory through its stylet. After 10–60 min of herbivory the whitefly becomes viruliferous and after ingestion there is a latency period for the virus to be transmissible to other plants. This latency period varies between begomoviruses between 8 and 19 h (Rosen et al. 2015). The virus circulates in the insect moving from the midgut to the epithelial cells and haemolymph to the saliva. From the saliva during herbivory, it can be passed on to a new healthy plant. Besides vectors, ToLCNDV has been reported to be transmitted mechanically (Lopez et al. 2015) in melon and through seeds in zucchini (Kil et al. 2020) and chayote (Sangeetha et al. 2018) plants. The symptoms of ToLCNDV in several hosts include curling of the leaves, vein thickening, darkening of leaf margins, reduction of leaf area, short internodes and severely stunted plants (Zaidi et al. 2017).

Table 2. Genomic components of ToLCNDV and functions of encoded proteins (Lee et al. 2020)

Genome component	ORF/Encoded protein	Function
DNA-A	AC1/Replication-associated protein (Rep)	Initiates replication of the viral genome by binding tothe viral DNA (Fondong et al. 2013)
	AC2/Transcriptional Activator Protein (TrAP)	Virus pathogenicity, gene activation, suppression of gene silencing, activation of the transcription of the genes coding for coat protein and movement protein, alter host responses including jasmonate signaling (Fondong et al. 2013), counter hypersensitive response (Hussain et al.2007)
	AC3/Replication enhancer protein (REn)	Enhances viral DNA accumulation and symptomdevelopment in plants. Interacts with Rep. (Fondong et al. 2013)
	AC4/Viral effector	Interacts with host AGO4 and precludes viral DNAmethylation (Fondong et al. 2013)
	AV1/Coat protein (CP)	Structural protein of ToLCNDV particles, involved inviral movement (Fondong et al. 2013)
	AV2/Pre-coat protein	Pre-coat protein. Involved in viral movement. Main pathogenicity factor involved in suppressing plant antiviral RNAi mechanisms (Basu et al., 2018). Mutations in this protein result in low ToLCNDVaccumulation (Fondong et al. 2013)
DNA-B	BV1/Nuclear shuttle protein (NSP)	Directs viral DNA to the nucleus, involved into cell tocell movement, pathogenicity determinant (Fondong et al., 2013; Hussain et al. 2007)
	BV2/Movement protein (MP)	Required for virus cell-to-cell and long-distance movement, interacts with the NSP in bipartitebegomoviruses. (Fondong et al. 2013)

In melon symptoms have been reported 9 days post inoculation with whiteflies (Ruiz et al. 2017). Melon exhibits severe symptoms including curled leaves with yellow mosaic and most importantly, cracking of the fruits (Fig. 1) (Sáez et al. 2017). Research on the identification of ToLCNDV resistant cucurbit cultivars is limited. Therefore, measures of integrated management that would require a knowledge of the ecology and the variability of the virus are crucial. In 2016, four years after the first detection of the virus in the Mediterranean basin Fortes and co-workers conducted a genetic diversity and phylogenetic analysis study of ToLCNDV that helped to distinguish the Mediterranean strain from the Asian/Indian (Fortes et al. 2016). The study showed a uniform viral population throughout Spain that could be explained by the population bottleneck of moving of the virus to a new area. Furthermore, the similarity of four regions of DNA-A between the Spanish and the Indian isolates were low as well as most of the regions of DNA-B which showed less than 90% similarity. Begomoviruses are very prone to recombination and pseudo-recombination events that can alter their pathogenicity and virulence significantly (Juarez et al. 2019). The mixed infections where two viruses share the same niche can buffer these events. ToLCNDV-ES

is hypothesized to be the result of such a recombination event (Fortes et al. 2016). Juarez et al. conducted in 2019 a similar genetic diversity study and confirmed that the ToLCNDV population in Spain was genetically very homogeneous and clearly different from the Indian ones. Panno et al. in the same year also found a low genetic diversity of ToLCNDV in Italy among the Italian isolates and high similarity of these isolates to the ones from Spain, Morocco and Tunisia. This further suggests that the common origin is indeed ToLCNDV-ES (Panno et al. 2019). These data show that the virus is currently still at an introductory phase in the European continent and why its genetic variability is still low in Europe. Geminiviruses can exploit the host mechanisms in order to promote their proliferation and virulence. Viruses can (i) alter host gene expression, (ii) interfere with hormonal signaling, (iii) change host protein degradation pathways and (iv) interfere with cellular metabolism, and (v) counter antiviral gene silencing (Ramesh et al. 2017) Antiviral gene silencing is an important plant defense mechanism against viruses since plants lack the ability to produce antibodies.

Upon viral infection double stranded RNA (dsRNA) of the virus is cleaved into 21–22 nt small interfering RNAs (siRNAs) or 19–24 nt microRNAs (miRNAs). During post transcriptional gene silencing (PTGS) these siRNAs form a complex with Argonaute proteins and cleave cognate mRNAs in the cytoplasm. During transcriptional gene silencing 24nt siRNAs are interacting with AGO or methyltransferases in order to induce viral DNA methylation in the nucleus (Schwab et al. 2006). ToLCNDV has also been shown to possess mechanisms for evading antiviral defenses. For example, AC2 protein of the virus has been shown to act as silencing suppressor on the host miRNA machinery. This is happening by directly interacting with the plant's Argonaute protein 1 (AGO1) which can cleave viral mRNAs during antiviral defense, inhibiting AGO1 action (Kumar et al. 2016).

Apart from PTGS, ToLCNDV can suppress also TGS by interaction of its AC4 protein with AGO4 that is involved in DNA methylation (Vimutha et al. 2018). Finally, ToLCNDV besides suppressing TGS and PTGS, the main plant antiviral mechanism, should also be competent for mediating in plant physiology in various ways mentioned above similar to other Geminiviruses.

Yield loss and control methods

Open field melon cultivation in central Spain has been reported to reach losses up to 20% due to the virus (Saez et al. 2017). The cracking of the melon fruits is an important factor since it makes them non-marketable, moreover apart of the cracks, fruits don´t reach marketable size and internal quality is very poor as they do not ripe properly. The European Plant Protection Organization has placed the virus in its alert list in order

to prevent the spreading in the rest of Europe [https://www.eppo.int/]. The main way that the virus is spreading in nature is through whiteflies (*Bemisia tabaci*). Therefore, limiting the populations of these insects is crucial for controlling the virus. Isolated nurseries for growing plants such as greenhouses and screenhouses that are insect free and insecticide applications could limit the contact of the plants with potential viruliferous whiteflies. Removing plants that are infected with the virus quickly or to remove plant remainders and other plant hosts around the area that whiteflies can use as shelters could also be helpful to reduce epidemics. An integrated pest management strategy suggested monitoring of insect populations and planning of the cultivation periods outside of fight activity periods of *B. tabaci* (Bragard et al. 2020). Eventually, large efforts are carried out by the plant breeding industry for introducing tolerant or resistant cucurbit varieties that could be widely adopted to limit the economic loss due to ToLCNDV in Europe.

Figure 1. ToLCNDV symptoms in melon: Mosaic and curling of the leaves as well as crackling of the fruits.

Host resistance to ToLCNDV

Genetic resources of resistance

Germplasm screenings have resulted in the identification of resistant cucurbits sources against ToLCNDV. In total, 36 resistant/tolerant accessions were identified in C. melo groups corresponding to *C. melo* (26 accessions, of which taxonomy groups were not shown in the corresponding reports), C. melo var. acidulous (2), *C. melo* var. kachri (2), *C. melo* var. ibericus (1), *C. melo* var. momordica (4), and *C. melo* var. tibish (1) (Table 3) (López et al. 2015; Romay et al. 2019; Rosa et al. 2018; Sáez et al. 2017). Resistant accessions belonging to C. melo var. momordica (i.e., Kharbuja, PI 124,112, and PI414723), *C. melo* var. kachri (i.e., WM9 and WM7), and *C. melo* (i.e., IC-274014,

PI 282,448, AM 87 and PI 179,901) were asymptomatic or exhibited mild symptoms. Virus accumulation was either not detectable or diagnosed at a low amount (López et al. 2015; Romay et al. 2019; Sáez et al. 2017). Accession Kharbuja exhibited no or mild viral symptoms but contained high virus accumulation which makes it a tolerant source to ToLCNDV (López et al. 2015; Sáez et al., 2017). Accessions PI 164,723 and PI 313,970 showed a recovery phenotype after infection and it was confirmed by Polymerase Chain Reaction (PCR) analysis that the virus content gradually decreased (Romay et al., 2019). In addition, we summarized susceptible accessions to avoid redundant screening efforts. The list includes 90 C. melo accessions (76 of them were susceptible to the isolate ND2014-1 V, the other 14 to ES13-35) and C. melo var. cantalupensis (3 to ES13- 35) (Table 3) (Supplementary Table 1) (Romay et al. 2019; Rosa et al. 2018).

Genetics of ToLCNDV resistance

To support breeding programs, decent knowledge on the genetic basis o To support breeding programs, decent knowledge on the genetic basis of the resistance to ToLCNDV in a few promising melon accessions has been established through quantitative trait loci (QTL) mapping approaches (Romay et al. 2019; Sáez et al. 2017). Segregating populations (F2 and BC1) were developed by crossing parental lines WM 7 (resistant) and C. melo cultivar C. melo subsp. melo Piñonet Piel de sapo (PS) (susceptible) (Sáez et al. 2017). Mapping analysis resulted in the identification of one major dominant locus on chromosome 11 (three overlapping QTLs, ToLCNDVSy15_11, ToLCNDVSy30_11, and ToLCNDVVT30_11), and two minor QTLs on chromosomes 2 (ToLCNDVVT30_2) and 12 (ToLCNDVSy15_12, ToLCNDVSy30_12, ToLCNDVVT30_12) (Fig. 2). The SNP marker D16 which is closely linked to the resistance gene on chromosome 11 can be used in marker-assisted breeding for ToLCNDV resistance in melon (Sáez et al. 2017) (Fig. 2). Using the same resistant and susceptible lines, two candidate genes CmARP4 and CmNAC were identified and transcript amount was differentially higher in the inoculated susceptible genotype (Piel de Sapo) when compared to the inoculated resistant line WM 7 (Román et al. 2019). Overexpression of NAC domain protein in tomato (SlNAC1) resulted in substantial increase in TYLCV accumulation, and CmNAC, which play a function similar to SlNAC1, located on chromosome 7 (Selth et al. 2005) (Fig. 2). The co-location of CmARP4 with the minor QTL on chromosome 2 indicates that the accumulation of transcripts of this gene might be involved in ToLCNDV accumulation and degree of symptoms development (Fig. 2). Accessions IC 274,014 possessed the highest resistance level because all the tested individuals were asymptomatic with no detectable virus accumulation (Romay et al. 2019). ToLCNDV resistance is explained by one recessive locus begomovirus resistance-1 (bgm-1) and two dominant genes Begomovirus resistance-2 (Bgm- 2), and ToLCNDV, according to

phenotypic results of F2 population (Romay et al. 2019). Recently, four patents were issued on ToLCNDV resistant fragments (five QTLs) in C. melo accessions NCIMB 42,585 (QTL-5), NCIMB 42,506 (QTL-11), NCIMB 42,705(QTL-1) and NCIMB 42,625 (QTL11 & QTL12), respectively. The resistance QTL-5 on chromosome 5 is flanked by markers KASP06 and KASP01 and it was successfully introgressed into commercial susceptible cultivars (Nunhems B.V. Patent: US20190225983A1) (Fig. 2). The QTL-11 is located on chromosome 11 between markers melon_ sbg_617_42 and melon_sbg_16835_17 and has been implemented in breeding programs as well (Vilmorin SA Patent: US20200178485A1) (Fig. 2). Moreover, QTL-1 is identified on chromosome 11 and flanked between makers SED ID No.1 and SED ID No.9 (Rijk Zwaan Patent: WO2018219861A1) (Fig. 2). The ToLCNDV resistant plants have an introgression fragment on chromosome 11 comprises QTL11 acting in a recessive manner and/or on chromosome 12 contains QTL12 presenting in a partially dominant manner (Nunhems B.V. Patent: US2020040355) (Fig. 2)

Table 3. Summary of previously identified sources of *Cucumis melo* groups species resistant to *tomato leaf curl New Delhi virus*

Isolate	Botanical group	Resistance accessions	References
ND2014-1V	*C. melo*	LM47, LM96, LM78, LM77, LM8, LM66, LM98, LM89, LM93, LM60, LM95, LM62, LM52, LM57, LM55, LM75, LM70, LM73, LM91, LM90	Rosa et al., 2018
Spanish isolate	*C. melo var. momordica*	Kharbuja, PI 124112, PI 414723	López et al., 2015; Sáez et al., 2017
	C. melo var. kachri	WM9, WM7	
	C. melo var. ibericus	Pinonet Piel de sapo	
ES13-35	*C. melo*	AM 87, IC-274014, PI 282448, PI 179901, HSD 2445-005, HSD 2458 B	Romay et al., 2019

Understanding the genetic control of ToLCNDV resistance in relative species could be helpful for resistance gene identification in melon. An extensive Cucurbita spp. collection was screened in 2016 for ToLCNDV in Spain including *C. pepo, C. moschata* and *C. maxima* and wild species using mechanical and whitefly inoculation. From all these species examined only *C. moschata* accessions were found resistant to the virus (Saez et al. 2016). QTL analysis of two resistant *C. moschata* accessions coming from this screening namely PI 604,506 and PI 381,814 yielded the same monogenic recessive factor on chromosome 8, controlling both symptom expression and virus accumulation (Sáez et al. 2020). The chromosome 8 candidate region of *C. moschata* is syntenic to the region in chromosome 11 of melon, previously described as responsible for ToLCNDV

resistance with defined QTLs (Fig. 2) (Vilmorin SA Patent: US20200178485A1; Sáez et al. 2017). Transcriptomic analysis that was performed in the WM- 7 melon which carried the chromosome 11 QTL indicated genes that were differentially expressed after ToLCNDV infection and thus might play a role in ToLCNDV susceptibility (Saez et al. 2021). These top candidate genes are involved in important functions for antiviral defense such as jasmonic acid signaling pathways, photosynthesis, RNA silencing, sugar transport (Saez et al. 2021). Among melon and *C. moschata* resistance against ToLCNDV has also been reported in cucumber. Three cucumber accessions originating from India CGN23089, CGN23423 and CGN23633 were found highly resistant to the virus after mechanical inoculation. After examining the inheritance of the resistance found in CGN23089 by crossings with susceptible accessions the authors concluded in a recessive monogenic resistance on chromosome 2. The resistance includes a significant sharp reduction in viral titers and asymptomatic or mild symptomatic plants (Sáez et al. 2020). Finally, more recent research in Cucurbita spp. revealed a single dominant gene for ToLCNDV resistance in the *C. moschata* accession BSUAL-252 that originates from Japan after screening with the virus (Masegosa et al. 2020).

Susceptibility gene impairment as a new way to control diseases

Breeding for dominant versus recessive resistance

Traditionally, resistance has been introgressed into varieties by using resistance genes (R- genes). The procedure initiates with interspecific hybridization between the cultivated variety and wild relatives progressing with successive backcrossing generations of the progeny with the elite line. This method has been widely adopted in plant breeding and used to confer resistance to various plant pathogens despite the drawbacks that are associated with it such as the limited durability of the resistance in many cases. Multiple backcrossing generations accompanied with the respective genotyping/phenotyping screenings for each generation delay significantly the final product which is often falling behind to the evolutionary arms race with the ever-evolving pathogens. As a consequence, the newly introduced resistance lasts only for a short while on the field before it is overcome by new pathogen races with the need of constantly introducing new resistance genes (Sun et al. 2014). Nevertheless, there are examples of durable R-gene resistance and also against viruses such as the Tm-22 which is used for many decades in tomato against Tobacco Mosaic Virus (Lanfermeijer et al. 2005). A few R-genes have been identified for dominant resistance against TYLCV in tomato such as the Ty-1/Ty-3 loci that code for RNA-dependent RNA polymerases and Ty-2 which codes for a NLR protein as well as Ty-6 and Ty-4 loci (Caro et al. 2015; Shen et al. 2020; Gill et al. 2019). Similarly, several QTLs for ToLCNDV resistance described above could harbor R-genes that should be identified in order to be exploited

by plant breeding. An alternative approach for obtaining resistance is deployment of impaired susceptibility genes (*S*-genes) in the host (Pavan et al. 2010), also known as recessive resistance breeding. *S*-genes are hijacked by the pathogen virulence factors upon several stages of infection in order to promote its establishment and sustenance in the host plant. Van Schie and Takken (2014) distinguished three different temporal stages on which susceptibility genes act contributing to the successful disease establishment: (a) early infection process, (b) regulation of host defenses and (c) later pathogen sustenance.

S-genes that are exploited by viruses have also been identified with one of the most known categories of them being the translation initiation factors for Potyvirus susceptibility. These proteins are binding to the 5' end cap of mRNAs and play an important role in the initiation of their translation. Translation initiation factors interact with the genome linked viral protein of the virus (VPg) which is located to the 5'end of viral RNA and is crucial for viral infection (Wang et al. 2012). For Begomoviruses, such as TYLCV, the recessive locus *ty-5* is one of the best known recessive loci for resistance. The locus was generated in *Solanum peruvianum* by a natural mutation in *PELOTA* (Lapidot et al. 2015). *PELOTA* contributes to Geminivirus susceptibility also in pepper apart from tomato. A loss of function protein that was identified in pepper random mutagenesis population was found to be resistant to other Geminiviruses such as *Pepper Yellow Leaf Curl Indonesia Virus* and *Pepper Leaf Curl Virus* (KEYGENE N.V. Patent: WO2019122374A1) (Koeda et al. 2021). As far as ToLCNDV is concerned there is no research currently specifically identifying S-genes but only speculation regarding candidate genes (Roman et al. 2019; Saez et al. 2017). For example, the aforementioned identified recessive ToLCNDV resistance QTL in chromosome 11 of melon as well as the respective recessive QTL in chromosome 2 of cucumber should both harbor impaired susceptibility genes. More research is needed e.g. fine mapping to identify those genes.

Recessive resistance by disrupting S-genes

Natural or artificial disruption of the genes mentioned above can confer durable and broad spectrum resistance in melon. A characteristic example of the durability of this resistance is the powdery mildew resistance in barley, based on the disrupted *mlo*-gene, which is used for over 70 years (Sun et al. 2014). Artificial disruption of S-genes in plant breeding industry has been mainly focused on Random Mutagenesis and TILLING (Targeting Induced Local Lesion IN Genomes). Random mutagenesis includes the exposure of plants under x-rays, gamma-rays or chemicals like ethyl-methane sulfonate (EMS) in order to induce random mutations in their genome (Tadele et al. 2016). Detection of the random mutations follows with TILLING which is a DNA-screening

technique that identifies point mutations occurring in a specific gene. The method is based on the formation of heteroduplexes between alleles during PCR and subsequent recognition and cleavage of their mismatch point by nucleases (Tadele et al. 2016). TILLING is a quite popular reverse genetics technique as it can be applied to a large array of plant species with genome size or ploidy not being limiting factors (Kurowska et al. 2011). However random mutagenesis can have various drawbacks. Detection of the desired mutants in an EMS population is a laborious and time consuming procedure. Furthermore, since the mutations are happening randomly the method can result into many unwanted off target mutations that can affect other crop traits (fitness, yield etc.) (Liu et al. 2017).

Clustered regularly interspaced short palindromic repeats/CAS (CRISPR/CAS) is one of the latest advances on genome editing technology which allows disruption of S-alleles (Zaidi et al. 2017). The method is based on the procaryotic immunity that can degrade invading DNA whether is originating from attacking bacteriophages or plasmids. CRISPR/Cas utilizes two components to cleave target DNA, single guide RNAs (sgRNAs) and Cas enzyme which is a DNA endonuclease coming from bacteria. SgRNA is a ~ 100nt synthetic RNA which on its 5' has a 20nt sequence that is complementary to the target genomic DNA sequence with a PAM (protospacer adjacent motif). Adjacent to this gRNA target site, should be a PAM site (protospacer adjacent motif) of a few nt to allow the DNA double stranded breaking activity by the Cas enzyme. The sgRNA forms a loop that binds to the Cas enzyme and creates a complex in order to guide the Cas enzyme to the cleavage site. Cas has then the ability to induce double stranded breaks (DSB). After the DSB the cell initiates the repair of the DNA through non- homologous end joining (NHEJ) or homology directed repair (HDR). NHEJ which is the most common repair mechanism can result in small insertions, deletions and single nucleotide polymorphisms (SNPs) that can lead to gene knockouts (Liu et al. 2017). The CRISPR/Cas complex is usually introduced stably into the plant using a plasmid through *Agrobacterium* mediated transformation which can later be crossed out in order obtain non-transgenic plants. Also methods for transient introduction of the CRISP-Cas complex exist. The main advantage of the method compared to TILLING is the prevention of off targets mutations that are occurring through random mutagenesis. In this way we can add important agronomic traits to cultivar while at the same time we secure its existing genetic background.

CRISPR/Cas9 has already been applied in cucumber, melon and watermelon (Chandrasekaran et al. 2016; Hoogvorst et al. 2019; Tian et al. 2017). However, research is still limited due to the fairly recent introduction of CRISPR/Cas9 and to the fact that cucurbits are recalcitrant plants for agrobacterium mediated transformation needed

for CRISPR/CAS9 editing. In cucumber Chandrasekaran et al. in 2016 edited *eIF4E* (eukaryotic translation initiation factor 4E) gene which was a known S-gene (Nicaise et al. 2003; Julio et al. 2015) for potyviruses in other plant species and conferred resistance to *Zucchini yellow mosaic virus*, *Papaya ring spot mosaic virus-W*, and the Ipomovirus *Cucumber vein yellowing virus*. In the same way Pechar et al. in 2021 edited the melon *eIF4E* in order to achieve resistance to potyviruses. Very recently, another group (Wan et al. 2020) using Crispr/Cas9 edited *Clpsk1* previously known to attenuate immunity against *Botrytis cinerea* and *Hyaloperonospora arabidopsidis* in arabidopsis and achieved *Fusarium oxysporum* f.sp. *niveum* resistance in watermelon.

CRISPR/Cas9 wide application in plants for disruption of S-genes and disease resistance begun the previous decade. CRISPR/Cas in melon is still in an initial phase probably due to the difficulty in *Agrobacterium* mediated transformation. Many already proven S-genes for resistance have been published in the literature for various plants species. Knocking out the homologues of those genes in melon and other cucurbits using CRISPR/Cas9 could confer resistance to these pathogens. The role of genes located in recessive resistance QTLs for these diseases could also be elucidated by testing impaired function mutants. Finally, it must be noted that editing susceptibility genes with Crispr/Cas in order to create these mutants can lead to pleiotropic phenotypes and can very often be lethal as these genes most of the times have key roles in plant physiology.

Conclusion

Here, we have presented a compilation of the potential ToLCNDV resistance in cucurbits based on available literature. Host resistance is generally the most favorable control method when considering environmental, economic, and social reasons. The key to the success of melon breeding aiming at durable and broad-spectrum resistance relies on (1) the development of high- throughput and accurate artificial inoculation method per disease; (2) consistent disease scores implemented to indicate the resistance level for each disease; (3) broad-spectrum resistant cultivars to multiple diseases; (4) taking into account resistance durability through resistance genes pyramiding; (5) in view of resistance-breaking and lack of resistance sources, utilization of impaired susceptibility genes offers a novel alternative strategy for melon resistance breeding.

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Supplementary Table 1. Previous published data on susceptible *Cucumis melo* sources against Tomato leaf curl New Delhi virus

Strain	Botanical group[a]	Resistance accessions	References
ND2014-1V	*C. melo*	CVMV, Piñonet, LM9, LM15, LM16, LM18, LM20, LM21, LM25, LM26, LM27, LM29, LM30, LM35, LM36, LM37, LM38, LM39, LM40, LM41, LM42, LM43, LM44, LM45, LM46, LM63, LM67, LM68, LM72, LM80, LM81, LM82, LM85, LM87, LM88, LM92, LM97, LM24, LM61, LM33, LM34, LM22, LM12, LM14, LM23, LM79, LM74, LM11, LM19, LM7, LM28, LM64, LM65, LM31, LM13, LM56, LM69, LM4, LM3, LM1, LM32, LM6, LM84, LM5, LM51, LM10, LM71, LM76, LM86, LM53, LM17, LM59, LM54, LM2, LM58, LM50 Ouzbèque 1, Ouzbèque 2, Isabelle, Anso 77, AR Hale's Best Jumbo, Canton, HSD	Rosa et al., 2018
ES13-35	*C. melo*	93-20-A, PI 161375, PI 164323, PI 179905, PI 234607, PI 255478, PI 482420, PI 236355	Romay et al., 2019

Chapter 4

DNA primase large subunit is an essential plant gene for geminiviruses, putatively priming viral ss-DNA replication

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Abstract

The family of *Geminiviridae* consists of more than 500 circular single-stranded (ss) DNA viral species that can infect numerous dicot and monocot plants. Geminiviruses replicate their genome in the nucleus of a plant cell, taking advantage of the host's DNA replication machinery. For converting their DNA into double-stranded DNA, and subsequent replication, these viruses rely on host DNA polymerases. However, the priming of the very first step of this process, i.e., the conversion of incoming circular ssDNA into a dsDNA molecule, has remained elusive for almost 30 years. In this study, sequencing of melon (*Cucumis melo*) accession K18 carrying the *Tomato leaf curl New Delhi virus* (ToLCNDV) recessive resistance quantitative trait locus (QTL) in chromosome 11, and analyses of DNA sequence data from 100 melon genomes, showed a conservation of a shared mutation in the *DNA Primase Large subunit* (*PriL*) of all accessions that exhibited resistance upon a challenge with ToLCNDV. Silencing of (native) *Nicotiana benthamiana PriL* and subsequent challenging with three different geminiviruses showed a severe reduction in titers of all three viruses, altogether emphasizing an important role of *PriL* in geminiviral replication. A model is presented explaining the role of *PriL* during initiation of geminiviral DNA replication, i.e., as a regulatory subunit of primase that generates an RNA primer at the onset of DNA replication in analogy to *DNA Primase*- mediated initiation of DNA replication in all living organisms.

Introduction

The family of Geminiviridae consists of more than 500 viral species which infect numerous monocot and dicot plants. Geminiviruses are divided into 14 genera based on genome organization, transmission vectors, and host range. The Begomovirus genus is the most diverse one and contains more than 400 whitefly transmitted species (Fondong, 2013; Fiallo-Olivé et al., 2021), many of them being plant pathogenic, with a major economic importance in the agriculture of tropical and subtropical regions (Fiallo-Olivé and Navas-Castillo, 2020).

The genome of geminiviruses varies in size from ~2.5 to 5 kb, and is organized in one (monopartite) or two (bipartite) circular single stranded (ss)-DNA molecules of approximately 2.5 kb, that are encapsidated into twinned icosahedral protein particles (Krupovic et al., 2009). Only begomoviruses include species with two genomic components, denoted DNA-A and DNA-B, which contain six respectively two open reading frames (ORFs). Proteins encoded by DNA-A are involved in genome replication (REn, replication enhancer protein; Rep, replication-associated protein; TrAP, transcriptional activator protein), particle assembly and insect transmission (CP, coat protein), control of gene expression, and modulation of host defense responses (AC2, AC4), whereas proteins encoded by DNA-B are required for intra- intercellular movement in host plants (MP, movement protein; NSP, nuclear shuttle protein) (Briddon et al., 2010). Except for a sequence of approximately 200 nucleotides, called the common region, A and B components hardly share sequence homology. The common region contains various regulatory elements (e.g. iterons, TATA box) involved in replication and transcription, and a stem-loop structure that is essential for replication. The stem-loop structure contains a highly conserved nonanucleotide sequence (5'-TAATATTCA-3') at its top, presenting the origin of viral replication, and is found in all geminiviruses (Bhattacharjee and Hallan, 2022). Upon feeding of viruliferous whiteflies onto healthy plants, virions introduced into the host's cytoplasm uncoat and release their ss-DNA, which traffics to the nucleus for replication (Kumar, 2019).

Geminiviruses replicate their genomes initially with the conversion of ss-DNA into a double stranded (ds) replicative intermediate (RI) that is used as template for rolling circle replication (RCR) which generates progeny single stranded DNA molecules (Bernardi and Timchenko, 2008). Geminiviruses do not encode DNA polymerases for replication of their DNA genome but rely on DNA polymerases encoded by the host. DNA polymerase a is responsible for generation of dsRIs by creating the complementary strand of ss-DNA whereas polymerase d promotes subsequent accumulation of new

geminiviral ss-DNA from the dsRI template (Wu et al., 2021). RCR is initiated by Rep with the introduction of a nick within the nonanucleotide sequence of a dsRI molecule (Ruhel and Chakraborty, 2019). Apart from the essential functional role of Rep in replication initiation, Rep is also responsible for recruiting (Gong et al., 2021; Kushwaha et al., 2017) host factors involved in ubiquitination to promote trimethylation of histones on viral replisomes, leading to enhanced viral genome transcription. Viral genes are clock- and anti-clockwise oriented on the DNA genome and become expressed via bidirectional transcription of dsDNA molecules (Fondong, 2013).

Resistance to the geminivirus ToLCNDV has been investigated in cucurbits (Saez et al., 2021) since the virus is a major threat to these crops (Desbiez et al., 2021; Fortes et al., 2016; Juarez et al., 2014; Panno et al., 2016; Orfanidou et al., 2019; Zaidi et al., 2017). In melon (*Cucumis melo*) ToLCNDV resistance is attributed to two minor QTLs in chromosomes 2 and 12 and one major recessive QTL in chromosome 11: 30,112,563-30,725,907 nt (melonomics v.4) (Sáez et al., 2017; Roman et al., 2019; Romay et al., 2019; Saez et al., 2022) whereas in Cucurbita moschata the same type of resistance was mapped in a major recessive QTL on chromosome 8 (Saez et al., 2016; Sáez et al., 2020). This region of C. moschata is highly syntenic to chromosome 11 of melon, again at the similar (overlapping) locus ch11:30,045,251-31,108,850 nt. Within this syntenic region, two C. moschata genes (CmoCh08G001790, CmoCh08G001780) showed high impact mutations in the resistant parents, presumably leading to a knock-out of the gene, while another two (CmoCh08G001760, CmoCh08G001720) exhibited moderate changes that could alter the biochemical properties of the protein. The homologues of these genes in melon are MELO3C022313 (putative transmembrane protein), MELO3C022314 (putative F17L21.8), MELO3C022315 (MADS-box transcription factor 8like) and MELO3C022319 (DNA primase large subunit), respectively. Due to the synteny of the QTLs between C. moschata and melon any changes in the aforementioned melon homologues could also be associated with resistance to ToLCNDV.

In our study, we evaluated these four candidate genes. Our plant genomic and functional data analyses revealed that plant DNA primase is essential for geminivirus pathogenicity. This enzyme is part of the DNA Primase complex, that synthesizes RNA primers for initiation of a DNA strand that is complementary to a single stranded DNA template. A model is presented to explain the role of melon DNA primase in geminiviral ss-DNA replication.

Materials and methods

Whole genome sequencing of K18 resistant melon line and SNPs detection

Leaf material from the ToLCNDV resistant line K18 carrying the recessive QTL in chromosome 11 and the susceptible line of similar genetic background K15 but lacking the ch11 QTL, was provided by Nunhems B.V in order to extract genomic DNA (gDNA) for whole genome sequencing (WGS). 200 mg of tissue per genotype were ground to fine powder using liquid nitrogen. The protocol of Healey et al. (2014) was followed for isolation of highly concentrated, high quality gDNA. 1.2 ug of non-contaminated, non-degraded gDNA, was sent to Novogene Cambridge Genomics Center, United Kingdom, for library preparation and WGS (PE150, Q30≥80%), using the Illumina Novaseq 6000 platform. Raw reads were aligned using Bwa mem with less stringent settings (-A1 -B1 -E1 -O1 -M) to reference genome Melon_v4.0 (Castanera et al.,2020), which includes the chloroplast and mitochondrial genome sequences. Duplicate reads were tagged using Mark Duplicates from Picardtools. Samtools was used for post-processing of the alignment files and Alfred for computing quality statistics of the alignment files (Danecek et al., 2021). Variants of chr 11 of both samples were joined called using FreeBayes (min-mapping-quality 2) and filtered using vcflib (vcffilter -f "QUAL > 1 & QUAL/AO > 10 & SAF > 0 & SAR > 0 & RPR > 1 & RPL >1"). SNPeff was used to predict the effect of protein-coding SNVs on the structural phenotype of proteins (De Baets et al., 2012).

Virus induced gene silencing in Nicotiana benthamiana and viral inoculations Plant material

115 *N. benthamiana* plants were grown at Unifarm, Wageningen University & Research. The greenhouse growth conditions were 24°C - 18°C day/night temperature with a photoperiod of 16 hours and 60% relative humidity (RH) for optimal germination and growth.

VIGS constructs

A 321bp region of the *N. benthamiana* DNA primase large subunit (NbPriL) coding sequence targeting both gene transcript variants (Niben101Scf00366g01013.1, Niben101Scf01950g03015.1, Sol Genomics Network) was amplified through PCR with primers (ForwardNbPriL:5'-AGGATGCAACTTGGTCTATTTCTC-3', ReverseNbPriL:5'- TTGTCTAACACATCCTCAACTGCT-3'). The region was chosen based on the best scoring of Sol Genomics Network VIGS tool for optimal virus induced gene silencing. The PCR product was purified and cloned into a pENTR™/ D-TOPO™® vector with the cloning pENTR Directional TOPO cloning kit. Using the Gateway™ LR Clonase™ Enzyme Mix the cloned insert was incorporated into the

pTRV2 vector. Agrobacterium tumefaciens strain C58C1 was transformed using heat shock method with the pTRV2:NbPriL vector for downstream VIGS assays. In the same way the TRV : GUS and TRV : PDS constructs were previously generated.

Agroinfiltration with the VIGS construct

Agrobacterium tumefaciens cultures transformed with the various TRV2 constructs (TRV : NbPriL, TRV : NbGUS and TRV : NbPDS) were mixed in 1:1 ratio with TRV1 cultures until a final OD600 of 2 was reached and were eluted into MMA buffer (10 mM MES pH 5.6, 10 mM MgCl2, 200 Mm acetosyringone) (Norkunas et al., 2018). GUS encodes the beta-glucuronidase enzyme and is a known reporter gene that is not found in N. benthamiana genome (Hull and Devic, 1995). Two-week-old N. benthamiana cotyledons were syringe infiltrated with these mixtures. In plants with the TRV: GUS construct, the plant silencing machinery was turned on without any of the native plant genes being silenced. *PriL* silenced plants were compared to GUS silenced plants. Silencing of the N. benthamiana PDS gene was used as a positive control of silencing as successful silencing of this gene leads to a characteristic clearly visible developmental phenotype (photo bleaching). 40 plants were infiltrated with TRV : NbPriL, 40 plants with TRV : NbGUS and 5 with TRV : NbPDSA. tumefaciens cultures whereas the remaining 30 plants were kept wild type (WT).

Viral inoculations

As soon as the PDS silenced plants exhibited the photobleaching phenotype (2 weeks post infiltration) showing successful silencing, agro-inoculation (Peyret and Lomonossoff, 2015) with various geminiviruses took place. Agrobacteria cultures (OD600 = 2) transformed with viral vectors of BCTV (Stanley et al., 1986), TYLCV (Morilla et al., 2005) or ToLCNDV (Lee et al., 2020) were eluted in MMA buffer and syringe infiltrated into 1-2 fully grown leaves of N. benthamiana. Eight plants were used per virus per VIGS construct, and six plants per virus for the WT controls. Eight TRV : GUS, eight TRV : *PriL* and 6 WT plants remained non-inoculated as controls. Viruses were maintained in different experimental blocks to prevent cross contamination, and the plants within each block were completely randomized.

DNA and RNA extraction and quantification of viral titers and *PriL* transcripts

Young leaf tissue (non-infiltrated, non-inoculated) from all plants was harvested after the appearance of the first systemic viral infection symptoms. TYLCV inoculated plants were harvested 7 dpi as they started to exhibit symptoms earlier, whereas ToLCNDV and BCTV plants were harvested 10 dpi. The tissue was ground into fine powder using metal beads and Qiagen Tissue Lyser II Cat. No./ID: 85300.

DNA extraction

Total DNA was extracted from the collected samples in order to determine viral DNA titers. For DNA extraction 200 mg of ground tissue per sample was mixed with 400 ml working buffer solution. The working buffer solution was prepared for 40 samples and contained: 12.5 ml extraction buffer (0.35 M Sorbitol, 0.1M Tris-HCl pH 8.0, 5 mM EDTA pH 8.0), b) 12.5 ml lysis buffer (0.2 M Tris-HCl pH 8.0, 0.5M EDTA pH 8, 2 M NaCl, 2% CTAB), c) 5ml sarcosyl 5% (w/v), d) 0.15g NaHSO3, e) 0.6g PVP-40. RNase A 20mg/ml was also added into the buffer mix working solution, and mixed. Samples were incubated at 65°C for 1h. After incubation, two equal volumes of chloroform: isoamylalcohol (24:1) were added for extraction. After vortexing for 30s and subsequent centrifugation for 5 min at 13.000 rpm, the aqueous phase was collected and transferred to a clean microcentrifuge tube. An equal volume of cold isopropanol was added, and the tube inverted several times for DNA precipitation. DNA was pelleted by centrifugation for 5 min at 13.000 rpm. After isopropanol was discarded, DNA pellets were washed with 70% ethanol, and subsequently dried at room temperature for 15 min. DNA was resuspended in sterilized water and stored at
-20°C for further analysis, RNA extraction RNA was extracted from plants in order to determine transcription levels of NbPriL after different treatments. RNA extraction was carried out using TRIzol according to the manufacturers' protocol (Invitrogen). In brief, 1 ml of Trizol was added to each ground sample and the samples were vortexed for 30s. Next, 200 mL of chloroform was added, and samples again vortexed for 15s, followed by a centrifugation at 13.000 rpm for 30 min at 4°C. From the aqueous phase 400 mL was transferred to a clean microcentrifuge tube and 400 mL of isopropanol was added and mixed. After incubation for 10 min at room temperature, RNA was pelleted during centrifugation at 13000 rpm for 20 min at 4°C, and the pellet washed with 75% ethanol, air dried and subsequently resuspended in 50-70 mL of sterilized water. Thermofisher DNase I was used to remove (residual) DNA from the purified RNA samples. First strand cDNA synthesis was performed using BioRad iScript cDNA synthesis kit according to the manufacturers' protocol.

Quantification of gene transcripts and viral titers

Quantitative real time PCR (qRT-PCR) was performed to determine gene expression levels and viral titers. Quantitative RTPCRs were performed in technical triplicates, using a Bio-Rad CFX96 C1000 Thermal cycler with BioRad iQ SYBR Green supermix. Two sets of primers for the NbPriL gene were used to quantify the total expression of the gene due to the two transcript variants Niben101Scf00366g01013.1 and Niben101Scf01950g03015.1. Primers for viral titer quantification were designed based on the genomic sequence of each virus (Supplementary Table 1) using total DNA extracted from the plants as input. NbPriL total expression was calculated taking into

account the CQ values of both transcript variants. NbPriL expression levels as well as viral titers were normalized to the housekeeping gene NbAPR (Solyc02g093150.2) (Liu et al., 2012). The 2^{-DDCt} method (Livak and Schmittgen, 2001) was applied to assess relative transcript levels and viral titers. 2^{-DDCt} was calculated in Microsoft excel and t-test was used to discern the significant differences between control and test samples CQ values.

Disease assays on melon accessions

Having identified four mutations in *PriL*, leading to an amino acid substitution in the resistant melon parent K18, their occurrence was analyzed in the *PRiL* gene of 100 melon accessions, as sequenced by Demirci et al. (2021). Eighteen melon accessions of the Center of Genetic Resources, the Netherlands (CGN), carrying at least one of the SNPs present in K18 were grown in a multispan greenhouse with passive ventilation through zenithal windows and cooling system for temperature control at SAKATA, Almeria, Spain Twelve replicates per accession were used for the assay. The same number of susceptible control "Coliseo" melon plants as well as the resistant control "GRAND RIADO" melon plants were added. Temperature range during the assay was 20-28 °C and relative humidity 60-90%. Each plant genotype was maintained in a different experimental block. Whiteflies collected from zucchini plants infected with ToLCNDV were released onto 1 week old plants (5-10 adults/ plant). 20 days post inoculation access of viruliferous whiteflies, plants were scored on disease severity. Taking into account the systemic symptoms of ToLCNDV in melon as well as the overall fitness of the plant, a scale of 1-10 of disease score was used (1: fully asymptomatic – 10: fully symptomatic). 3-D modelling of PriL-PriS protein models.

The coordinates of the melon PriS and PriL three-dimensional models were obtained from the AlphaFold database (accession codes A0A5A7T8Y3 and A0A1S3CBL6, respectively) (Jumper et al., 2021). Then melon PriS, PriLNTD and PriLCTD, domains were individually fitted into the model of human primase initiation complex (Baranovskiy et al., 2016b) using the "align to molecule" function in PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.). The linker between PriLNTD and PriLCTD was rebuild and refined using Coot (Coot3Emsley et al., 2010). The sites for the binding of metal ions and substrates are well conserved between the melon and human primases, providing their straightforward transfer from the human to the melon model.

Results

Sequencing of a ToLCNDV resistant melon source shows four changes in DNA primase large subunit coding sequence

Plant material of melon accession K18 carrying the ToLCNDV recessive resistance QTL was sequenced in order to examine potential changes in the four top candidate gene coding sequences compared to the susceptible K15 background. No high impact changes that could result into early stop codons and gene knock outs were found in any of the four candidate genes examined in K18. A more detailed look revealed no changes whatsoever in the coding sequence of the first candidate i.e. MELO3C022313 compared to the susceptible reference genome. The coding sequence of the second candidate, MELO3C022314, showed two SNPs, one synonymous T-C 30,276,085 nt and one moderate impact SNP G-C 30,275,808 resulting into a conserved L-V aa change in position 100 of the protein. A high impact, frameshift mutation (TA insertion, ch11: 32734434, v.3.6) that could result to a knock-out protein was previously found in MELO3C022314 of CGN24928 accession. However, subsequent disease assays showed that plants of this accession carrying the frameshift mutation in MELO3C022314 were susceptible to ToLCNDV, indicating that this gene is unlikely the susceptibility gene. As a potentially loss of function MELO3C022314 protein could not confer resistance to the virus, this gene was excluded as candidate susceptibility gene. The coding sequence of the third candidate MELO3C022315 revealed only one synonymous nucleotide substitution (A-G, ch11: 30,297,365 nt) which does not impact the encoded protein sequence. However, and interestingly, five changes were found in the coding sequence of the last candidate gene, MELO3C022319. One of the nucleotide substitutions was synonymous, having no effect on the produced protein, whereas the other four resulted into amino acid changes in the DNA primase large subunit (PriL) protein (Table 1). Taken altogether, changes in the DNA primase large subunit most likely caused for the resistance mapped in chromosome 11.

Silencing *PriL* resulted in reduced viral titers of ToLCNDV and two other geminiviruses in *Nicotiana benthamiana*

We knocked down the *PriL* homologue (NbPriL) by virus induced gene silencing vector in Nicotiana benthamiana, and subsequently challenged the plants with ToLCNDV. In order to investigate the involvement of *PriL* in the susceptibility to geminiviruses at family and genus level two other distinct geminiviruses were included: Tomato yellow leaf curl virus (TYLCV) (Geminiviridae: begomovirus) and *Beet curly top virus* (BCTV) (Geminiviridae: curtovirus).

N. benthamiana plants verified for a reduction in the level of NbPriL transcripts exhibited a characteristic phenotype that appeared 8-9 days post infiltration with the TRV : NbPriL = construct. This phenotype was consistently observed in all replicates and included shorter internodes and stunting that persisted throughout the experiment. Despite the stunted phenotype of plants infiltrated with TRV : NbPriL, when compared with plants infiltrated with a TRV : NbGUS negative control construct, the former plants were able to survive without any further defects (Figure 1A).

Two weeks post infiltration with the VIGS constructs, N. benthamiana plants were agro-inoculated with infectious clones of ToLCNDV, TYLCV and BCTV. In plants that had received the silencing construct TRV-NbGUS disease symptoms started to show up one week post inoculation with TYLCV, consisting of leaf curling and light yellowing of the upper leaves. Symptoms were more prominent in TRV : NbGUS infiltrated plants than in non-silenced wild type N. benthamiana. Three days following the symptoms in TYLCV challenged plants, first symptoms were observed in ToLCNDV (Figure 1B) and BCTV challenged plants, and similarly revealed mild leaf curling and yellowing of the upper leaves, especially in the TRV : GUS plants. However, and in contrast, during this entire period of monitoring no symptoms appeared upon a challenge with any of the geminiviruses in plants silenced on *PriL* by the TRV-NbPriL construct.

Upon the onset of the first viral symptoms in TRV : GUS plants (i.e. 7 dpi for TYLCV and 10 dpi in ToLCNDV and BCTV), expression levels of NbPriL and viral titers were determined. As expected, *PriL* expression levels in plants that had received TRV : *PriL* were significantly reduced compared to the control plants containing TRV : GUS (Figure 1C) and WT plants ($p < 0.05$). When viral titers were determined, significantly lower titers were observed for all viruses in TRV : PriL treated plants compared to the negative control, TRV : GUS treated plants ($p<0.05$) (Figure 1D). A further look showed that in PriL-silenced plants TYLCV viral titers were higher than the other two viruses ($p<0.05$). Altogether, silencing of *PriL* led to significant reduction in viral titers of all three geminiviruses, strengthening its role as a susceptibility factor for geminiviruses.

DNA sequence data of 100 melon genomes show a conservation of PriL alleles with a shared mutation among accessions with ToLCNDV resistance

ToLCNDV disease assays were performed on accessions carrying at least one of the four mutations found in the PriL coding sequence of K18 to investigate whether, and if so, which mutations conferred ToLCNDV resistance, similarly to the K18 accession. ToLCNDV disease assay data were collected from greenhouse screenings in Spain using whitefly inoculations. In 18 out of the 100 accessions one or more mutations were present in PriL that were also observed in K18 and leading to an amino acid

substitution. Those alleles contained all the mutations as present in K18 (ABCD) or only some of them (ACD, AB, C, D, B, BD, BCD) (Table 1; Supplementary Table 2).

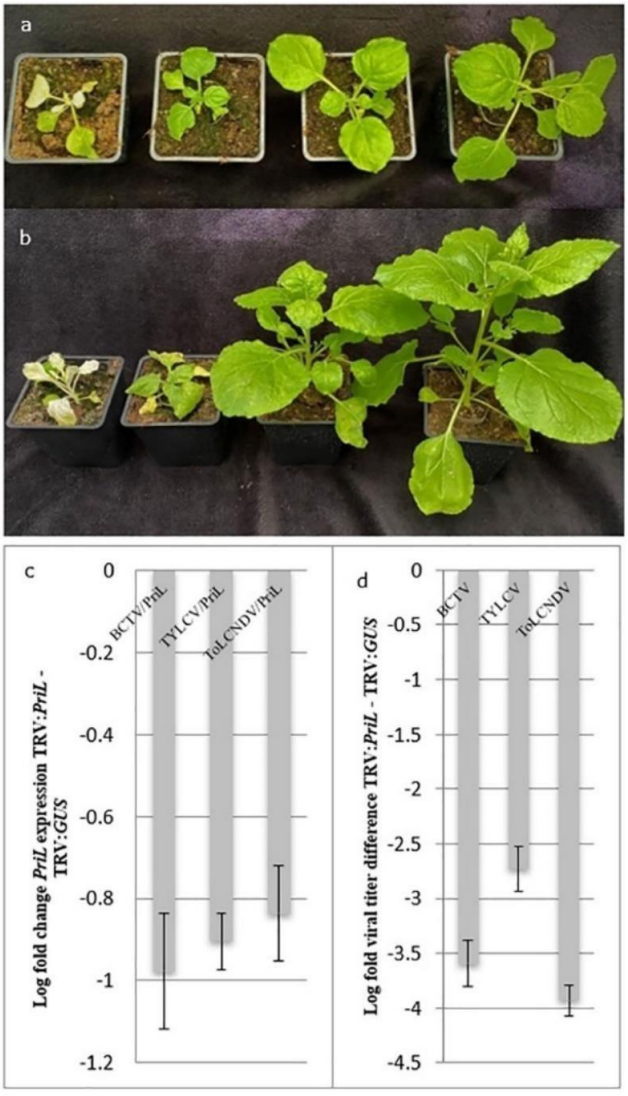

Figure 1. (a) *Nicotiana benthamiana* plant phenotypes 14 days post infiltration with VIGS constructs. From left to right: TRV: Nb*PDS*, TRV: Nb*PriL*, TRV: *GUS*, Wild Type (WT). **(b)** ToLCNDV inoculated *N. benthamiana* plant phenotypes 10 days post inoculation. From left to right: TRV: Nb*PDS*, TRV: Nb*PriL*, TRV: *GUS*, Wild Type (WT). Inoculation with the geminiviruses TYLCV and BCTV led to similar phenotypes compared to ToLCNDV. **(c)** Average log fold *PriL* expression change of TRV : *PriL* plants, inoculated with the different viruses, compared to TRV: *GUS* plants. **(d)** Average log fold difference in viral titers between TRV: *PriL* and TRV: *GUS* plants. Significant difference between TRV : *PriL* and TRV : *GUS* ($p < 0.05$).

From all four mentioned amino acids changes only mutation A consistently associated with ToLCNDV highly resistant phenotypes (symptomless). All evaluated accessions that harbored the Y to H substitution on position 4 (A) of PriL appeared to exhibit resistant phenotypes to ToLCNDV comparable to the resistant control genotype. Two accessions were carrying the same aa change according to sequencing data, however on a heterozygous state, and appeared to be susceptible to ToLCNDV. One accession carried mutation A in a homozygous state but only about half of the plants that were phenotyped exhibited highly resistant phenotypes to the virus. The rest of the CGN140853 plants were exhibiting fully or intermediately resistant phenotype scores (D.I. with 10 = fully symptomatic and 1 = fully symptomatic: 7.0 in 3/18 plants, 5.0 in 3/18 plants and 3.0 in 2/18 plants, 1.0 which means fully asymptomatic in 10/18 plants). Mutation B exhibited a correlation with phenotypes of intermediate resistance, independently of the presence of mutation A (Supplementary Table 2).

Table 1. Overview on the four types of changes observed in the amino acid sequence of DNA Primase Large subunit (PriL) of melon accession K18, carrying the ToLCNDV recessive resistance QTL on chromosome 11. We used melonomics genome version 4.0.

Change	SNP/genomic position	Amino acid change/ position in protein	Polarity change
A	T-C/30,355,908	Y-H/4	Polar to basic polar
B	C-T/30,354,702	P-L/125	Polar to non-polar
C	T-G/30,354,543	S-R/143	Polar to basic polar
D	C-A/30,354,303	A-E/165	Non-polar to acidic polar

From these observations we conclude that the amino acid substitutions from tyrosine to histidine (Y-H) at position 4, and from proline to leucine (P-L) at position 125 are the most likely changes in PriL in the resistant genotype K18, leading to loss of its function for the virus, but maintenance of the protein's function for the host plant.

Discussion

The investigation into the genetic cause of recessive resistance to ToLCNDV on chromosome 11 of melon identified the *DNA primase large subunit* (*PriL*) gene as the most likely candidate. We found non-synonymous mutations in the *PriL* gene in the resistant K18 melon line, which were also present in multiple other resistant melon accessions with diverse genotypes. The resistance of these accessions was determined through disease index scoring. In future studies, the quantification of viral titers could be included to confirm that the observed resistance is reflected in reduced viral titers.

To investigate the functional role of *PriL* in ToLCNDV susceptibility a VIGS approach was implemented in *N. benthamiana* (Senthil-Kumar and Mysore, 2014) due to the inefficiency of the method in melon. Efficient silencing of the native PriL homologue in *N. benthamiana* resulted into characteristic developmental phenotypes but also into significantly reduced viral titers for all evaluated geminiviruses ToLCNDV, TYLCV and BCTV. In future experiments the effect of PriL-silencing will be extended to other viruses (e.g. RNA viruses) that do not rely on PriL as a susceptibility factor for their multiplication, to further substantiate the findings from this study.

Our findings indicated a high correlation of melon's PriL A type mutation (involving a Y-H substitution, Table 1) with ToLCNDV resistant phenotypes. There was one exception to this correlation, which was observed with accession CGN140853. Although the plants in this accession were homozygous for the A-type mutation, they were still somewhat susceptible to ToLCNDV, although their susceptibility was reduced compared to the susceptible control. Since resistance against ToLCNDV in melon is known to be controlled by more than one QTL (Sáez et al., 2017), it could be that the minor QTLs in chromosome 2 and 12, with an additive effect on resistance, are absent in this particular accession resulting into a more susceptible phenotype. Moreover, the resistance in chromosome 11 could be the result of more than the A- type mutation in PriL. Type B mutation (Table 1) could be another factor of resistant phenotypes. The B mutation gave indications of conferring intermediate resistance (independently of the A presence) in CGN140808 and CGN140870, but the correlation of the mutation with the effect was weaker. Altogether, the results indicate that PriL plays a major role in the pathogenicity of ToLCNDV and other geminiviruses in melon. Eventually, at least two amino acid changes can alter PriL in a way that still supports the protein's role for the plant but hamper viral replication.

The plant replication machinery includes several essential genes that are required for DNA replication of the plant. Apart from DNA polymerases, DNA primases are also essential components for DNA replication of the plant. Their role is to synthesize RNA primers that polymerases subsequently elongate on both DNA template strands of the replication fork for the generation of new dsDNA, since polymerases alone are unable for de novo dsDNA synthesis (Frick and Richardson, 2001; Kuchta and Stengel, 2010). Eukaryotic primases are heterodimers consisting of two distinct subunits: a small one, *DNA Primase Small subunit* (*PriS*), which carries the active site for RNA primer (~9 nt) synthesis, and a large one, *PriL*, which determines the activity of the primase and the transfer of the synthesized primer to Polymerase a (Pola) (Baranovskiy et al., 2016b). In organisms with dsDNA, primer synthesis happens only once in the continuous leading strand replication, which becomes extended at its 3' terminus by Pola and Polє for the

formation of the complementary strand. In the lagging strand the orientation is opposite. Consequently, RNA primers need to be newly generated constantly, with following extension by Pola and Polδ leading to Okazaki fragments. With this discontinuous strand replication, sequential Okazaki fragments altogether generate the complementary strand of the lagging strand (Zhou et al., 2019).

Considering the fundamental role of *PriL* in cell replication, the developmental phenotype that was observed after silencing of NbPriL was expected since plant DNA replication was likely inhibited to some extent. Although the development of a normal phenotype was compromised after silencing of NbPriL, the residual expression levels (~50% transcript availability) of NbPriL were still enough to maintain viability and functionality of the plants throughout the experiment. Another indication of the significance of PriL in plant viability was the absence of high impact mutations (e.g. coding sequence deletions, insertions) that could lead to a knock- out of the protein in any of the 100 melon genotypes examined. Normally, plants have only one gene copy of *PriL*. Loss of function of that gene would be lethal, explaining the absence of high impact mutations in PriL in the 100 melon genomes. This implies that only subtle mutations in PriL would be allowed, such as amino acid substitutions.

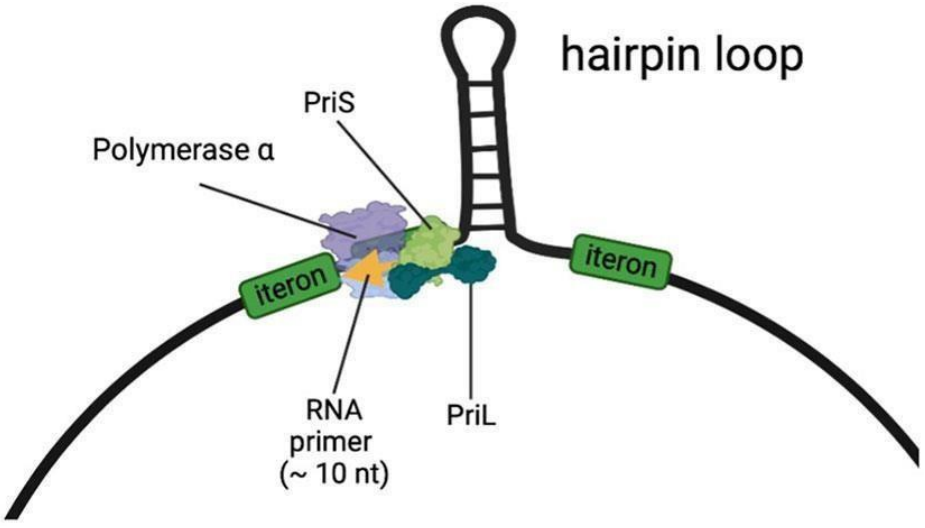

Figure 2. A model for the DNA Primase complex, consisting of PriL, PriS, and DNA polymerase α, producing an RNA primer near the hairpin loop of the ss-DNA of geminiviruses. DNA polymerase α consists of a catalytic subunit (p180) and an accessory subunit (p70). We hypothesize that in the resistant melon one or more mutations in PriL hamper the production or extension of the primer on the viral DNA, but still supporting the primer production in DNA replication forks of the host plant.

Remarkably, despite the thorough studies (Wu et al., 2021) on the role of host polymerases in geminiviral replication there were still no suggestions, comments or experimental data that refer to a possible involvement of host encoded DNA primases in replication of viral DNA. The only study that gets close to this point goes back 30 years ago: Saunders et al. (1992) observed that the conversion of ss- into dsDNA of the geminivirus *African cassava mosaic virus* (ACMV) initiated in a region very close to the hairpin loop with the conserved nonanucleotide sequence. There, a small RNA nucleotide sequence was preceding the complementary DNA strand, at the start of this complementary strand. This observation pointed to the possibility of priming with a RNA oligonucleotide, of which the origin and generation have remained elusive so far.

Based on findings from this study it is now tempting to hypothesize that PriL together with PriS from the host generates an RNA oligonucleotide as the very first step to prime the complementary strand on the single stranded DNA of the virus, thus enabling the conversion of incoming ssDNA into dsDNA (Figure 2). Whereas the 'natural' location of the DNA primase in living organisms is close to the replication fork, the single-stranded DNA geminiviruses do not have such replication fork. However, all geminivirus DNA components contain a hairpin loop in the common region with the conserved nonanucleotide sequence "TAATATTAC" at its top (Figure 2), which resembles a replication fork of DNA replication complexes (Heyraud et al., 1993). The base of this hairpin loop could present the initiation site for DNA Primase to start RNA oligomerization. Docking of the host DNA primase just upstream of the hairpin loop would be in agreement with the findings from Saunders et al. (1992), who mapped an RNA primer at this site. Since all Geminiviruses need the conversion from incoming single-stranded to double-stranded DNA, and all geminiviruses contain the structurally highly conserved region with a hairpin loop, it is likely that priming by DNA primase at the hairpin structure is generic to all geminiviruses, irrespective of the host. This also turns PriL into a susceptibility factor for geminiviruses that do not limit to melon and opens the possibility for its exploitation in other important crops as well.

Mechanistically, it is yet still unclear how the changes detected in melon PriL specifically would inhibit its functionality towards the virus. The PriL protein comprises of two domains, an N-terminal (PriLNTD) and a C-terminal domain (PriLCTD) with a long, flexible linker of 18 residues in between (Baranovskiy et al., 2016a). PriLCTD is attached to the eukaryotic DNA of the lagging strand, supporting PriS with the initiation of the primer synthesis, while the PriLNTD is attached to PriS which is elongating the primer by adding nucleotides (Baranovskiy et al., 2016a). It is possible that ToLCNDV ss-DNA interacts with the PriL close to the C terminus of the protein as this is the part that has DNA binding abilities and is unique in higher eukaryotes (Klinge et al.,

2007; Weiner et al., 2007; Holzer et al., 2017; Holt et al., 2018). Mutations in this part could inhibit protein-viral DNA affinity and subsequently the efficacy of priming for viral DNA replication. However, the Tyr4His mutation in melon that associates with the resistance to the virus is located in the N-terminal of the protein (position 4). Mutations in the N-terminal could affect the interaction of the protein with the PriS or the Pola (Baranovskiy et al., 2015), and in case of the latter abrogate extension of the RNA primer by Pol a. Alternatively, binding of other virulence factors (Fondong, 2013) of ToLCNDV to the N-terminal domain of PriL (PriLNTD) could be prevented and affect the functional activity and integrity of the primase/elongation complex. Considering that at this very first step in viral DNA replication, viral genome transcription has not yet occurred, and viral proteins thus are not synthesized, making the latter explanation less likely.

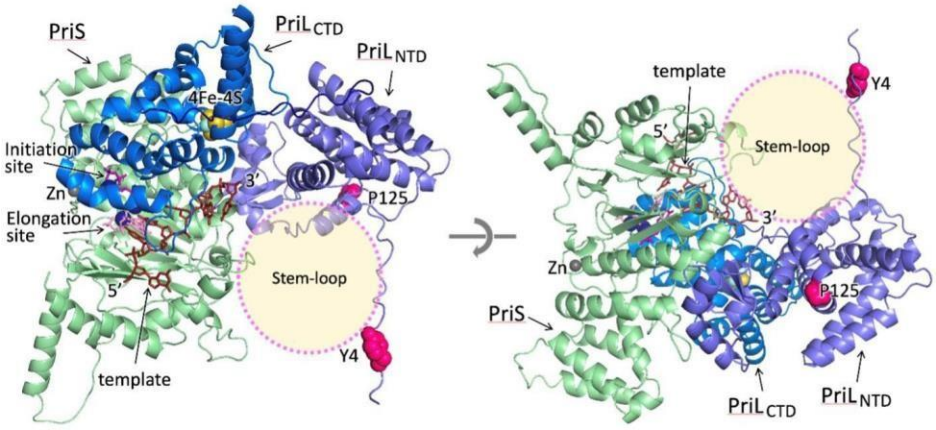

Figure 3. The model of the melon primase initiation complex. For clarity, two different views from opposite directions are shown. The potential stem-loop docking site is indicated by a transparent circle. The two amino acid substitutions in PriL in the resistant melon (Tyr4 and Pro125) are shown as circles in magenta.

To understand the possible implications of PriL mutations for the discrimination between melon and geminivirus DNA replication, the model of the melon's primase initiation complex was constructed. The model shows that the 3'-end of template DNA protrudes toward the open space at the junction of PriS and PriLNTD (Figure 3). The major difference between the DNA templates of melon and geminivirus is a conserved 11 bp long stem-loop structure in a geminivirus circular ssDNA. Indeed, the space at the junction of PriS and PriLNTD is a potential site for the docking of the stem-loop upon initiation of primer synthesis on the geminivirus circular DNA. Intriguingly, the Tyr4 and Pro125 are close to this area and their mutations may affect the primase interaction with the stem-loop, causing disruption of a delicate initiation process. The effect could be direct, by disrupting the local docking surface by Pro125Leu mutation or the primase

interaction with the stem-loop by the Tyr4His mutation. Alternatively, the other viral or melon protein may facilitate the recruitment and holding of geminivirus stem-loop, and the Tyr4His and Pro125Leu mutations disrupt primase interaction with that accessory protein. Further studies are required to discriminate between these mechanisms.

Conclusion

The results presented here provide supporting evidence for a role of PriL as an S-gene for geminiviruses. This evidence is based on sequence data analysis of the resistant melon accession K18 with the recessive QTL for resistance on ch11, and on DNA sequences of 100 other melon accessions and concomitant virus challenging assays to analyze their susceptibility or resistance to ToLCNDV. Additional support was obtained by silencing of PriL in *N. benthamiana* leading to reduced viral titers for three distinct geminivirus species. For almost 30 years the very first step in the conversion of ss-DNA to ds-DNA of geminiviruses has remained unclear. Based on the findings from this study, a role for the host DNA primase in the initiation of geminiviral DNA replication is postulated. How the Tyr4His and Pro125Leu amino acid substitutions within PriL of melon accession K18, carrying the Tomato leaf curl New Delhi virus (ToLCNDV) recessive resistance QTL in chromosome 11, is correlated to the resistance to ToLCNDV remains an experimental challenge for the future.

Data availability statement

The original contributions presented in this study are publicly available. The DNA sequence reads of the QTL region of the sequenced melon accessions can be found at European Nucleotide Archive (ENA) (https://www.ebi.ac.uk/ena/) under accession number PRJEB59465.

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Supplementary material

Supplementary Table 1. Primers used for qRT-PCR.

Primer Set	Target	Forward	Reverse
Nb*APR2* (Liuet al., 2012)	*Solyc02g093150.2*	5'- CATCAGTGTCGTT GCAGGTATT-3'	5'- GCAACTTCTTGGGTT TCCT CAT-3'
Nb*PRIL* 1013	*Niben101Scf0036 6g01013.1*	5'- TCTGGAGGATTTT GAGTTTTATGCCAT- 3'	5'- TGCCTGCGGATGCCT CATA TTTT- 3'
Nb*PRIL* 1015	*Niben101Scf0195 0g03015.1*	5'- CCGCCATTTTCAG TATTCGACGAAATT-3'	5'- TACAGAACGATAGAG AGGCAGAGT-3'
ToLCN DV A (Simonet al., 2017)	ToLCNDV genomic component A	5'- CATTATTGCACGA ATTTCCG-3'	5'- ATCGTAGCCGACTGT GTCT G-3'
TYLCV	TYLCV genomic sequence	5'- TTCGTCTAGATATTCC CTATATGAGGAGGTA-3'	5'- GGCAAGCCCATTCAA ATTAAAGG-3'
BCTV	BCTV genomic sequence	5'- TTGGGAAGAACAAA TGGAG AACTCATTG-3'	5'-ATAGAGTAAAGCATT CTCC TTCACGTCTTC-3'

Supplementary Table 2. *PriL* alleles detected in various CGN accessions and the resulted phenotype after a challenge with ToLCNDV.

***PriL* allele**	**Phenotype (Number of accessions with the allele)**	**CGN accession (D.I score)**
C	Fully susceptible (5/6), Intermediately susceptible (1/6)	CGN14805 (6.6), CGN14806 (4.8), CGN140858 (7.4), CGN140865 (7.8), CGN140866 (7.8), CGN140872 (7.8)
D	Fully susceptible (2/2)	CGN140807 (9), CGN140874 (6.4)
B	Intermediately resistant (1/1)	CGN140870 (1.8)
BD	Intermediately resistant (1/1)	CGN140808 (1.8)
ACD	Highly resistant (4/5), Intermediately resistant (1/5)*	CGN140839 (1.0), CGN24602 (1.0), CGN24621 (1.0), CGN24624 (1.0), CGN140853 (2.9)*
BCD	Intermediately susceptible (1/1)	CGN140868 (4.0)
AB	Highly resistant (1/1)	CGN140857 (1.0)
ABCD	Highly resistant (2/2)	K18, CGN140871 (1.0)

The average ToLCNDV phenotypic disease score is given per accession and refers to a scale of 1 (highly resistant, no symptoms) to 10 (highly susceptible, severe symptoms). The resistant melon control genotype "Coliseo" exhibited a score of 1.5 whereas the susceptible melon control "Grand Riado" exhibited a score of 7.0. DI ≤ 1.5 = Highly resistant, 1.5<DI ≤3.0 = Intermediately resistant, 3.0<DI ≤ 5 = Intermediately susceptible, 5<DI ≤ 10 = Fully susceptible.

Chapter 5

Cucumis melo PELOTA is a susceptibility factor for tomato yellow leaf curl virus

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Abstract

Geminiviruses constitute one of the most important and diverse groups of plant pathogenic viruses affecting a wide variety of crops. *Tomato yellow leaf curl virus* (TYLCV) is a geminivirus that mainly affects solanaceous crops and can be destructive for tomato cultivation. The investigation into resistance against the virus in wild tomato germplasm has yielded significant findings regarding genetic loci. Among these loci, the *ty-5* locus stands out as the sole known recessive locus identified thus far. The resistance occurs by a single nucleotide substitution in *PELOTA*, a conserved gene associated with multiple cellular processes including translation and reproduction. It has been shown that mutant *pelota* whether in tomato or other solanaceous crops is an inhibiting factor for the proliferation and spread for other geminiviruses too, apart from TYLCV. *Tomato leaf curl New Delhi virus* (ToLCNDV) is another related geminivirus that severely affects cucurbit production, especially melon, reducing the yield or downgrading the fruit quality. It is unknown whether melon *PELOTA* functions as an anchor for geminiviral pathogenicity and whether mutations could result into resistance in geminiviral pathogens important for these crops, e.g. ToLCNDV for melon, similar to Solanaceae. In the current research we show that complementation of melon *PELOTA* in a *ty-5* background restores susceptibility for TYLCV both in viral titers and symptoms, revealing the broad role of this gene in geminiviral susceptibility independent of the host plant.

Introduction

Geminiviridae is a viral family consisting of at least 14 genera of plant pathogenic species affecting various monocot and dicot crops. Begomovirus is the most diverse genus of the family and of the entire virosphere including at least 400 distinct species (Fiallo-Olive et al., 2020). Transmission of begomoviruses occurs naturally in the field by viruliferous *Bemisia tabaci* whiteflies which feed on the healthy plants (Czosnek et al., 2017). Similar to other geminiviruses, begomoviruses consist of circular ss-DNA genomes that are encapsidated into twinned icosahedral protein particles, however it is the only genus of the family that contains viruses with bipartite genomes (Fiallo-Olive et al., 2020). One of the most significant species of the genus is *tomato yellow leaf curl virus* (TYLCV) which is a major viral pathogen of cultivated tomato causing stunting, curling of the leaves as well as yellowing which subsequently leads to yield loss. Apart from tomato, the virus has been reported to cause leaf curl disease in other vegetable crops like pepper, bean, and cucurbits (Morilla et al., 2005; Navas-Castillo et al., 1995; Zhu et al., 2020). TYLCV has a monopartite genome of 2.8 kb that contains six open reading frames (ORFs) and an intergenic region (Yan et al., 2021). ORFs V1 and V2 are located on the virion-sense strand coding for the viral capsid protein (CP) and a silencing suppressor protein, respectively. On the complementary strand, the four ORFs C1, C2, C3 and C4, code for replication initiator protein (Rep), transcriptional activator protein (TrAP), replication enhancer protein (REn) and C4 protein respectively (Zhao et al., 2020; Gill et al., 2019).

Resistance against TYLCV has been extensively investigated in tomato germplasm over the years and several genes have been reported for dominant resistance against the virus. The TYLCV resistance loci *Ty-1* and *Ty-3*, which are allelic variants of *RNA-dependent RNA polymerases* (El Sappah et al., 2022) were introgressed from *Solanum chilense* LA1969 and LA2779 accessions respectively (Caro et al., 2015; Verlaan et al., 2013). The *Ty-1* locus has been associated to an increased transcriptional gene silencing antiviral defense response through elevated accumulation of viral small interfering RNAs vsiRNAs (Voorburg et al., 2021). The *Ty-3* locus is speculated to act in an analogous way improving RNAi response considering that is coding for a different allelic variant of the same gene (Voorburg et al., 2021). The *Ty-2* locus which was introgressed from *S. habrochaites* codes for a typical nucleotide- binding leucine-rich repeat (NLR) protein. NLRs usually recognize the pathogen by interacting with its avirulence factors, an event which culminates into a hypersensitivity response (HR) that limits the pathogen into an isolated necrotic area (Maekawa et al., 2012). Although no visible HR is observed in the *Ty-2* resistant plants possibly due to the localization of the virus into the phloem tissue, the Ty-2 and Rep/C1 co-expression can lead to an HR

in *Nicotiana benthamiana* (Shen et al., 2020; Yang et al., 2014). Eventually, *Ty-4* is a minor locus for resistance against TYLCV isolated from *S. chilense* LA1932 accession and *Ty-6* is a major locus for the resistance which originated from LA1938 and LA2779 accessions (Ji et al., 2009).

Apart from the introgression of classical dominant resistance genes an alternative method of conferring resistance against plant viruses is by recessive resistance breeding which is based on disruption of susceptibility (S) genes. S-genes are host genes that are hijacked by the pathogen's molecular machinery before they can successfully establish an infection and proliferate. Viruses being obligate parasites depend on host genes to survive and proliferate. Mutations in S-genes generated naturally or artificially, often lead to the inability of the pathogen to successfully employ or use the coded protein resulting into resistance (Van Schie & Takken 2014).

Considering begomoviruses such as TYLCV, the recessive locus *ty-5* is one of the major recessive loci for resistance. The locus was first described in *S. peruvianum* accession TY172 which carried a natural mutation in the first exon (*T-G*, position 47) of *PELOTA* (Lapidot et al., 2015) resulting to a *V-G* amino acid change in position 16 of the coded protein. PELOTA is a conserved protein among many organisms including archaea (Ragan et al., 1996), fungi (Davis and Engebrecht, 1998), plants (Caryl et al., 2000; Lapidot et al., 2015) and mammals (Shamsadin et al., 2000; Shamsadin et al., 2002). The protein is associated with the RNA quality control mechanisms and recycling of ribosomes. However, its general role in plants remains elusive (Ding et al, 2018). Apart from tomato, *PELOTA* is known to contribute to geminivirus susceptibility also in pepper (Koeda et al. 2021). A loss of function protein that was identified in a pepper random mutagenesis population led to resistance to other geminiviruses such as *pepper yellow leaf curl Indonesia virus* (PYLCIV) and *pepper leaf curl virus* (PepLCV) (Koeda et al. 2021). Over the last years more evidence has emerged regarding the conserved functional role of PELOTA for more geminiviruses apart from TYLCV. It has been shown that *ty-5* lines of tomato exhibit resistance against *tomato yellow leaf curl China virus* (TYLCCNV) / *tomato yellow leaf curl beta satellite* complex as well as *tomato leaf curl Yunnan virus* (TLCYnV) (Ren et al., 2022). The same researchers showed a partial resistance of the line against *beet curly top virus* (BCTV) which belongs to another geminiviral genus (Curtovirus).

Cucurbitaceae are one of the most important families of cultivated plants worldwide and are susceptible to a wide range of diseases including geminiviruses (Weng et al., 2012). Reports of TYLCV in cucurbits are scarce as the virus is not a major pathogen in these crops. However, other geminiviruses like *tomato leaf curl New Delhi virus*

(ToLCNDV) can affect them causing extensive disease epidemics of large economic impact. ToLCNDV is a relatively new virus in Europe that severely affects cucurbit crops (Fortes et al., 2016), especially melon and zucchini. The virus is prevalent in India infecting mainly solanaceous crops (Moriones et al. 2016), however it has been expanding its genetic diversity, infecting new hosts in different areas of the world. In 2012, it was introduced in the Mediterranean basin where it caused large epidemics in zucchini crops in Spain (Juarez et al. 2014) and since then ToLCNDV is considered as one of the major pathogens for open field and greenhouse grown cucurbits causing curled leaves with yellow mosaic, short internodes and stunting and most importantly, cracking of the fruits in melon (Siskos et al., 2022).

Due to the accumulating evidence of a functional conservation of *PELOTA* for various geminiviruses independently of plant species, the role of the melon homologue in Geminivirus resistance was also examined. The aim of the research is to investigate the role of *PELOTA* as a susceptibility factor of geminiviruses in cucurbit crops.

Materials and Methods

Alignment of the PELOTA proteins from different geminiviral hosts and investigation of mutations in 100 CGN melon genomes

PELOTA proteins from different plant species were extracted from NCBI. The multiple alignment of all PELOTA protein sequences was performed using the COBALT multiple alignment tool NCBI. The examination of SNPs in the 100 melon genomes was performed with the same parameters described in the MLO chapter.

Cloning of CmPELO (MELO3C008594) to a plant overexpression vector

Seeds of "Rampicante Zuccherino" commercial melon cultivar were sown in Unifarm climate chamber of Wageningen University & Research, the Netherlands. The growing conditions were 25 °C with a 16/8 h day/night cycle and a relative humidity of 75%. RNA isolation and cDNA synthesis were performed as previously described (Siskos et al., 2023). The coding sequence of CmPELO (MELO3C008594) was amplified from cDNA with primers 5′- caccATGAAGATCGTACGCAAACA-3′ (Forward) and 5′-TTACATTTCAATGTCTTCGAGATCCG-3′ (Reverse) using ThermoScientific Phusion High-Fidelity DNA Polymerase.

CmPELO PCR products were incorporated into to the Gateway-compatible vector pENTR/D- TOPO (ThermoFisher Scientific, U.S.A.). Subsequently, an LR reaction followed to transfer the CmPELO into the binary vector pK7WG2, which harbors the

constitutively active 35S cauliflower mosaic virus promotor for *in planta* expression as well as the *nptII* selectable marker gene for kanamycin resistance.

Complementation of ty-5 tomato with melon *PELOTA* homologue

150 seeds of *S. lycopersicum* carrying both alleles of the ty-5 locus as well as the same amount of *S. lycopersicum* cv. Moneymaker with the wildtype *PELOTA* were used for this experiment. The seeds were disinfected for 20 min in 1% NaOCl with 3 drops of Tween-20, using tea- strainers and sterile buttercups containers. NaOCl was removed rinsing the seeds four times in sterile water. After sterilization, 25 seeds were sown in germination medium (GEM) [(2.2 g MS ½ conc, 10 g sucrose, pH 5.8, 8 g Micro agar)/L]. For synchronization of germination, the seeds were stored first at 4°C for 4 days and subsequently, they were transferred in a growth chamber at 24°C for 7 days.

A week later, explants were cut from cotyledons by discarding the tip of the cotyledon. The cotyledons were placed up-side-down (adaxial side in contact with medium) on petri dishes with SIM + AS medium [(4.3 g/L MS salts, 108.73 mg/L Vitamins Nitsch, 30 g/L sucrose, pH 5.8, 8 g Micro agar/L, Duchefa, http://www.duchefa-biochemie.com/]. After autoclaving the medium, 1.5mg/L zeatine riboside, 0.2mg/L IAA and 100μM acetosyringone were added to it. Approximately 50 explants were placed in each petri dish, on top of two sterile filter paper discs and left for 2 days at 24°C in the light. *Agrobacterium tumefaciens* cells (AGL1) transformed with the pK7WG2:CmPELO overexpression construct were grown overnight in 10 ml LB medium. Bacterial pellets were acquired by centrifugation for 15 min at 4000 rpm, and the pellet was resuspended in inoculation medium (MS salts+vit.4.4 g/L, glucose30 g/L, pH: 5.2) until an optical density of O.D. ±0.3-0.4 was reached. After two days of preculture the explants were placed up-side down in the inoculation medium without the filter-discs and remained in the infection medium for 15-20 min at room temperature with occasional swirling. Subsequently, the explants were blotted on a sterile filter paper to remove excessive bacteria and placed back in the preculture plates where they remained for 2 days at 24°C in the light. Two days later, they were transferred to autoclaved SIM + SEL medium [4.3g MS salts, 108.73 mg Vitamins Nitsch, 30 g sucrose, pH 5.8, 8 g Micro agar/l, 1.5mg zeatine riboside, 0.2 mg/l IAA, 50 mg/l kanamycin, 200 mg/l cefotaxime, 100 mg/l vancomycin]. The petri dishes were incubated in a growth chamber at 24°C in the light and medium was refreshed every 10 days. The first regenerant shoots appeared after a month. The regenerant shoots that were rooting were transferred to higher culture dishes with SIM + SEL, without hormones. Transformed shoots were detected by PCR on the coding sequence of melon *PELOTA*.

Evaluation of TYLCV and BCTV susceptibility restoration in Cm:PELO overexpressing *ty-5* tomato

Individual T1 transformants of both Cm:PELO overexpressing *ty-5* and MM plants were grown at Unifarm greenhouse of Wageningen University & Research, the Netherlands for the generation of T2 families by self-pollination. 25 to 30 seeds per transgenic family (ty- 5:CmPELO, 35MM:CmPELO, 36MM:CmPELO) were sown in soil and plantlets were examined for the segregation of the transgene through PCR. Nearly all plantlets were found transgenic suggesting multiple insertions of the transgene into several sites of the genome. As susceptible control, 15 seeds of cultivar Moneymaker (MM) were sown. As resistant controls, 15 seeds of TYLCV resistant *ty-5* tomato, the same background used for the complementation, were sown.10 days after germination Agrobacteria cultures (OD600:2) transformed with viral vectors of BCTV (Stanley et al., 1986) and TYLCV (Morilla et al., 2005) were eluted in MMAi buffer and syringe infiltrated into cotyledons of tomato. Seven plants were used per virus from ty-5:CmPELO plants, six plants per virus from the MM:CmPELO as well as five plants per virus for *ty-5* and MM controls. Viruses were maintained in different experimental blocks to prevent cross contamination, and the plants within each block were completely randomized. Symptoms of viral diseases were scored 10 and 15 dpi using a 0-10 scale, where 0 represents a complete lack of symptoms and 10 fully diseased plants. For all plants of the T2 families as well as the resistant and susceptible control plants young leaf samples were harvested and immediately frozen in liquid nitrogen 15 days post inoculation for DNA/RNA extraction.

DNA extraction

For total genomic plant and viral DNA an extraction CTAB based protocol was used after the tissue was ground to powder with metallic beads in QiagenTissueLyser II . 500 µl CTAB buffer [100ml 1M TRIS, 140 ml 5M NaCl, 740 milli-q water, 20ml 0.5 M EDTA pH:8.0, 2% CTAB] with 1 µl RNase (2mg/ml) per ml of CTAB was added to each individual ground sample. The samples were mixed for 3 minutes using a shaker and they were incubated for 60 minutes in 65 °C. After incubation, the samples were cooled for 30 minutes in ice water. 250 µl of isoamyl alcohol (24:1) were added to each tube and the suspension was mixed by inverting the tubes for 40 times. Centrifugation of samples at 4000 rpm for 15 minutes followed in order to separate the organic from the aqueous phase and 400 µl of the latter were collected through pipetting and transferred to a new Eppendorf tube. 200 µl of isopropanol was added to the collected aqueous phase and the suspension was mixed briefly. Centrifugation at 4000 rpm for 15 min followed. The supernatant was discarded, and the precipitated pellet was washed with 300 µl of 70% ethanol and centrifugated at 4000 rpm for 30 minutes. Pellets were dried at room temperature and dissolved in 30 µl of milli-Q water.

RNA extraction

RNA was extracted from young leaves 15 dpi in order to determine CmPELO expression in transgenic plants. RNA extraction was conducted using Trizol according to the manufacturers' protocol (Invitrogen). In brief, 1 ml of Trizol was added to each ground sample and the samples were vortexed for 30s. Next, 200 µL of chloroform was added, and samples again vortexed for 15s, followed by a centrifugation at 13.000 rpm for 30 min at 4 °C. From the aqueous phase 400 µL was transferred to a clean microcentrifuge tube, and 400 µL of isopropanol was added and mixed. After incubation for 10 min at room temperature, RNA was pelleted during centrifugation at 13000 rpm for 20 min at 4 °C, and the pellet washed with 75% ethanol, air dried and subsequently resuspended in 50-70 µL of sterilized water. Thermofisher DNase I was used to remove (residual) DNA from the purified RNA samples. First strand cDNA synthesis was performed using BioRad iScript cDNA synthesis kit according to the manufacturers' protocol.

Quantification of CmPELO transcripts and viral titers

Quantitative real time PCR (qRT-PCR) was performed to determine gene expressions levels and viral titers. Quantitative RT-PCRs were performed in technical triplicates, using a Bio-Rad CFX96 C1000 Thermal cycler with BioRad iQ SYBR Green supermix. Primers targeting the coding sequence of CmPELO (Cm*PELO*qF: 5'-TGGCCGTCACCGTTAGAAAA-3', Cm*PELO*qR: 5' – GCAGGATCAGAAGCTTGATGG – 3') were used to quantify the total expression of the gene. Primers for viral titer quantification were designed based on the genomic sequence of each virus (TYLCV F: 5'- TTCGTCTAGATATTCCCTATATGAGGAGGTA-3', TYLCV R: 5'- GGCAAGCCCATTCAAATTAAAGG-3', BCTV F: 5'- TTGGGAAGAACAAATGGAGAACTCATTG-3', BCTV R: 5'-ATAGAGTAAAGCATTCTCCTTCACGTCTTC-3') using total DNA extracted from young leaves as input. Cm*PELO* expression levels as well as viral titers were normalized to the housekeeping gene Sl*TIP41*F: 5'-ATGGAGTTTTTGAGTCTTCTGC-3', Sl*TIP41*R: 5'- GCTGCGTTTCTGGCTTAGG-3'(Lacerda et al., 2015). The $2^{-\Delta\Delta CT}$method (Livak et al., 2001) was applied to assess relative gene transcript levels and viral titers. $2^{-\Delta\Delta CT}$was calculated in Microsoft Excel and t-test was used to discern the significant differences between control and test samples CQ values.

Results

ty-5 PELOTA carries an amino acid change in a motif conserved in multiple plant families

The ty-5 locus detected in wild Solanum species carries a Valine to Glycine (V-G) amino acid change that is responsible for the resistance to TYLCV and other

geminiviruses (Lapidot et al., 2015; Ren et al., 2022). Interestingly, alignment of tomato PELOTA protein with the one from melon and cucumber, apart from the general high homology of the proteins (99% coverage, 82.59% aa percentage identity for melon and 92% coverage, 76.42% aa percentage identity for cucumber), revealed a functional conservation of the ‘’susceptible’’ Valine amino acid in all three species. Moreover, the same amino acid participates in a conserved GSVK motif among all three species. Additionally, alignment of the PELOTA proteins of other Cucurbitaceae and Solanaceae species as well as species of two more plant families (Brassicaceae, Fabaceae) that are in the host range of TYLCV and other geminiviruses showed a conservation of the GSVK motif in all PELOTA proteins aligned (Figure 1). In addition to the previous, 100 CGN melon genome sequences were examined in order to examine the presence of natural mutations that would result in a knockout protein. No high impact mutation that would result in a knockout (frameshift mutation) was found in any of the 100 melon genomes examined. Additionally, no mutations were detected inside the coding sequence of the conserved GSVK motif in any of the 100 melon genomes indicating that the motif in cucurbit germplasm is unchanged.

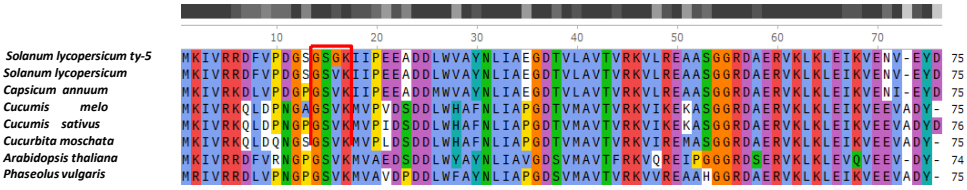

Figure 1. PELOTA protein alignment of plants from four different plant families (**Solanaceae**: *S. lycopersicum*, *C. annuum*, **Cucurbitaceae**: *C. melo*, *C. sativus*, *C. moschata*, **Brassicaceae**: *A. thaliana*, **Fabaceae**: *P. vulgaris*. Highlighted in red the conserved GSVK motif.

Functional analysis of melon *PELOTA* by complementation of ty-5 tomato

CmPELO restores TYLCV symptoms in *ty-5* tomato

In order to examine the functional role of *C. melo PELOTA (*Cm*PELO)* in susceptibility for geminiviruses the melon gene homologue was stably integrated into the genome of *ty-5* tomato. The purpose was to complement the *ty-5* non-functional *pelo* with the melon functional allele and subsequently challenge the plants with geminiviruses to examine any potential restoration of susceptibility to geminiviruses. CmPELO (MELO3C008594) was amplified from cDNA of a commercial geminivirus-susceptible melon accession, “Rampicante Zuccherino”. The PCR product was of the expected size (1.140 bp) and the DNA sequence was identical to gene in the reference genome of the susceptible genotype ‘DHL92’.

T2 families were obtained from two individual transformants. From each T2 family, 24 plants were sown and screened by PCR for the presence of the overexpression construct. 23 plants out of the 24 sown of the T2 individual populations obtained from the CmPELO transformants carried the transgene, suggesting multiple insertion sites per individual primary transformant. Similar segregation rates were observed for the two "Moneymaker" (MM) T2 individual populations that were created as controls. No developmental phenotype whatsoever was observed in the transgenic lines. T2 families were inoculated with the two geminiviruses TYLCV (*Begomovirus*) and BCTV (*Curtovirus*), belonging to two distinct genera, and symptoms were scored at two different time points, 10 and 15 days post inoculation, based on a 0-10 scale, with 0 being completely free of viral symptoms and 10 fully symptomatic.

At 10 dpi the most prominent TYLCV symptoms were observed in the MM overexpression families 35 and 36 as well as in the wildtype (WT) MM plants (Figure 2B). The symptoms included yellowing and a slight curling of the upper leaves in the plants inoculated with TYLCV or BCTV. No significant differences were observed between the MM overexpression lines and the wild type lines for both viruses regarding symptoms at that point. ty-5:CmPELO plants exhibited significantly higher TYLCV disease scores ($p<0.05$, ANOVA) than the ty-5 control plants that remained asymptomatic at 10dpi while they did not differ significantly from the PELOTA overexpressing MM or WT MM families (Figure 1A). This indicates restoration of susceptibility to TYLCV by *PELOTA* from melon in the ty-5 background of tomato, when looking at symptoms.

At 15 dpi, the differences between the disease scores of ty-5:CmPELO in comparison with 36MM:CmPELO and 35MM plants became prominent with the disease progressing significantly faster in the last two genotypes ($p <0.05$, ANOVA) while there was no statistical difference with the WT MM plants ($p=0.052>0.05$, ANOVA). The TYLCV symptoms at 15 dpi included characteristic small top leaves that were becoming yellow between the veins and general stunting. Additionally, the disease was more severe in the ty-5: CmPELO compared with the ty-5 plants which remained almost without symptoms. Ty-5 plants inoculated with TYLCV exhibited minor symptoms e.g. yellowing without curling of the leaves or stunting as in the rest of the genotypes (Figure 2A).

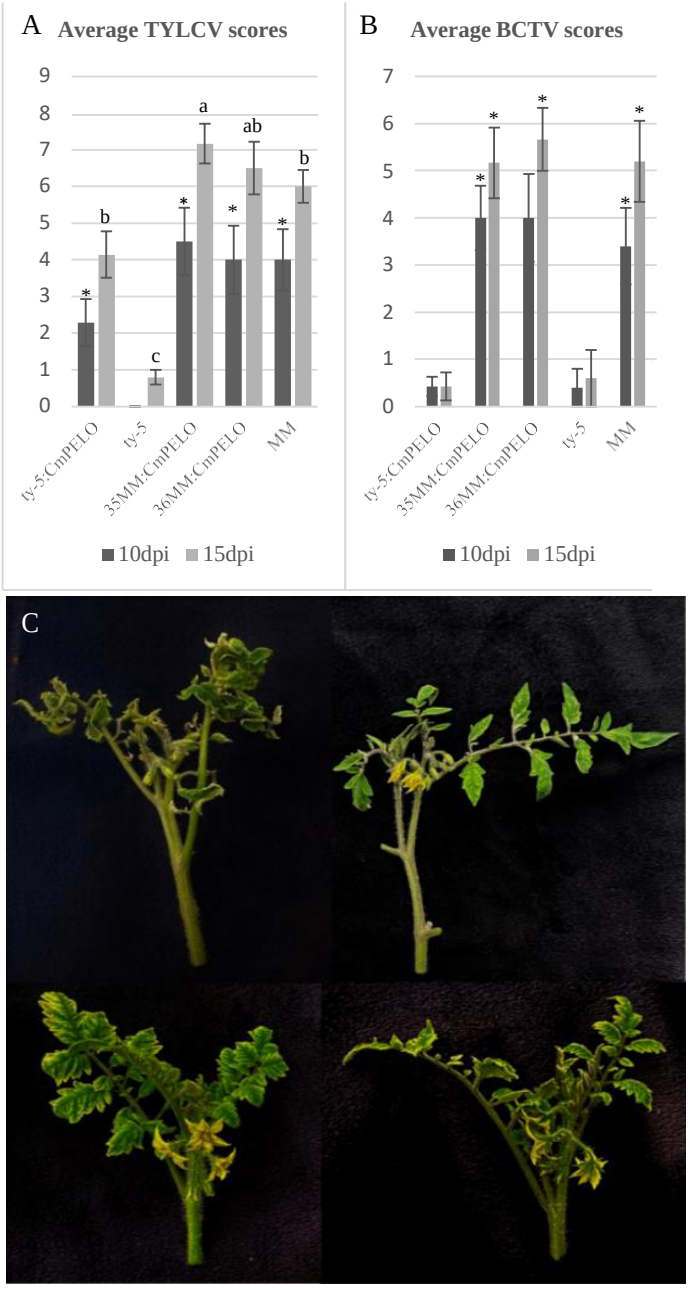

Figure 2. A. Average TYLCV disease scores at 10 and 15 dpi. Asterisk indicate significant differences between all genotypes from the ty-5 plants at 10dpi; a, b, c indicate the different groups formed according to statistical difference, with ab being statistically similar both to groups a and b (ANOVA, $p<0.05$). **B.** Average BCTV disease scores at 10 and 15 dpi. Asterisks indicate significant differences between the ty-5 and the rest of the genotypes (ANOVA, $p<0.05$) **C.** TYLCV disease symptomatology in different genotypes at 30dpi. Upper (left to right): ty-5:CmPELO, ty-5, Lower (left to right): MM:CmPELO, MM.

On the other hand, regarding BCTV, at 10 dpi, ty-5:CmPELO plants did not differ from the ty-5 control plants which remained almost asymptomatic with a slight yellowing of top leaves. The only significant differences at 10 dpi instead were detected between the ty-5/ty- 5:CmPELO and the MM (transgenic and WT) plants ($p<0.05$, ANOVA). (Figure 1B). Remarkably, *PELOTA* from melon did not restore the susceptibility to the geminivirus BCTV in the ty-5 tomatoes.

For BCTV at 15 dpi the disease did not progress in the ty-5: CmPELO genotype and remained similar to the ty-5 plants. The disease progressed normally in the MM plants whether they were expressing the CmPELO or not. The symptoms in these plants included curling of the upper leaves, yellowing and stunting (Figure 2B).

Finally, at 30 dpi, TYLCV inoculated ty- 5:CmPELO plants exhibited symptoms similar to MM plants, leaf curling and yellowing as well as short internodes and dwarfing. The ty-5 genotype exhibited normal growth quite similar to the non-inoculated ty-5 plants exhibiting only minimal yellowing of the top leaves (Figure 2C). ty-5:CmPELO plants inoculated with BCTV remained with the same infection levels as the ty-5 ones with light yellowing and slightly shorter internodes than the non-inoculated ones, whereas the MM exhibited severe dwarfing and yellowing symptoms.

CmPELO contributes to higher TYLCV viral titers in *ty-5* CmPELO over- expressing lines but not to higher BCTV titers

The systemic viral titers of TYLCV and BCTV were quantified through qPCR 15 days post inoculation when there was a clear segregation in symptoms between the different genotypes. Regarding TYLCV inoculated plants, significantly higher viral titers ($p<0.05$) were observed in ty-5:CmPELO genotype compared to the non-complemented ty-5 genotype (Figure 3 A, C). Moreover, the viral titers in the ty-5:CmPELO genotype were similar to the ones detected in MM plants. The difference was significant between the non- complemented ty-5 genotype and MM plants considering viral titers with the latter containing higher amounts of TYLCV transcripts regardless of whether they were complemented with CmPELO or not. No significant difference in viral titers was observed between the different MM:CmPELO lines as well as between these lines and wild type MM lines. Contrary to TYLCV, BCTV titers exhibited no significant difference between the CmPELO complemented and the non- complemented *ty-5* plants which both remained with low viral titers (Figure 3B, C). Eventually, BCTV viral titers were equally higher in MM plants whether they were complemented with melon *PELOTA* or not.

We conclude that *PELOTA* from melon can act as a susceptibility gene in tomato for the begomovirus TYLCV, but not for curtovirus BCTV, although both viruses belong to the geminiviridae.

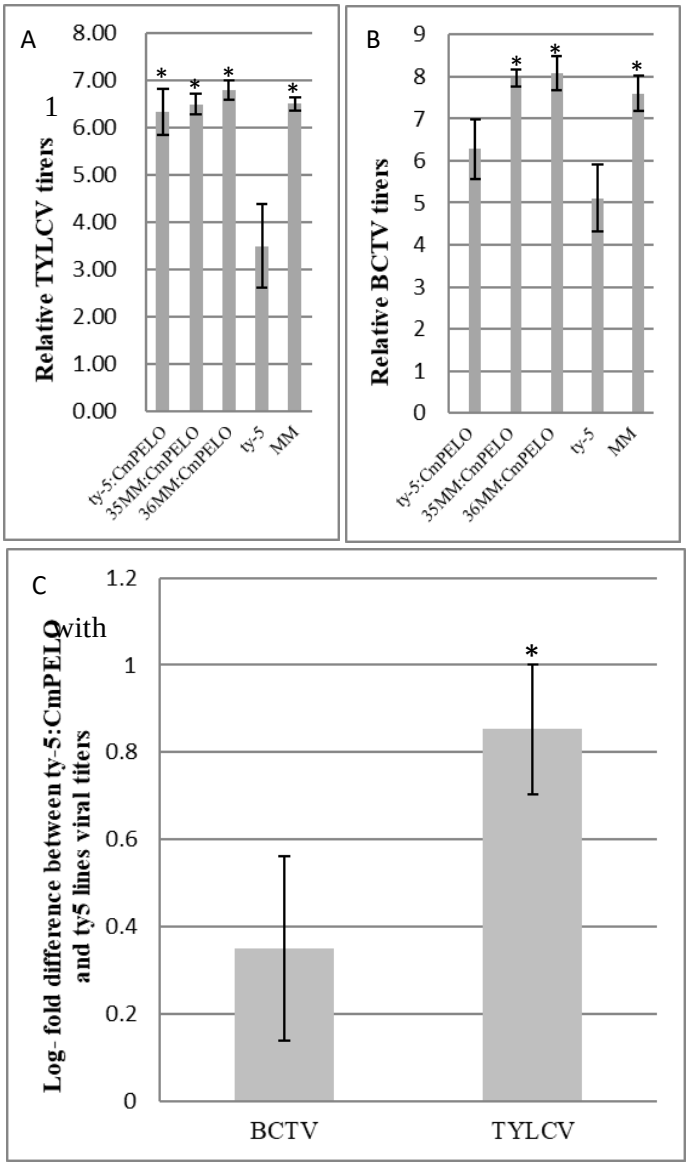

Figure 3. A. Average relative TYLCV viral titers at 10 and 15 dpi. **B.** Average relative BCTV viral titers at 10 and 15 dpi**.** Asterisks indicate significant differences between the ty-5 and the rest of the genotypes (t-test, $p<0.05$) **C.** Log- fold viral titers difference between ty-5:CmPELO and ty-5 lines for BCTV and TYLCV. Asterisks indicate significant differences betweenty-5:CmPELO and ty-5 lines (t-test, $p<0.05$.

Discussion

In the current research we demonstrated the ability of melon PELOTA to confer susceptibility to TYLCV, an important begomovirus affecting mainly solanaceous crops. Research for begomovirus resistance in cucurbits over the last decade, initiating from the detrimental effects of ToLCNDV epidemics in European melon cultivation, has become a priority. One of the most known tomato recessive resistance loci against TYLCV which is a related begomovirus to ToLCNDV is produced by a single nucleotide polymorphism in *PELOTA (*Lapidot et al., 2015*)*. Loss of function in homologous genes of other plant species has been effective for resistance against TYLCV as well as other geminiviruses revealing the putatively universal role of this gene in geminiviral susceptibility in different plant species (Ren et al., 2022).

The protein PELOTA is conserved across all eukaryotes and archaea due to its vital role in the mRNA surveillance mechanisms of the cell (Szádeczky-Kardoss et al., 2018; Arribere & Fire, 2018). These mechanisms are essential prerequisites for the accurate biosynthesis of protein products (Kong et al., 2021). The inevitable production of aberrant mRNAs results either, in mRNAs with premature or lacking stop codons, or in mRNAs with secondary structure formations and other abnormal modifications that are stalled during translational elongation. Monitoring and rescue of stalled ribosomes is occurring through the mediation of PELOTA which initiates the recycling process of mammalian, plant and yeast cells (Shoemaker et al., 2010; Hilal et al., 2016; Kong et al., 2021). Apart from the *PELOTA* mutation effect in plant resistance against geminiviruses, in Drosophila, pelo- loss of function mutants exhibit resistance against various RNA and DNA viruses e.g *drosophila C virus* (DCV) and *invertebrate iridescent virus* (IIV), respectively (Wu et al., 2014). Additionally, it is suggested that the normal viral replication upon the absence of the protein is disrupted due to the lack of available free ribosomes, as a result of the inhibited ribosomal recycling, and the subsequent inefficient translation of viral proteins (Wu et al., 2014). Other studies in various host-virus interaction systems, whether it is *Aedes aegyptii* mosquito - dengue virus or mammalian cells- bovine viral diarrhea virus show that reduction of the PELOTA protein bioavailability leads to reduced viral replication.

The pleiotropic effects of a loss of function PELOTA varies according to organism and the effect on plants remains unknown. In yeast and Drosophila loss of function results in mitotic arrest whereas it is lethal in mice as in mammals as the protein is necessary for genetic stability (Kong et al, 2021). The pepper pepy-1 similar to the *ty-5* locus do not result in loss of function proteins but still encode PELOTA despite the aminoacid changes that are present. From the functional role of the protein in plant physiology it could be

hypothesized that it is necessary for normal plant development. In support of the previous, no frameshift mutations were detected in any of the 100 melon genomes examined. Additionally, the PELOTA GSVK motif seems to be a conserved domain among several plant families and evolutionary it could be playing a significant role for the functionality of the protein. Given the fact that peppy-1 mutation is on different domain than ty-5 it could be speculated that there are more domains other than GSVK that are necessary for a PELOTA to be functional for the virus (Koeda et al., 2021). The high conservation of GSVK in many plant families as well as the absence of mutations in this domain in 100 melon genomes examined could reveal some hints on the important functional role of this domain for plants. However, given the role of this domain for TYLCV susceptibility as well as the fact that ty-5 locus does no result to a loss of function protein, it remains unclear why it is not found changed in more plants. Due to the absence of natural mutations that could result into a knockout allele in any of the 100 CGN melon sequences examined as well as due to the cumbersome procedure of a CRISPR/Cas9 knockout we opted for overexpression of the Cm*PELO* allele in *ty-5* tomato. Subsequent inoculation with TYLCV and BCTV followed. Due to the lack of available ToLCNDV agro-infectious clones isolated from tomato a clone of the virus that was isolated from infected cucurbits (Lee et al., 2022) was used. However it was not able to produce any effect on tomato plants even though it was quite effective in *N. benthamiana* inoculation (Siskos et al., 2023). A repetition of the inoculation with an agroinfectious clone that is able to infect tomato is needed in order to be able to study the effect of CmPELO in ToLCNDV susceptibility.

Regarding TYLCV, there was a clear restoration of susceptibility in the ty-5 tomato that was overexpressing Cm*PELO* both in symptoms and viral titers. Additionally, the level of restoration shows that the virus might be able to utilize both PELOTA proteins with a similar efficiency since the complemented ty-5 plants (melon PELOTA) exhibit similar viral titers as the MM tomato (tomato PELOTA). The alignment of the PELOTA protein sequences from the two plants, melon and tomato, reveals a high degree of similarity, with an identity of 89.2% and a query cover of 99%. Adding to the previous, considering that the virus can be pathogenic in both plants, it could be speculated that there could be an equal evolutionary adaptation of the virus on the host targets of both plants including PELOTA. It is also noteworthy that viral titers were observed in the ty-5 tomato even though they remained significantly lower than the moneymaker plants and the ty-5 plants over expressing Cm*PELO*. This result which has been observed also in other studies (Ren et al., 2022), could indicate that a non-functional *PELOTA* does not completely stop viral proliferation but inhibits it in means that normal plant development is not disturbed. The minimal TYLCV symptoms that were observed in some replicates of the ty-5 tomato remained minor throughout the course of experiment supporting the previous claim.

Considering BCTV, a CmPELO effect was not observed regarding restoration of susceptibility both in symptoms and viral titers, making doubtful whether the gene is necessary for the virus in melon pathogenicity. Literature evidence suggests that BCTV can cause mild disease symptoms in cucurbits however these crops are generally poor hosts for the virus in general, especially as far as melon is concerned (Chen et al., 2009).The weak adaptation of the virus to melon could also mean a poor adaptation and incompatibility with host's machinery including *PELOTA*. Additionally, the *ty-5* locus is able to confer partial resistance to BCTV (Ren et al., 2022) possibly indicating that the virus is not as dependent as e.g. TYLCV to tomato PELOTA protein availability for its pathogenicity. Therefore, considering the previous it would be logical that melon PELOTA is an even less necessary accessory for the virus. Based on the inability of melon PELOTA to restore susceptibility in ty-5 background it would be intriguing to examine whether *PELOTA* of cucurbits could be one of the limiting factors in the poor adaptability of the virus in these plants. In that case, complementation of melon plants with the tomato gene homologue or with a *PELOTA* homologue of a plant species from the main host range of the virus (e.g sugar beets) could lead to increased viral proliferation in melon.

Eventually, our findings suggest that a non-functional melon *PELOTA* could potentially confer melon resistant to TYLCV since it is an S-gene for the virus and potentially to other relative begomoviruses like ToLCNDV. Such a non-functional locus resulting either from genetic engineering (i.e. CRISPR/Cas9), random mutagenesis (EMS) or natural introgression could be an important asset for melon breeders in controlling ToLCNDV.

Conclusion

The role of *Cucumis melo* PELO (Cm*PELO*) in susceptibility to several geminiviruses was examined through complementation of ty-5 background tomato and subsequent agro- inoculation with TYLCV,BCTV and ToLCNDV. Our results demonstrate that Cm*PELO* is able to promote susceptibility at least to TYLCV, as evidenced by the significant increase in disease severity index (DSI) and higher viral titers of the *ty-5* CmPELO transformants compared to the non-transformed *ty-5* controls. On the other hand, Cm*PELO* does not seem to contribute to susceptibility to BCTV. PELO-based resistance may be a common occurrence in plant species and mutations in *PELOTA* homologs may lead to recessively inherited resistance to different geminiviruses in different crops. The previous would be a milestone for combating ToLCNDV disease in melon production. Further research is needed to fully understand the potential of melon

PELOTA as an S-gene for more geminiviruses apart from TYLCV and particularly for ToLCNDV.

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Chapter 6

A natural insertion in melon's *MLO1* gene homologue leads to partial resistance to powdery mildew

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Abstract

Powdery and downy mildew (PM and DM) are two major diseases that severely affect cucurbit crop cultivation. Research for durable resistance is the key for an efficient disease free crop production. Recessive resistance which is based on loss of functional susceptibility (S) genes offers an important lead in the evolutionary arms race between plant and pathogens. Using re- sequencing data from 100 melon genomes, allele-mining on known S genes for both mildews were performed. We identified accessions carrying loss-of-function mutations in a melon homologue of an already known S gene, *TCP14*, for DM in Arabidopsis, as well as of one Clade V *MLO* gene that has been previously associated with PM susceptibility in cucumber, *CmMLO1*. Using a segregation population, we showed that a two-nucleotide insertion in the coding sequence of the *CmMLO1* gene co-segregated with partial resistance to *Podosphaera xanthii*, which is the causal agent of PM in cucurbits. The findings further confirm earlier speculations regarding the general role of this specific gene in PM susceptibility. At the same time, the mutated allele could be an extra asset utilized in melon breeding programs for the improvement of plant fitness and resilience under high PM pressure field conditions.

Introduction

Cucurbit crops are susceptible to a variety of fungal, oomycete and viral diseases that can cause significant yield losses, decreased crop quality, and reduced economic returns for growers. Powdery and downy mildews, which can infect a wide variety of both monocot and dicot plant species, are the two main devastating foliar diseases in cucurbit crops.

Cucurbit downy mildew (CDM) is a plant foliar disease that is caused by the oomycete *Pseudoperonospora cubensis* (Palti & Cohen, 1980). Infected plants exhibit chlorotic and angular lesions on the adaxial side of the leaves. Chlorotic lesions become necrotic and eventually necrosis expands in the whole leaf and in extension to stems and the whole plant (Savory et al., 2011). *P. cubensis* is classified to 10 pathotypes depending on the ability to infect different cucurbit crops (Cohen et al., 2003; Cohen et al., 2015; Thomas et al., 2015). Infectivity of the pathogen is at large extent dependent on its ability to secrete RXLR type effectors (Tian et al., 2011) into the cytoplasm of host cells during infection process in order to exploit host machinery for its establishment and proliferation. Resistance to CDM can be achieved by modifying plant susceptibility (S) genes that encode such host targets. There is a wide array of plant genes that are reported to be S genes for downy mildew (DM) in various DM-host plant pathosystems. For example, the *DMR6* gene coding for a protein that converts salicylic acid (SA) a major defense signaling hormone to 2,3-dihydroxybenzoic acid (2,3-DHBA). Loss of function of this gene results into DM resistance in multiple plant species e.g. arabidopsis, tomato, grapevine, barley (Van Damme et al., 2008; Van Schie and Takken, 2014; Thomazzela et al., 2021; Pirello et al., 2022). Other well-known S- genes to DM from *A. thaliana* are a) *AGD5* coding for GTPase-activating protein at the trans-Golgi network (Stefano et al., 2010), *MKP1* coding for Mitogen-Activated Protein Kinase Phosphatase (Escudero et al. 2019) as well as *NUP133* (Ried et al. 2019) coding for nucleoporin protein. Loss of function of these genes leads to resistance to DM in Arabidopsis. In cucurbits, *TCP14* encoding a leaf-specific transcription factor has been shown to contribute to susceptibility to foliar diseases like CDM (Zheng et al. 2019).

Cucurbit powdery mildew (CPM) is a plant disease caused by the biotrophic ascomycete fungus *Podosphaera xanthii* and one of the most common and economically important diseases in cucurbit crops (Rabelo et al., 2017). While three distinct fungal species are able to infect cucurbits, i.e., *Leveillula taurica*, *Golovinomyces orontii* and *Podosphaera xanthii*, the latter is the most prevalent one (Glawe, 2008; Lebeda et al., 2016). Plants infected with *P. xanthii* exhibit reduced yield, primarily caused by the reduction of photosynthetic activity since the pathogen can cover the complete leaf surface. Losses

come also from damages in stems, leaves and fruits which reduce plant fitness and the overall quality of production (Kristkova et al., 2009). Disease symptoms of CPM are easy to identify since plants are often covered with characteristic white powdered fungal growth (Figure 1). In addition to the leaves, the stems or petioles can be covered with spores; CPM infection can spread and eventually cover the whole plant.

More than 200 *Cucumis melo* CPM resistant accessions to different PM races have been identified over the years with 25 dominant loci clustered mostly in chromosomes 2 and 5 while seven resistance QTLs have also been reported (Cui et al., 2022). Apart from dominant resistance two recessive resistance loci have been reported in melon wild species; a loss of function *MLO* (mildew resistance locus O) gene as well as a locus designated as *pm-s* (Cui et al., 2022) in PI 313970. Loss of function of *MLO* genes can result in resistance to PM in various crops of different plant species. Natural mutations in *MLO* were initially found in Ethiopian barley in the middle of the previous century and was exploited by barley breeding for more than forty years exhibiting exceptional durability (Kusch et al., 2017). Since then, natural loss of function of MLO genes for PM resistance has been reported in germplasm of several crops (Bai et al., 2008; Berg et al., 2015;) and were also induced artificially (Yan et al., 2022; Pessina et al., 2016;). *MLO* is a family of plant genes coding for proteins that consist of seven transmembrane domains and a calmodulin binding domain (Jones et al., 2017). Ca^{2+} influx is a key component for plant defense signaling during pathogen invasion and it is monitored by calmodulin binding proteins such as MLO. *MLO* gene families are categorized in seven distinct clades based on phylogenetic analysis. *MLO* genes from clades IV (for monocot species) and V (for dicot species) participate in PM susceptibility (Berg et al., 2017), and shown to be negative regulators of immunity and their expression is induced by PM infection (Kim et al., 2002).

Research on Cucurbitaceae has revealed several *MLO* gene homologues, including the 16 *MLO* homologues that are identified in melon (*Cucumis melo*), 14 in watermelon (*Citrulus lanatus*) and cucumber (*Cucumis sativus*) and 18 in pumpkin (*Cucurbita pepo*) (Iovieno et al., 2015; Zhou et al., 2013). Despite the large numbers of *MLO* genes in the different cucurbit genomes only a few of them belong to the clade V which is responsible for PM susceptibility. In cucumber, three clade V MLO gene homologues were previously reported namely *CsaMLO1 (Csa1M085890), CsaMLO8 (Csa5M623470) and CsaMLO11 (Csa6M292430)* (Berg et al., 2017). A transposable element insertion that was detected in the *CsaMLO8* gene of the wild cucumber accession PI 215589 was associated with hypocotyl resistance to *P. xanthii*, highlighting the role of this gene in CPM infection (Berg et al., 2015). The same loss of function mutation on *CsaMLO8* was also detected in multiple other CPM resistant cucumber

accessions exhibiting a conservation in the plant's germplasm (Nie et al., 2015a; b). In melon, natural mutations in one of the three Clade V MLO gene homologues, *MELO3C012438* were found in CPM resistant wild melon species (Hong et al., 2015).

In addition to MLO, other gene categories have been identified to be required for susceptibility to PM and loss of function of these genes has been shown to lead to PM resistance in various crops. *PMR4* codes for a callose synthase protein that reinforces plant cell walls by depositing callose at the site of the pathogen penetration, making them more resilient to infection (Martínez et al. 2020; Nishimura et al., 2003). Since a loss of function in such a gene would be paradoxical to lead to resistance researchers showed that callose deposition also negatively affects salicylic acid levels, which is a major defense signaling hormone of the plants. Additionally, *PMR5* which codes for an acetylation protein and *PMR6* which codes for pectate lyase are also correlated to PM susceptibility and mutants of both genes in *A. thaliana* exhibit resistance to PM (Vogel et al. 2004; Vogel et al. 2002). Eventually, two more well-known PM S genes in *A. thaliana* that are also involved in SA regulation are the enhanced disease resistance genes *EDR2* and *EDR4* (Wu et al. 2015; Nie et al., 2012).

In the current research we investigated the presence of natural loss-of-function mutations in known candidate susceptibility gene homologues of 100 re-sequenced melon genomes available at Center for Genetic Resources, the Netherlands (CGN) (Demirci et al. 2021). The aim of the research was to provide the breeding industry with promising insights on natural durable recessive resistance sources against PM and DM.

Materials and Methods

Investigation of known S-gene homologues from Arabidopsis in melon

Coding sequences of several S-genes reported in Arabidopsis and retrieved from NCBI were aligned against melon genome version 3.6 (Cucurbit genomics database) in order to determine the candidate homologues in melon based on the top hits. The candidate genes were selected based on certain criteria such as: a) mutant S-gene alleles confer full resistance to PM or/and DM b) no orthologous genes present in the genome c) low fitness cost upon loss of function. Melon homologues of MLO clade V genes were retrieved after blasting the known Clade V cucumber protein homologues against melon genome version 4.0 proteins (Cucurbits Genomic Database). The top hits for each homologue were aligned and a maximum likelihood phylogenetic tree was constructed to further confirm the homology using MEGA software alignment tools.

CGN resequencing data examination for high impact mutation detection in candidate susceptibility genes

For all accessions Illumina paired end reads were mapped to the *Cucumis melo* v 3.6.1 reference genome using bwa mem v0.7.17-r1188 and then sorted and indexed using SAMtools v1.8. For each gene of interest, the alignments overlapping the locus were extracted using SAMtools. Duplicate reads were then marked using Picard MarkDuplicates v2.22.1 with options:
ASSUME_SORT_ORDER=coordinate,
OPTICAL_DUPLICATE_PIXEL_DISTANCE=2500
VALIDATION_STRINGENCY=SILENT, MAX_SEQUENCES_FOR_DISK_READ_ENDS_MAP=50000 (DEFAULT), MAX_FILE_HANDLES_FOR_READ_ENDS_MAP=8000 (DEFAULT), SORTING_COLLECTION_SIZE_RATIO=0.25,
TAG_DUPLICATE_SET_MEMBERS=false,
REMOVE_SEQUENCING_DUPLICATES=false, TAGGING_POLICY=DontTag DUPLEX_UMI=false,
REMOVE_DUPLICATES=false,
ASSUME_SORTED=false, DUPLICATE_SCORING_STRATEGY=SUM_OF_BASE_QUALITIES,
PROGRAM_RECORD_ID=MarkDuplicates,
PROGRAM_GROUP_NAME=MarkDuplicates,
READ_NAME_REGEX=<optimized capture of last three ':' separated fields as numeric values> MAX_OPTICAL_DUPLICATE_SET_SIZE=300000,
VERBOSITY=INFO,
QUIET=false COMPRESSION_LEVEL=5, MAX_RECORDS_IN_RAM=500000, CREATE_INDEX=false CREATE_MD5_FILE=false, GA4GH_CLIENT_SECRETS=client_secrets.json USE_JDK_DEFLATER=false USE_JDK_INFLATER=false. fi. Using the GATK v4.1.3.0 HaplotypeCaller variants were called using default settings. Subsequently, SNPeff v4.3t was used to determine variant effects and to add ANN, LOF and NMD tags to the vcf INFO field.

Detection of the CGN140842 mutation and generation of the F2 population

Seeds of CGN140842 were sown in a Unifarm greenhouse climate chamber at 25°C, relative humidity 75%, and 16 hour-photoperiod. Following DNA isolation from cotyledons using the DNA extraction method described before (Siskos et al., 2023) the genomic locus flanking the insertion was amplified by PCR using primers CmMELO1F: 5’- GAAGCTATCGACTTCACTCTTGTA-3’, CmMELO1R 5’-TTATGATCTATCAGGGTGCTTGTAC-3’. PCR products were sent for Sanger

sequencing in order to determine the presence and the zygosity of the mutation in the seed batch. Plants carrying the mutation in homozygous state were crossed with SAKATA X0790 Piel de Sapo PM susceptible melon to produce the F1 and by subsequent selfing, the F2 populations.

Functional analysis of natural mutation present in CGN140842

Genotyping, disease assays and scoring

The CGN140842 F2 population was grown at Unifarm climate chamber with conditions that were mentioned above. Genotyping of ~100 F2 plants was performed in order to detect the segregation ratio of the mutant mlo1 allele in the population with PCR and Sanger sequencing using the primers mentioned above. Following genotyping, 28 plants from each F2 population genotype (MLO1/MLO1, MLO1/mlo1, mlo1/mlo1), five plants of CGN140842 parent (resistant control) and five Rampicante Zuccherino commercial melon line plants (susceptible control), were grown in individual plastic pots and maintained according to standard procedures. Drip irrigation was used in order to prevent spread of PM in water. A completely randomized block design was used to arrange the plants in the chamber, minimizing environmental effects. As soon as the plants reached the first true leaf stage PM inoculation took place by spraying with SAKATA PM race Px3.5 spore suspension. The inoculum was prepared by washing 10 PM infected leaves in 150ml of water until a concentration of $4x10^4$ conidia per ml was reached. Subsequent dilution to a final volume of 1.5 L created the final sprayed inoculum suspension. Scoring of disease progress took place 7, 10 and 14 dpi while a 0-5 scale was used with 0 representing asymptomatic plants and 5 fully symptomatic ones. A single factor ANOVA statistical test was used to determine significant differences in symptom scores between the different genotypes considering a p value <0.05.

Results

Re-sequencing data of 100 melon genomes reveal high impact mutations in the coding sequence of a known Arabidopsis S - gene for DM

The melon homologues of candidate S-genes (Table 1) for PM and DM already identified in Arabidopsis (and other plants) were examined utilizing resequencing data of 100 melon CGN re-sequenced genomes for the presence of high impact mutations in their coding sequence. Naturally occurring high impact mutations in those genes would ideally result into non- functional proteins. For DM S-genes homologues no high impact mutations were detected in the coding sequences of *AGD5* (MELO3C018195), *NUP133* (MELO3C024202) and *MKP1* (MELO3C023830)

in any of the 100 melon genomes that were examined. The same applied for all the Arabidopsis S-genes homologues for PM namely *IDD4* (MELO3C022568), *PMR4* (MELO3C013621), *EDR2* (MELO3C013803), *EDR4* (MELO3C020021) as well as *IAN9* (MELO3C005230) that was previously reported as a S-gene for hemi-biotrophs. The only arabidopsis S- gene homologue that did exhibit several high impact mutations in multiple CGN accessions was *TCP14* (MELO3C025629). More specifically, insertions of one or multiple nucleotides were detected in accessions 140862, 140866, and deletions of one or more nucleotides were detected in 140805, 14058, 140872, 24602, 140862, 140866, 140874 and 140806 (Table 1). The effect of these changes in the resistance against CDM were not examined in any of the aforementioned accessions in the current research, due to difficulty of production of reproducible DM inoculum for melon.

Table 1. Melon homologues of known Arabidopsis S-genes for powdery mildew and downy mildew.

Pathogen	Gene name	High impact change	Position (nt, genome v.3.6)	CGN accession
Downy mildew	*AGD5*	-	-	-
	NUP133	-	-	-
	TCP14	GGGAGTTCAGTAATCAA CGCCCGGCGAAATCAGC AAAACCGGGAGTAAGA AC CTGTGGTGAT -> G	4,807,424	140805, 14058,140872, 24602,140862, 140866
		TGG –> T	4,807,430	140862, 140866
		TAATCAACGCCCG GC-> T	4,807,434	140862, 140866
		CA -> C	4,807,454	140862, 140866
		T ->TC	4,807,479	140862, 140866
		GTGAT -> G	4,807,481	140806, 140862, 140866
		CGATT -> C	4,807,535	140874
		GT -> G	4,807,564	140806
		T-> TGGGAGAGGCCGC ATTGGACGAAATT GAACAAGAAACA AC	4,807,566	140866
	MKP1	-	-	-
Powdery Mildew	*IDD4*	-	-	-
	PMR4	-	-	-
	EDR2	-	-	-
	EDR4	-	-	-
(Hemi)-Biotrophs	*IAN9*	-	-	-

High impact mutations detected in coding sequences of CGN melon accessions are shown along with their respective position in genome (melon genome version 3.6).

Re-sequencing data of 100 melon accessions reveal a high impact mutation in MELO3C005044 of CGN accession 140842

Apart from known Arabidopsis S-genes for PM and DM, melon homologues of loci associated with susceptibility in cucurbits were examined in the 100 melon genomes. The melon homologue (MELO3C012426) of Csa5M622830.1, which is a gene participating in a QTL for recessive resistance against CPM and CDM in cucumber, was examined for high impact mutations in the 100 melon genomes. No high impact mutations were detected whatsoever in any of the accessions examined, while multiple moderate impact changes were detected in various accessions.

The Cucumber Clade V MLO protein sequences of CsaMLO1 (Csa1M085890), CsaMLO8 (Csa5M623470) and CsaMLO11 (Csa6M292430) already known to contribute to PM susceptibility in cucumber (Berg et al. 2017), were aligned against the melon proteome in order to detect the respective melon homologue proteins that would share similar functions. The three Clade V melon homologues that were chosen based on protein alignment and phylogeny were: a) MELO3C005044 (CsaMLO1 homologue) b) MELOC012438 (CsaMLO8 homologue) and c) MELO3C025761 (CsaMLO11 homologue) (Fig.1). No high impact mutations were detected for MELO3C012438 and MELO3C025761 in any of the melon that examined. One high impact (frameshift) mutation (TA insertion, ch11: 4,012,907 nt, melon genome v.3.6.1) that could functionally inhibit MELO3C005044 was detected in *C. melo* subsp. *agrestis* accession CGN140842. Seedlings of CGN140842 were genotyped and the presence of the mutation was confirmed through Sanger sequencing in one or both alleles.

The mutation of *MLO* gene MELO3C005044 co-segregated with reduced CPM susceptibility in melon

In order to test the effect of the insertion in the *MLO* clade V gene MELO3C005044 on CPM susceptibility we generated F1 (CGN 140842 x X0790 Piel de Sapo) and F2 populations. Genotyping approximately 100 plants of the F2 population using markers flanking the mutation genomic locus revealed a 1 MLO1/MLO1: 2 MLO1/mlo1: 1 mlo1/mlo1 segregation ratio. We subsequently challenged the F2 population and the respective controls with *P. xanthii* and scored the progress of the disease 7-, 10- and 14-days post inoculation (dpi) (Fig. 2). Symptoms started to be visible around 3-4 dpi with small white powdery and light chlorotic spots on the upper side of the leaves, first in the *MLO1/mlo1* and susceptible control plants (SC), with mlo1/mlo1 plants and the parental CGN plants exhibiting similar symptoms shortly after (Fig 2). At 7dpi CPM was progressing on the same pace between the F2 plants independent of the mutation presence. The parental CGN plants remained less symptomatic compared to the F2 plants. However, the difference was significant only compared to the MLO1/mlo1,

MLO1/MLO1 and the SC ones ($p<0.05$). At 10 dpi symptoms progressed with colonies becoming bigger in size and increasing in numbers in most of the genotypes. The plants of the F2 started to segregate considering the disease severity with the mlo1/mlo1 exhibiting significantly less symptoms than the MLO1/MLO1 and the MLO1/mlo1.

The powdery spots that were detected in the CGN parental plants at 7dpi became smaller and fainter and the spread of the mycelium on other tissues was limited. A similar effect was observed in some mlo1/mlo1 F2 plants, but the effect was not consistent for all replicates. At 14 dpi the difference between the MLO1/MLO1 and the MLO1/mlo1 F2 plants followed the same trend as 7dpi with both genotypes exhibiting similar disease levels. The significant difference of these plants with the homozygous mlo1/mlo1 plants remained whereas the CGN parental plants remained in similar disease levels as 7dpi.

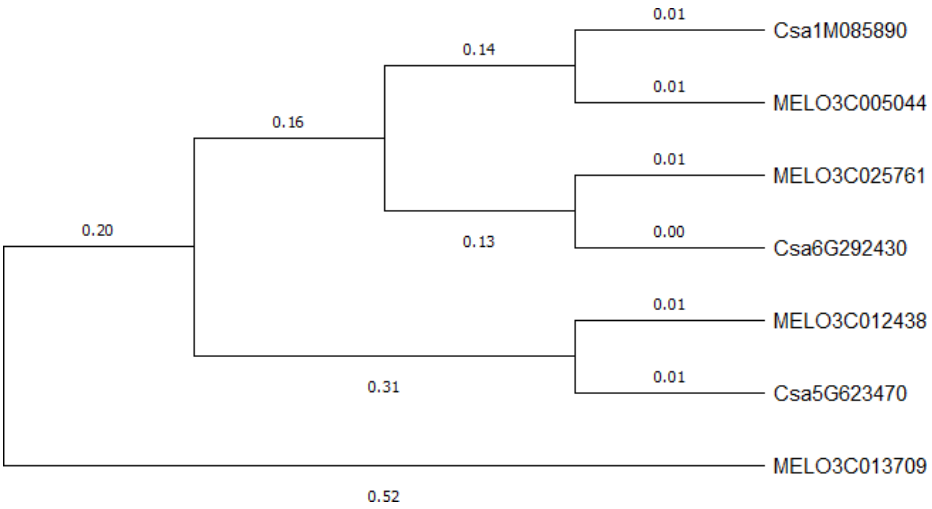

Figure 1. Protein phylogeny (Maximum Likelihood Tree) between known cucumber (Csa-) clade V MLO proteins Csa1M08590 (CsaMLO1), Csa5M623470 (CsaMLO8), Csa6M292430 (CsaMLO11) and melon (MELO-) MLO candidate orthologues.

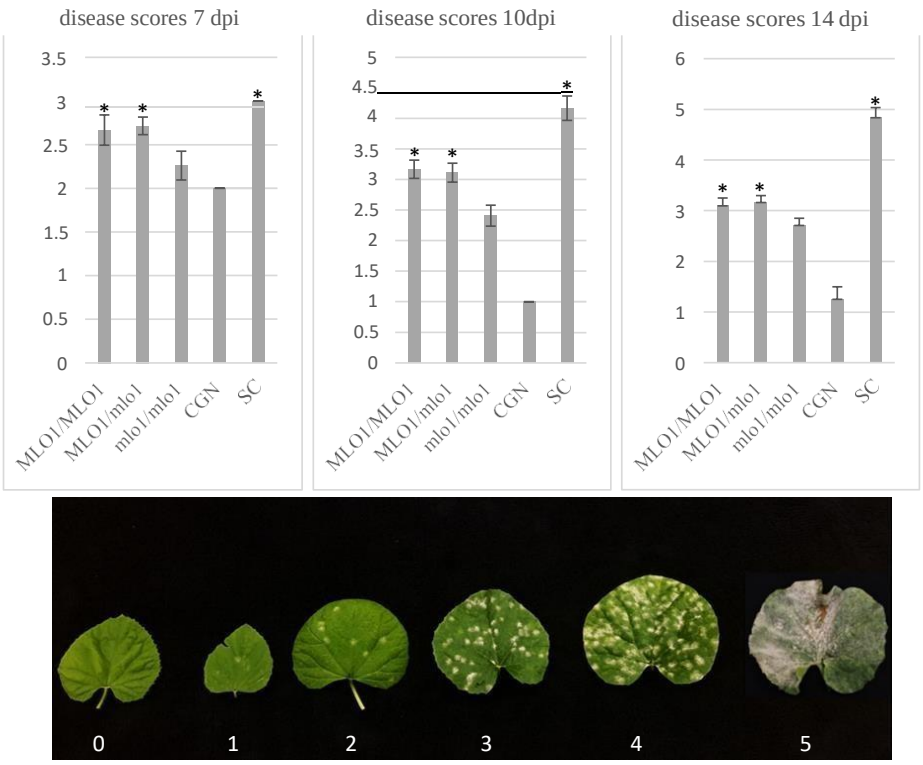

Figure 2. Disease index of different genotypes challenged with powdery mildew at 7 and10 dpi. A 0-5 scale of disease index was used with 0 representing absence of symptoms and 5 fully symptomatic leaves. Asterisks indicate significant differences in disease scores between plants that carry two mutant MLO1 alleles (CGN, mlo1/mlo) and plants that carry only one or no mutant alleles (MLO1/MLO1, MLO1/mlo1, SC) ($p<0.05$).

Discussion

Powdery and downy mildew constitute two of the most common pathogens in cucurbit crop cultivation. There is an everlasting need for generating resistant varieties either through introgression of resistance (R) genes from wild relative species or by impaired S-genes. Research on recessive resistance, which is a durable type of resistance based on non-functional S-genes, has produced a few promising results over the years (Van Schie & Takken, 2014). At the same time, there is a large pool of knowledge on genes for PM and DM susceptibility from studies in various other crops and especially *A. thaliana*. In the current research, we exploited existing knowledge on S-genes of other crops in order to apply it in the investigation of resistance against PM and DM in

cucurbits and specifically in melon. For this reason, we used the 100 re-sequenced CGN melon genomes for allele-mining on nine S-genes for PM and DM reported in literature for high impact mutations that impair their function.

One out of the nine candidate S- genes examined, *TCP14* (MELO3C025629), showed multiple high impact mutations in its coding sequence in multiple melon accessions. The mutations consisted of large deletions and insertions that could render the gene non-functional. There are approximately 21 *TCP* genes in Arabidopsis that participate in various physiological processes such as leaf and flower development, seed development and hormone signaling (Martin Trillo & Cubas, 2010, Kieffer et al., 2011, Palatnik et al., 2003, Ori et al., 2007). *TCP14* is a transcription factor that participates in immune and growth regulatory networks and is targeted by oomycete and bacterial pathogen effectors (Stam et al., 2021). In tomato it regulates the expression of defense genes however its activity is inhibited by binding to pathogen effectors leading to transcriptional changes that hamper immunity activation and favour the pathogen (Stam et al., 2021). In cucumber transcriptional analysis has revealed that the gene was negatively regulating the expression of a native R gene upon CDM infection (Zheng et al., 2019). Considering the specific targeting of this gene by different DM pathogens in distinct plant species we speculate that there is an evolutionary conservation of the *TCP14* function in DM pathogenicity. Therefore, any change in this gene could inhibit the interaction of the transcription factor with the pathogen effectors and in extent pathogenicity. The high impact mutations found in TCP14 coding sequences of many melon accessions are self-evidently not lethal for the plants probably due to the large abundance of similar genes from the same class that are present in the genome and are likely to complement its function. Due to the above it would be interesting to challenge the accessions mentioned above carrying mutations in the TCP14 gene with CDM to examine the effect on susceptibility.

There are several known and candidate susceptibility loci identified and studied in cucurbits including the candidate S-gene *Csa5M622830.1* which is a transcription factor in chromosome 5 of cucumber. A SNP in the 3' untranslated region (3'UTR) of the gene was correlated with resistance against CPM in cucumber and could also contribute to CDM resistance (Zhang et al., 2018), however the effect of the melon homologue is unknown. The absence of any high impact natural mutations in the coding sequences of 100 melon genomes raises doubts on whether this gene could result into viable melon plants upon knock out. An effector could potentially bind to the 3'untranslated region in order to alter the transcriptional regulation of the gene promoting pathogenicity without completely inhibiting its function for the plant. In that case the protein could still be accumulated in lower levels enough for the viability of the plant in the case it is

a necessary physiological component for the latter. Therefore, the absence of changes in the coding sequence of this gene were not unexpected. Finally, it would be interesting to examine changes in such regulatory areas as 3'or 5' UTRs and cis regulatory elements of this gene that could be binding hubs for pathogen effectors, something that was out of the scope of this research.

Three genes that were previously connected to PM susceptibility in cucumber are the Clade V MLO genes Csa1M085890 (CsaMLO1), Csa5M623470 (CsaMLO8) and Csa6M292430 (CsaMLO11). Due to the abundance of MLO genes in melon we opted for aligning the amino acid sequences of cucumber proteins against melon proteome to detect the most proximate functional homologues. From the three gene homologues detected in melon only MELO3C005044 (CmMLO1) was found to carry a high impact mutation in its coding sequence while CmMLO8 and CmMLO11 carried only moderate impact mutations. The two-nucleotide insertion detected in CmMLO1 results into a frameshift mutation and therefore probably a non- functional protein. Similarly, *CsaMLO8* has been previously reported to carry a transposon insertion in 31 cucumber accessions resulting into hypocotyl PM resistance (Berg et al., 2015). The loss of function of *CsaMLO8* mediated PM resistance caused by transposon insertion was related to cell wall apposition formation and cell death (Sun et al., 2023). Interestingly, contrary to cucumber, in melon, no high impact mutation was found in the CmMLO8 homologue which was unexpected taken into account the abundance of the mutated allele in cucumber germplasm. Moreover, the absence of high impact mutations in CmMLO11 in all melon accessions aligns with the respective absence of naturally occurring mutations in its cucumber homologue too (Tek et al., 2022).

CmMLO1 is located in chromosome 12 of melon at the position chr12 : 4012296 .. 4019430 (DHL92, v3.6.1). Melon chromosome 12 includes several reported QTLs for PM resistance (Fukino et al., 2008; Cao et al., 2021; Li et al., 2017; Toporek et al., 2021) but no QTL contains CmMLO1. The resistance conferred by the insertion mutation in CmMLO1 coding sequence was found to be partial, under optimal conditions for the disease. It has been reported that *CsaMLO1* and *CsaMLO11* play a minor role in PM susceptibility compared to CsaMLO8 (Berg et al., 2017). Our findings on the melon homologue of *CsaMLO1* are in alignment with the previous. Additionally, recent research in cucumber has shown that *CsaMLO1*:CRISPR/Cas9 knock-out cucumber lines exhibit quite higher penetration rates than *CsaMLO8* knock-out lines independent whether this mutation is present in combination with *CsaMLO1* and/or *CsaMLO11* loss of function mutations (Tek et al., 2022). In the same research it was shown that the resistance conferred by *CsaMLO8* is pre-invasive whereas the *CsaMLO1*/*CsaMLO11* resistance is post-invasive. The previous is another point of agreement with our findings

since the differences in disease scores between mlo1/mlo1 melon (CGN parent or F2) is increasing with the progress of the infection. The PM colonies in our mutant melon are initially similar in size and density as in the SC plants implying that the penetration is occurring but is not completely obstructed, whereas with the progress of time colonies become fainter, smaller and more localized. Despite the previous, it is noteworthy that CsaMLO1 is the only one of the Clade V genes upregulated 8 hours post inoculation with PM indicating an early participation of the gene in pathogenicity to PM (Schouten et al., 2014). Eventually, it would be insightful to perform histological examination on the infected leaves of mlo1/mlo1 in order to examine any potential formation of localized cell wall reinforcement at the attempted penetration sites (papillae) which is characteristic for MLO-based resistance. This examination could shed more light on the nature and the timing of *CmMLO1*'s contribution to PM pathogenicity.

Conclusion

Resistance against PM and DM in melon was investigated by examining the presence of naturally occurring high impact mutations in the coding sequences of known candidate S-genes in 100 melon genomes available at CGN. Out of the nine melon homologues of known Arabidopsis S- genes one gene associated with DM susceptibility was found to carry various insertions and deletions in its coding sequence in multiple melon accessions. It would be interesting to challenge these accessions with DM in order to examine whether the mutations segregate with resistance to the pathogen. Melon homologues of known Clade V cucumber MLO genes associated with *P. xanthii* susceptibility were examined for loss of function mutations over 100 melon accessions. One gene, *MELO3C005044*, which is the homologue of *CsaMLO1* was found to carry a 2-nucleotide insertion in its coding sequence. Partial resistance to PM was associated with the mutation in this gene. The locus can be exploited by melon breeders in order to improve the performance of commercial melon lines under PM pressure field conditions.

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Chapter 7

CRISPR/Cas9 genome editing for recessive resistance in cucurbits

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Abstract

CRISPR-Cas is one of the latest and most significant advances in genome editing, providing a powerful and precise tool for editing the genetic material of living organisms, including plants. CRISPR/Cas9 technology has been applied in various fields, including plant genetics and crop improvement. Cucurbits, which include plants such as cucumbers, melons and watermelons, have also been a subject of research utilizing CRISPR/Cas9 for genome editing. By targeting specific genes involved in disease susceptibility (S-genes), crops can be developed that are more resilient to diseases. Cucumber and melon suffer from mildew diseases (powdery/downy) and the last decade from a newly emerged virus, ToLCNDV, therefore knock outs of candidate S- genes in these crops could lead to resistance. In the current research a standard stable agrobacterium mediated transformation protocol of cucumber and melon was applied in order to deliver the CRISPR/Cas9 vector into the plant. Various mutations were induced in the genome of transgenic melon calli, however the regeneration of full plants from these calli was not possible confirming the already known difficulties of regenerating stably transformed events in these crops. Several actions to optimize the already existing transformation protocol such as removing abscisic acid (ABA) from the regeneration medium, increase of regeneration temperature, washing with antibiotics, improved the performance of the explants, nevertheless the generation of transgenic plants was still not possible.

Introduction

CRISPR/Cas9 is a groundbreaking gene editing tool that has revolutionized the field of genetics. It is a precise and efficient means of making targeted modifications to DNA sequences, and has the potential to cure genetic diseases, generate new crop varieties, and even eliminate some types of cancer (Adli, 2018). Several genome editing tools preceded the appearance of CRISPR/Cas9 in biotechnology research such as mega nucleases, Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). Nevertheless, their low genome editing efficiency, relatively high costs and/or their cumbersome practical application maintained their utilization in research limited. On the contrary, the introduction of CRISPR/CAS9 offered both a more robust and user friendly alternative that became accessible to a broader scientific public and pushed the boundaries of biotechnology research output into new groundbreaking levels (Gupta et al., 2014). The origins of the technology are found in the repeat elements of the *E.coli* genome. Contrary to typical tandem repeats they are separated by non-repeating DNA sequences known as spacers. Later these repetitive sequences were named as clustered regularly interspaced short palindromic repeat DNA sequences (CRISPR) (Gostimskaya, 2022). Interestingly, the spacer sequences were shown to be parts of viral DNA and other genetic elements (e.g plasmids) that were incorporated in past defensive encounters with a virus or invading plasmid. Additionally, these CRISPR elements are adjoining to various genes called CRISPR-associated (Cas) genes which code for enzymes that are able to cleave DNA. These enzymes which are acting in conjunction with CRISPR RNAs (crRNA) that are transcribed from the spacer sequences, protect the bacteria by cleaving any invading DNA whether viral or other by inducing double stranded breaks (DSB) (Ran et al., 2013) .

The transformation of CRISPR/Cas9 from a natural biological system to a genome editing tool occurred upon the discovery that a single guide RNA (sgRNA) could be generated by combining a crRNA with a targeting guide sequence and a trans-activating crRNA (tracrRNA) that anchors to Cas9 in order to induce precision targeting DNA cleavage in vitro. The sgRNA is around 100 nt long and consists of a 20nt sequence on its 5'-end that identifies the complementary target DNA sequence and is accompanied by 3nt NGG sequence called protospacer adjacent motif (PAM). The 3' of the sgRNA forms the loop that can anchor the Cas9 to the sgRNA and leads it to the point of cleavage. Cas9 consists of two domains the HNH and the RucV-like domain. The former is responsible for the cleavage of the DNA strand that is complementary to the sgRNA sequence while the latter catalyzes the cleavage of the non- complementary strand on the targeted DNA sequence. The Cas9 cleavage results in a DSB which is subsequently repaired by nonhomologous end-joining (NHEJ) or homology-directed repair (HDR) DNA repair mechanisms of

the cell. Non-homologous end joining which is the most common repair mechanism during a DSB often results in mismatches (SNPs) and indels (deletions/insertions) and consequently leads to gene knockouts. CRISPR/CAS9 has been extensively utilized for crop genome editing over the last decade for research and commercial purposes on a wide variety of plant species e.g *Arabidopsis thaliana* (Feng et al., 2013; Jiang et al., 2013; Li et al., 2013), *Brassica oleracea* (Lawrenson et al., 2015), *Nicotiana benthamiana* (Lowder et al., 2015; Yin et al., 2015), *Oryza sativa* (Jiang et al., 2013), *Zea mays* (Liang et al., 2014), *Vitis vinifera* (Ren et al., 2016), *Solanum lycopersicon* (Li et al., 2018). The first step of the CRISPR/Cas9 genome editing in plant breeding is the selection of the right genes to be edited in order to improve certain quality characteristics of existing commercial varieties or make the crops resilient to pathogens. Design of sgRNAs based on the coding sequence of the gene of interest follows and subsequently the sgRNAs are incorporated in a plasmid with Cas9. The delivery of CRISPR/Cas9 in plants occurs most commonly through stable agrobacterium mediated transformation of plant cells. Subsequent tissue regeneration from transformed/edited cells generates plants that are carrying the induced mutation in one or more alleles in the T0 lines. Null lines can be later used as pedigree in breeding programs that aim to improve commercial varieties.

Cucurbit crop cultivation is hampered by several pathogenic organisms that can reduce the yield and/or downgrade the quality of the fruits resulting into significant economic damage for the growers. Resistance breeding has been the main approach for limiting the damage caused by these pathogens either by introgression of resistance genes from wild relatives or by investigating mutations of certain plant genes that the pathogens exploit in order to proliferate, also known as susceptibility genes (S-genes) (van Schie & Takken, 2014). Mutations in these genes that can lead to knockouts and loss of function proteins, are particularly useful in the everlasting evolutionary arms-race between host plants and pathogens as they give an important lead to the former by shutting down major pathways of pathogenicity for the latter. Mutations of S-genes can be found in abundance naturally when examining the germplasm of several crops or they can be induced artificially through genome editing. CRISPR/Cas9 is currently used for this purpose in many crops including cucurbits mainly over the last couple of years (Table 1). One of the first applications of CRISPR/Cas9 in cucurbits for generating recessive resistance based on knocked out S-genes was reported in cucumber relatively recently in which by editing a translation initiation factor researchers (Chandrasekaran et al., 2016) managed to confer broad viral resistance to the plant. So far, the tool has been applied in other cucurbits (such as melon and watermelon) for quality and resistance breeding purposes however compared to other crops the application in cucurbits remains limited as it is inefficient and cumbersome. The recalcitrance of cucurbits in agrobacterium mediated transformation and regeneration is the main inhibiting factor in applying the technique in these crops (Zhao et al., 2022).

Table 1. Application of CRISPR/Cas9 in cucurbit crops for different trait purposes

Cucurbit crop	Gene	Trait	Reference
Cucumis sativus	eIF4E	Resistance to cucumber vein yellowing virus, zucchini yellow mosaic virus, papaya ring spot mosaic virus-W	Chandrasekaran et al., 2016
	MLO (-1,-8,-11)	Resistance toPodosphaera xanthii	Tek et al., 2022
	AKT1	Salt tolerance	Peng et al., 2022
Cucumis melo	CTR1, ROS1	Melon fruit ripening	Giordano et al., 2022
	eIF4E	Resistance to Moroccan watermelon mosaic virus	Pechar et al., 2022
	Fom-1 and Prv	Resistance to Fusarium races 0 and 2, and to papaya ring spot virus	Nizan et al., 2019
	NAC	Delay in melon climacteric fruit ripening	Liu et al., 2022
Citrullus lanatus	ClBG1	Decrease seed size, increase of seed germination	Wang et al., 2021
	GRF5	Transgenesis and genome editing enhancement	Pan et al., 2021
	PSK1	Resistance to Fusarium oxysporum	Zhang et al., 2020

Despite the elaborate pre-existing literature that is focusing solely on cucurbit transformation (Rajagopalan et al., 2005; Zhang et al., 2014; Nasanato et al., 2013; Du et al., 2022) more and more literature is emerging over the last years mainly focused on the application of CRISPR/Cas9 in cucurbits suggesting ways to overcome the difficulties at the regeneration stage when using agrobacterium mediated transformation (Hoogvorst et al., 2019; Pan et al., 2022; Nguyen et al., 2022; Xin et al., 2022; Tian et al., 2017). The aim of the current research is to apply the insights that were obtained from previous studies in cucurbit transformation as well as CRISPR/CAS9 genome editing in order to knock out candidate S-genes in cucumber and melon for three economically important diseases; powdery mildew (PM), downy mildew (DM) and tomato leaf curl New Delhi virus (ToLCNDV).

Materials and Methods

Generation of CRISPR/Cas9 constructs

Genomic sequences of candidate S-genes for powdery and downy mildew as well as ToLCNDV (Table 2) were retrieved from cucurbits genomic database, melon genome version 3.6 and cucumber Chinese long genome v3 (http://cucurbitgenomics.org/). SgRNAs were designed on the exonic regions of the genomic sequences of the genes with a preference on the first exons using CRISPR-P 2.0 online guide design tool. The

parameters for the guide design tool were a) ''NGG'' as PAM site b) U6 promoter c) a 20 nt sequence length d) *Cucumis melo* 3.5 melonomics as a target genome and *Cucumis sativus* phytozome v 9.0. Four sgRNAs per gene with the best scores and no-off targets individually promoted by U6-26 promoters were cloned into a Golden Gate expression vector that contained a nos-NPTII (kanamycin resistance) selection marker, a *35*S-GFP fluorescent marker, and a parsley ubiquitin 4 promoted SpCas9. The cloning procedure and the Golden gate plasmids used are described in detail by Santillan- Martinez et al., 2020. Subsequently, *Agrobacterium tumefaciens* competent cells strains EHA105 and GV3101 were transformed with the CRISPR/CAS9 vectors.

Stable agrobacterium transformation and regeneration of cucumber and melon plants

Several melon and cucumber accessions were selected for genome editing through CRISPR/CAS9 stable *agrobacterium*-mediated transformation (Melon: Charentais, Rampicante Zuccherino, Giallo d'Inverno, Cucumber: White Wonder, Marketmore, Johanna, FRANCHI seeds,). The basic criteria for the cultivar selection were a) the absence of any known resistance/tolerance against PM, DM and ToLCNDV as well as b) high homozygosity of their genome. Therefore cucumber and melon landraces were the most preferred choices for transformation. A protocol designed by Park et al. (2005) and later modified by Nanasato et al. (2020) was used with minor modifications for stable agrobacterium transformation of cucumber and melon. The coats of the melon seeds were removed, and seeds were sterilized for 1 minute in 70 % EtOH, followed by 10 minutes in 1% NaOCl and 10 minutes in 1% H2O2 using a tube- roller-system. Subsequently, the seeds were placed between two layers of three wet filter papers in order to germinate at 28 °C in the darkness. After four to five days when seeds had germinated, cotyledons were detached from the hypocotyl using a scalpel, and placed in infiltration medium (Murashige and Skoog basal medium including vitamins (MS+vit): 3 g/l, Sucrose: 2.5 g/l, Glucose: 2.5 g/L, MES: 2 g/L, Duchefa Biochemie, https://www.duchefa- biochemie.com/). *Agrobacterium tumefaciens (*EHA105, GV3101, AGL0) containing CRISPR-Cas9 constructs were diluted with infiltration medium until an OD600: 0.6 was reached and added to the cotyledons. Vacuum infiltration of the medium into cotyledons followed. The cotyledons were dried on filter paper and placed on co-cultivation medium (MS+vit: 3 g/L, Sucrose: 20 g/L, MES: 1 g/L, Micro Agar: 7 g/L, BAP: 2 mg/L Duchefa Biochemie) on top of three filter papers (Figure 1). Cotyledons were left on co-cultivation medium for two days in the dark, at room temperature. After two days, the cotyledons were placed on regeneration medium (MS+vit: 4.4 g/L, Sucrose: 30 g/L, Plant agar: 8 g/L, MES: 1 g/L, BAP: 2 mg/L pH 5.6, Duchefa Biochemie) at 28°C and 16 hours photoperiod. Regeneration medium was refreshed every 7-10 days.

Table 2. sgRNAs designed for various candidate S-genes of cucumber and melon

Crop	Gene	Gene code	Disease resistance	5'-tgtggtctcaATTG-sgRNA- gttttagagctagaaatagcaag-3'
Cucumis sativus	Cs*Bch5*	Csa5G622830.1	PM/DM	TATTGGCGGAGAATACCGAGTCAGAGAGCTCTGCCGACAAATACGGTTGGTTCAGGACTACG GGTGGTTGTTTCAGTGACAG
	Cs*TCP14*	Csa4G628330	DM	AGTCTAACACGACGATCCCTAATCGACGGCCTTACTAGGCTCCGACGGGTGAATAGAAACTA GCCGAACGCAAGATCTA
	Cs*MKP1*	Csa5G180300	DM	CATACCGAACAATCTGCACAATCCTGAAGTGAACAGAGAAGGTCAATATGTGAAGGCAGCAA GACCCGGATAAGAATTGTGGGT
	Cs*AGD5*	Csa3M851770	DM	CTGTAGTTTGCGGCCCAGCCTCCCCAGCAGCTGACTTGGCTG TGATTGTTGCTGAGCTAGCA CTCATGGCTGCAGCTAAATCT
	Cs*EDR2*	Csa3M006610	PM	TAGAAGAACTAGCATGAACGGCAGAAGAGCCTAAAAGCCCAGCGAGAAACTGGATAAATCA AT CGTGATGGTTCTCGAGACTGC
Cucumis melo	Cm*EDR4*	MELO3C020021		GCCTGGTTACCTTCAGAAGGCAGAGATGCTCCCGAACAAG CAGCTTGCTCATATAAAGTC
	Cm*NAC*	MELO3C010500	ToLCNDV	CGGCGGTAACTGCAAATCGGGCAATAATCGGCACCGAAATCCACTCCCAGCCGACCGGTT CTCGCAAGAAGAATAGTCTA
	Cm*ARP4*	MELO3C017295	ToLCNDV	GAAAACGCAAACTATATGT GGTTCCGCCGTGATAATAATGGTTCGGCAAGTAGCATGGATATG GTCGATAGCATA
	Cm*PELOTA*	MELO3C008594	ToLCNDV	ACGGCCATAACAGTGTCTCC TGGCTCTATTCTGCGGATACG ATAGCCGGCCCATGCTTGCG ATAATAAGTGTCGATGGAAC
	Cm*PMR4*	MELO3C013621	PM	AGGGAGCCATTGAACATACGG CGAGAGTGTCGATATTATCGGAGTTCCTCCAAACACGATG TGTGCTCTCGTCGGACCCATCAT
	Cm*MLO1*	MELO3C005044	PM	TTGCTCGAGCGACTTCCCAC CACAACATGTAGCTGCAACC TATATCATTGCTACTGACGG
	Cm*MLO8*	MELO3C012438	PM	TCATCCCGAGGCTGAACGAC AGAATGGTGAGGTACTGTCT GTAACTCTTCCCACCGATC
	Cm*MLO11*	MELO3C025761	PM	TGCCACGTGGCTGCCACGTC TGGTCGGAATCGACCAGGT GCACATTTGTCGGTACCAC
	Cm*AGD5*	MELO3C018195.2	DM	GACTTGGCTCCAGCAGCCATGGCCAACTTTGAGGAGAAACAT TGCATCATCAATCCAGGAAT

Histological analysis of transgenic calli and detection of CRISPR/Cas9 mutations

Calli that were able to survive kanamycin selection were exposed to UV light under a stereoscope (ZEISS SteREO Discovery.V8) in order to examine GFP expression. The fluorescent calli were grown in regeneration medium described above with absence of hormones, for shoot induction. Tissue from transformed fluorescing plant material (calli) was used for isolation of DNA with the protocol described by Siskos et al., 2023. Following DNA isolation PCR took place with primers amplifying the coding sequence of Cm*AGD5* in order to ligate the product to a pENTR™/D-TOPO vector. The purpose of the cloning was to be able to detect separate mutation events present in the pool of individually edited calli. Ligation product was used for *E.coli* DH5a transformation and subsequent screening led to a selection of ~20 positive colonies for Sanger sequencing following DNA isolation with QIAprep Spin Miniprep Kit. The reads from the edited calli were aligned to the wild type coding sequence of AGD5 using the BioEdit Sequence alignment editor software.

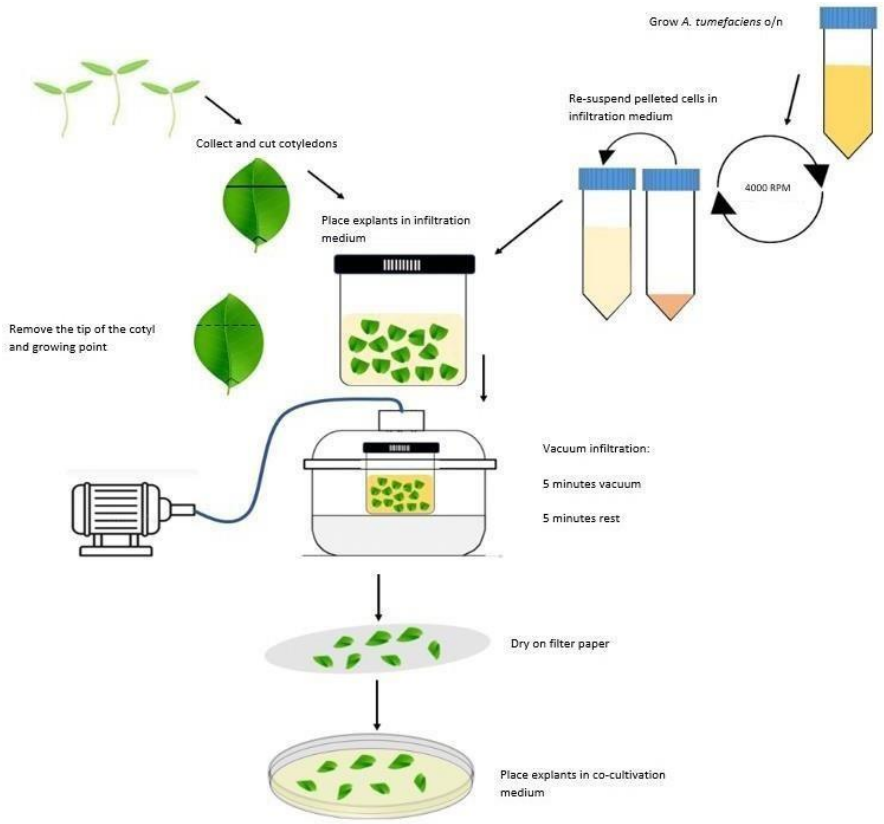


Figure 1. Agrobacterium mediated transformation protocol of cucumber and melon

Results

Temperature and media hormone modifications improve calli and shoot regeneration in melon

The optimization of the transformation protocol was necessary due to the limited shoot formation and regeneration that the Nasanato et al., 2013, protocol produced. Initially, two major limiting factors in cucumber and melon regeneration faced in the current study were a) the limited production of callus b) and the limited number of shoots produced from these calli. Callus and shoot regeneration are largely affected by the hormonal composition of the growth media used. Initially, two main hormones were used for callus and shoot regeneration media, BAP (2 mg/l) and ABA (1 mg/l) according to Nanansato et al., 2013. However, this resulted in our hands only in the emergence of relatively small calli from infiltrated cotyledons. Additionally, the produced calli often resulted into transgenic root formation instead of adventitious shoots (Figure 2). Cytokinins, including BAP, are plant hormones that have been shown to play a significant role in callus regeneration and are standard for many protocols for this purpose (Sahoo et al., 2011; Husaini et al., 2008). In cucumber tissue culture ABA has been used to enhance the formation of callus (Rai et al., 2011) and to improve the regeneration of shoots and whole plants from leaf explants (Gal-On et al., 2005; He et al. 2006; Rajagopalan and Perl-Treves 2005;) The exact physiological mechanism of ABA's contribution to shoot induction in cucumber remains unclear, however it is clearly connected to inhibition of adventitious roots. The ratio of cytokinin to auxin in the culture medium is another critical factor in callus induction and regeneration into shoots, with higher ratios of cytokinin promoting shoot regeneration and lower ratios favouring root regeneration (Su et al., 2011). Due to the inability of our material to efficiently regenerate calli and shoots and in order to limit the effect of rooting calli, since it is not clear how ABA might interact with cotyl auxin homeostasis, we opted for a complete removal of ABA from the regeneration medium and by keeping the BAP levels unchanged. The treatment had a positive effect both in calli and shoot regeneration and prevented the rooting of the calli. In addition to the hormones, the temperature that the explants were maintained in was another major factor that affected calli regeneration and shoot formation. Most of the protocols for cucumber and melon mention 25 ˚C as an optimal temperature of calli and shoot regeneration, however a higher regeneration rate was observed by maintaining the explants at 28 ˚C.

Figure 2. Transgenic roots emerging from transformed melon calli. Left normal light exposure, Right: UV:GFP filter exposure

Implementation of a washing step optimized selection of transgenic tissue

After the improvement of regeneration, the obtained calli growth was increased. The previous resulted into calli that were escaping kanamycin selection due to their high growth rate. Moreover, several parts that were escaping kanamycin, also because a large part of them were not directly in contact with the medium due to their fast-increasing size, were producing a large number of shoots that were non-transgenic. As a result, a more stringent selection of the non- transformed tissue was required.

Furthermore, the creation of additional edges in the plant's cotyledon was achieved through a minor incision in its center as it was observed that the transformation occurred primarily in the peripheral regions of the plant tissue, while the inner regions remained untransformed. To maximize the surface area of contact between the explants and the antibiotic, they were positioned in the medium in a manner that allowed a greater exposure to the antibiotic. To further enhance this contact, an additional treatment was administered prior to the transfer to the regeneration medium. The explants were rinsed with liquid Murashige and Skoog 20 medium (MS20), supplemented with 130 mg/L kanamycin, and 200 mg/L timentin, and subsequently incubated on a tube-roller-system for 15 minutes. After the washing process, the explants were dried on filter paper for 10 minutes before being placed on the regeneration medium. This approach yielded significant improvements in the selection against non- transformed tissue, as evidenced by the reduction of non-transformed tissue after three days (Figure 3).

Although regeneration rates remained similar between washed and non-washed explants in later stages, the total fluorescence in washed plants was visibly higher, suggesting

that the washing process after co-cultivation had a positive effect on the selection for transformed explants (Figure 3).

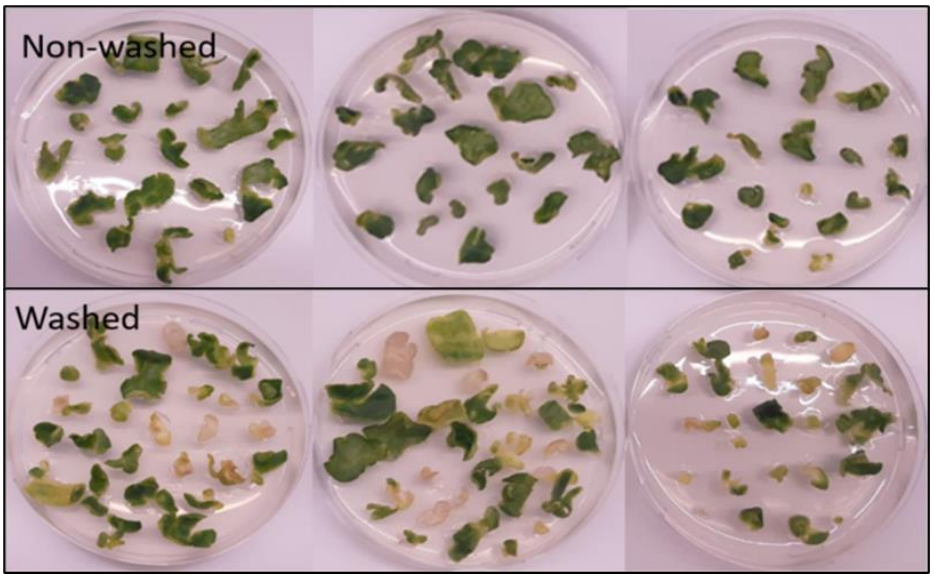
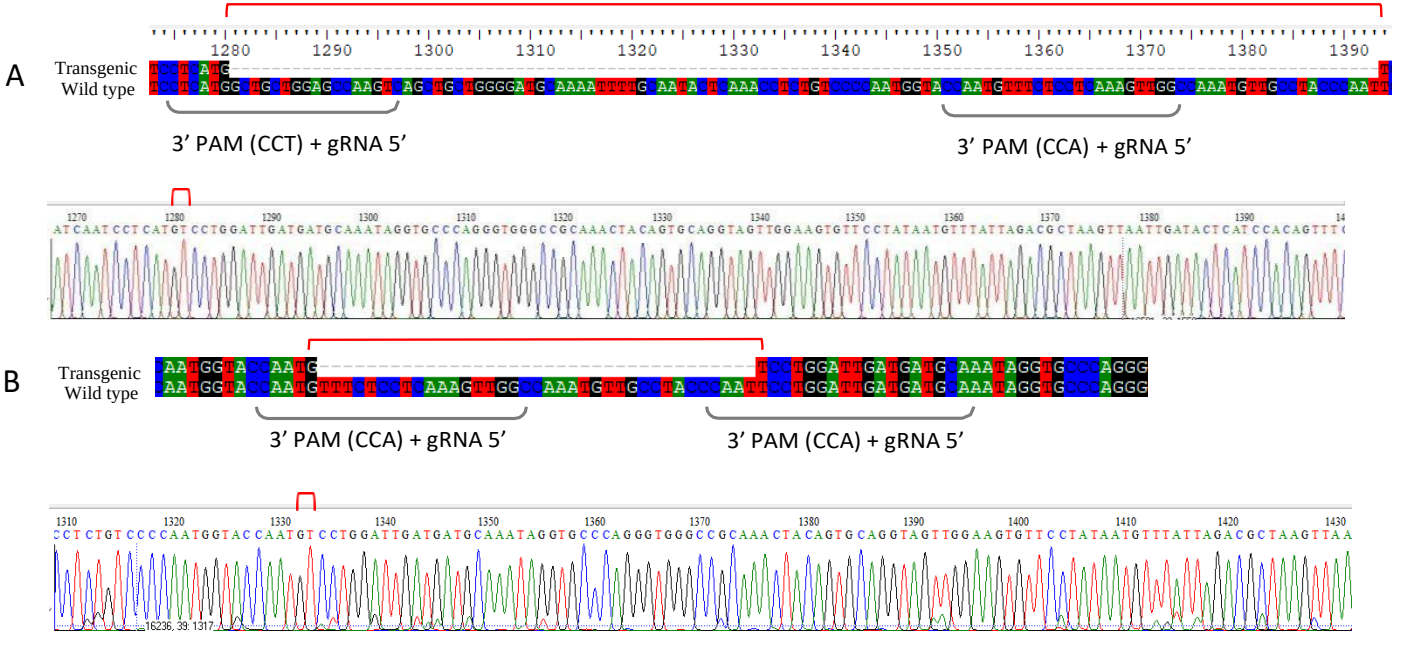


Figure 3. An additional washing step before putting the cotyledons on regeneration medium improves selection against non-transformed tissue. **Upper row:** explants after three days on regeneration medium, without washing step. **Lower row:** explants after three days on regeneration medium, washed before transplanting.

Genomic DNA from melon calli is successfully edited with CRISPR/Cas9 but shoot regeneration is not possible

Charentais melon cotyledons were stably transformed with several CRISPR/CAS9 constructs targeting the exonic regions of various candidate S-genes implementing the modifications above. Two to three weeks post infiltration the produced calli were examined under UV light stereoscope and the fluorescent parts expressing GFP were examined for mutations after PCR amplification and Sanger sequencing. Alignment of Cm*AGD5* Sanger sequencing reads against the WT coding sequence of the gene revealed a multitude of CRISPR/CAS9 induced mutations in harvested calli that were used for DNA isolation. Sequencing indicated the following edits in the exon region of the CmAGD5 genomic sequence: A) 114bp deletion by the 1st and 3rd guide B) 37 bp deletion by the 2nd and the 3rd guide, C) 77 bp deletion by the 1st and 2nd guide D) 3bp deletion by the 1st guide (Figure 3). The transgenic calli were maintained for more than 3 months in tissue culture without being able to produce any transgenic shoots that would allow a functional investigation of the mutation in disease resistance performance.

TCCAGTATGGTCTCTCCATTTGCTATGCATCAACAGCAACTTGCCATGCTAGCTCAGCAACAATCACTTCTCATGGCTGCAGCTAAATCTGGGGATGCAAAATTTAGCAATC
CTTCTCATCAGCAGCAACTTGCCATGTTAGCTCAGCAACAATCAAT**CCTCATGGCTGCTGGAGCCAAGTC**AGCTGCTGGGGATGCAAAATTTTGCAATACTCAAACCTC
TGTCCCCAATGGTA**CCAATGTTTCTCCTCAAAGTTGGC**CAAATGTTGCCTA**CCCAATTCCTGGATTGATGATGCAA**ATAGGTGCCCAGGGTGGGCCGCAAACTACA
GTGCAGGCAATGAACAGGGGGCTGGCAAATCCAGTTGGGAGTTCTGTTCCATGTCCAACATCTAGCCTTTATAGCCTCGGGCAAGTTTCTTCAGTGCCGGTGAATGGTGT
GACGCCCACAGCAAAAAATAAATCTCAATCAACAGCATCAGTATCATCAGCTACTCCTTCGCAATCGGGAAAAGAGTACGATTTCTCCTCTTTAACACAAGGTATGTTTTCA
AAACATTGA



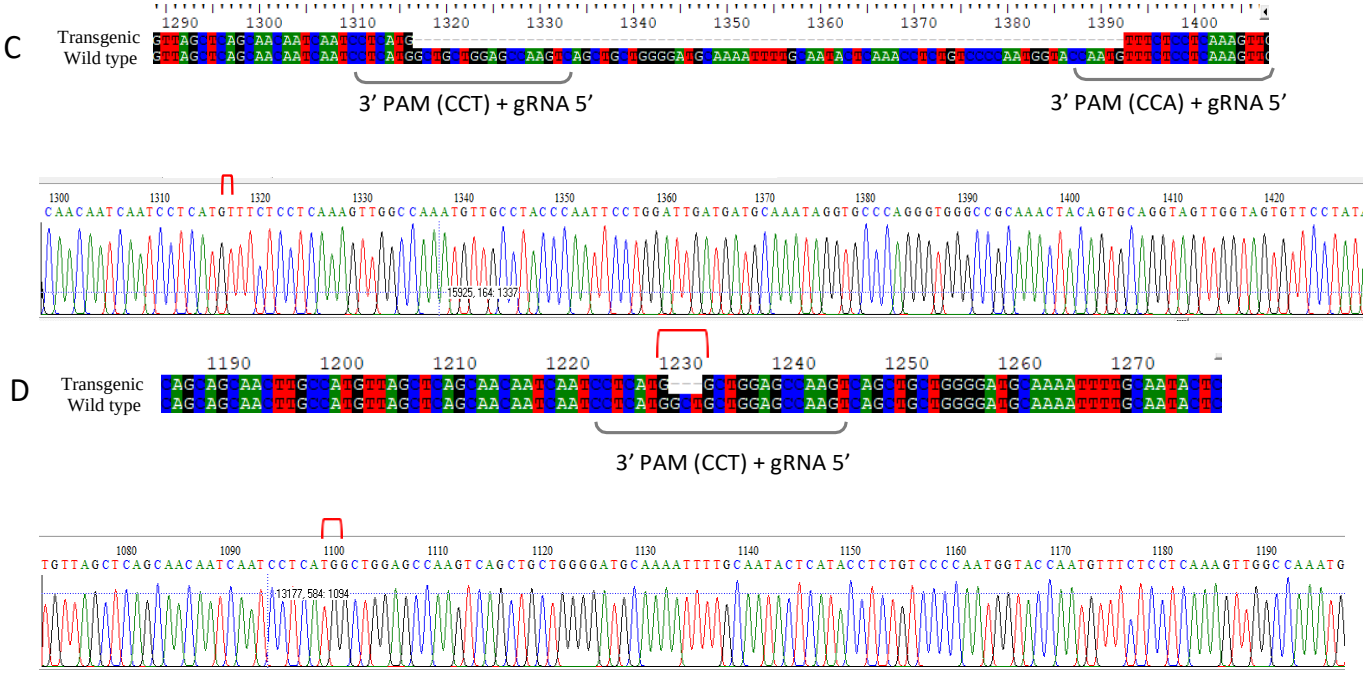


Figure 4. Cm*AGD5* exon sequence targeted with 3 sgRNAs highlighted in grey with PAM sites highlighted in yellow. Cas9 generated several mutations on transformed calli. A)114 bp deletion by the 1st and 3rd guide B) 37 bp deletion by the 2nd and the 3rd guide, C) 77 bp deletion by the 1st and 2nd guide D) 3 bp deletion by the 1st guide

Similar to the Cm*AGD5* calli, several other calli of cucumber and melon exhibited GFP fluorescence after agrobacterium mediated infiltration. Nevertheless, there was no emergence of fluorescent transgenic shoots that would develop into fully grown plants independent of the cultivar or any modifications applied in the hormonal composition of the regeneration medium in the protocol that was used. One exception were two shoot primordia that emerged from transgenic calli of Charentais melon transformed with Cm*PMR4* and exhibited GFP fluorescence (Figure 4). Despite maintaining these shoots for more than 5-6 months in tissue culture they never developed into fully grown plants (Figure 4).

Discussion

CRISPR/Cas9-based editing of plant genomes has significantly impacted the field of plant breeding and is enabling the development of novel crop varieties that exhibit enhanced resilience and productivity in the face of diverse environmental stressors, including pathogenic agents. Cucurbit cultivation is facing various economically important threats such as PM, DM and ToLCNDV. One potential strategy for establishing durable and broad- spectrum resistance against pathogens is through the disruption of host factors, known as S-genes, which are exploited by these pathogens to promote their virulence and proliferation. There are several known S-genes against the aforementioned pathogens that were identified in various plant species. These genes were either artificially or naturally disrupted resulting into resistance against a respective pathogen. Due to the functional construct containing GFP. From top to bottom: a) Normal light exposure b) UV:GFP filter exposure c) UV:RFP filter exposure conservation of certain S-genes in susceptibility across plant species (e.g., PMR4), the disruption of their homologues in cucurbits could also potentially result in resistance. Using CRISPR/Cas9 technology we attempted to knock out certain S-gene candidate homologues in cucumber and melon in order to functionally evaluate the effect of their loss of function in resistance against PM, DM and ToLCNDV in these crops.

Among the numerous delivery techniques available for this technology in plants, stable agrobacterium-mediated transformation represents a widely employed approach in contemporary research (Zhang et al., 2019). Although Agrobacterium-mediated transformation has shown significant success in *Arabidopsis thaliana*, its applicability in other plant species, such as pepper (Kim et al., 2020) or melon (Hooghvorst et al., 2019), remains inefficient and cumbersome. In these crops, the regeneration of transgenic plants typically necessitates the use of calli derived from explants, indicating that the effectiveness of this method varies across different plant species. Our study

confirms the above as the main inhibiting factor of efficiently generating fully grown edited plants. Several protocols have been published for stable agrobacterium-mediated transformation in cucurbits over the years e.g cucumber (Trulson et al., 1986, Fan et al., 2020; Nasanato et al., 2013; Zhao et al., 2022), melon (Dong et al., 1991; Guis et al., 2000; Valles et al., 1994; Zhang et al., 2013), watermelon (Cao et al., 2022; Vasudevan et al., 2021; Feng et al., 2021). Nevertheless, research that includes regeneration of stable transformation of these crops are infrequent due to the unavoidable low efficiency and challenging nature of this method.

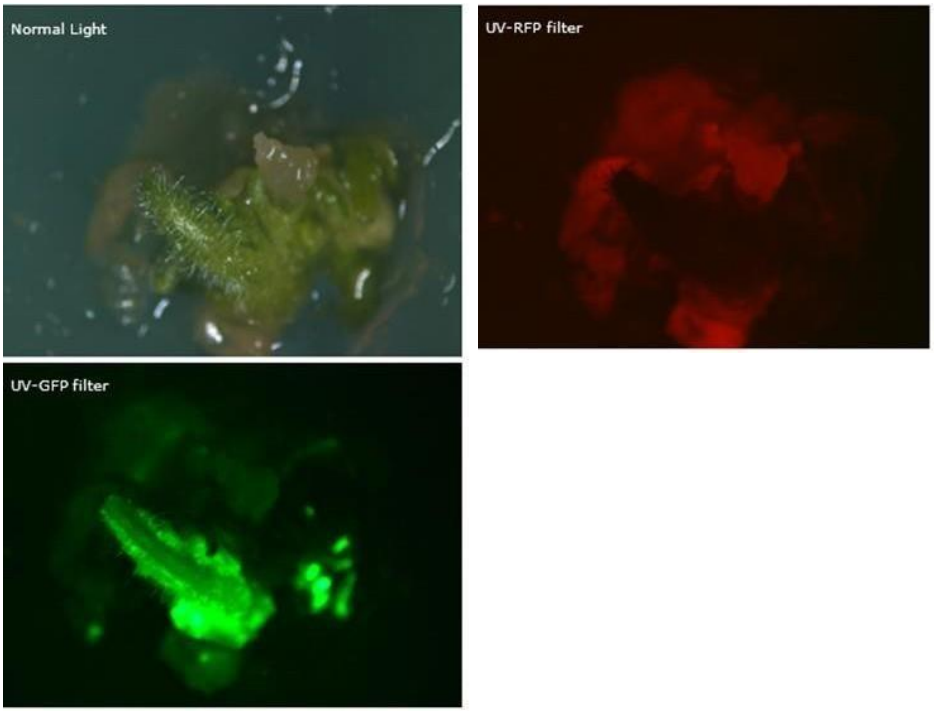


Figure 5. Stably transformed melon shoot primordia with CmPMR4:CRISPR/Cas9

The protocol of Nasanato et al., 2013 was utilized in this study due to its appealing high reported average transgenic efficiency for cucumber production (~12%). The protocol included two basic differences compared to other protocols. Firstly, infiltration of cucumber cotyledons with vacuum which promotes the efficient infiltration of Agrobacteria in the tissues and secondly the use of filter paper during co-cultivation which maintains the humidity at controlled levels and prevents bacterial outgrowth. The hormonal composition of regeneration medium included BAP and ABA similarly to various other protocols of cucurbit transformation. It is known that a high cytokinin:

auxin ratio promotes embryonic callus regeneration and shoot induction however a stress hormone as ABA is known to affect the homeostasis of auxin in the cotyls (Emenecker et al., 2020). The concentrations that are used from each of these two hormones vary between protocols from 1.5 mg/l BAP-1mg/l ABA (Zhao et al., 2022) which has been shown to contribute to maximum regeneration in cucumber (Zhang et al., 2017) to 1mg/L BAP- 1mg/ABA (Rajogapalan & Treves, 2005). In melon, protocols usually use 0.1 mg/L BAP-0.01 mg/L NAA (Bezirganoglu et al., 2014), 1.0 mg/L BAP-0.05 mg/L IAA (Zhang et al., 2014) as well as only 0.5mg/l BAP (Dong et al., 1991) without the addition of any ABA. In our material, whether cucumber or melon, the ratio of BAP to ABA was initially following the standard literature recommendations however it was observed that the regeneration was not optimal. Moreover, there was an increased production of transgenic roots from calli instead of shoots further indicating that the auxin levels were not balanced. ABA is a known plant hormone that has a synergistic role with cytokinins and an antagonistic with auxin, inhibiting root formation however, this is not always the case and it could be case dependent. Nevertheless, a suitable balance between auxin and cytokinin needs to be reached in order for optimal regeneration and rooting inhibition. The optimal regeneration was achieved by completely removing ABA from the medium and keeping only the cytokinin BAP in concentration 1.5-2 mg/l. Based on the variation between protocols regarding cytokinin-auxin ratio in the literature it is probable that the hormonal fine-tuning is not standard for all genotypes and different conditions may cause different reactions in different hormonal treatments. Moreover, high levels of ABA could be antagonistic to cytokinins such as BAP. Additionally, it is noteworthy to mention that the use of an auxin, IAA, in concentrations of 1mg/l or 0.5mg/l in combination with 2 mg/l BAP did not produce a different effect considering regeneration, in our hands.

Most of the cucumber and melon transformation protocols recommend maintaining the explants at 25 ± 2°C for callus and shoot regeneration. Cucumber and melon are warm season crops therefore vegetative growth is favored by relatively high temperatures. Melon has a reported optimal growth between 29 and 35°C (Hartz et al., 2008) therefore it was doubtful whether lower tissue temperatures exploit the full growth potential of these crops when on callus or regenerated shoot stage. Due to the above reasoning, it is quite probable that altering the temperature of the growing chamber from 25 to 28°C significantly increased the vegetative growth of calli and shoots of melon in the current study. Following the optimization of regeneration, due to the high growth rates, there was a large increase in the untransformed callus biomass which was able to escape kanamycin selection. Untransformed parts not only resulted in false positive regenerated shoots but also in outgrowth against the transgenic part of the calli.

The large sizes of calli very often disrupted the normal contact of big parts of the callus with the medium resulting in a) reduced or no selection of the parts that were not coming in contact with the medium b) no nutrient uptake for the transgenic part of the callus inhibiting its growth. The solution for the previous was the implementation of a washing step with a high dosage of kanamycin in order to maximize selection against the non-transformed tissue since early steps of regeneration. At the same time, a high dosage of timetin against remaining agrobacteria and drying with filter paper improved the outgrowth of agrobacteria which was more frequent at 28°C compared to 25°C.

Regeneration of transgenic calli was monitored with GFP fluorescence under a UV stereoscope. Apart from antibiotic washing method the non-transgenic and thus non-fluorescing parts were removed with a scalpel regularly. Nevertheless, the fluorescing parts were usually not able to produce shoot primordia and almost all of them were emerging from the non-transgenic parts. As an exception, two fluorescing shoot primordia were produced from transgenic part however they were unable to grow further despite the treatment with 1, 2 mg/l GA3 or absence of hormones and/or selection. One reason behind the lack of further growth could be a damaged or absent apical meristem. Indeed, when observed under a stereoscope there was no clearly visible apical meristem present in the produced shoot primordia however due to their limited size it might have been difficult to distinguish. In addition to the previous, cucurbits are plants that have a high degree of genetic plasticity and are prone to somaclonal variation, thus they are more likely to undergo genetic changes during the tissue culture process which can result in changes to the plant's growth and development (Moreno & Roig, 1990).

A final factor that could have affected the ability to regenerate transgenic plants is the genotype of the plant material. The genotype of the plants utilized for stable agrobacterium transformation was selected based on their lack of any resistance against PM, DM and ToLCNDV. For this reason landrace melon and cucumber lines that have not been through intensive breeding were taken over in this research. There are various melons or cucumbers used by different researchers. For cucumber, the following genotypes have produced transgenic plants: *C. sativus* cv. Shinhokusei No. 1 (Nasanato et al., 2013), Deltasta, Guonong25, cucumber line 3413, 3407, 3461, Chinese long: 461, 9930, R1407, R470, Xintaimici genotypes (Zhang et al, 2017) , Changchunmici (Zhao et al., 2022), Illan (Chandrasekaran et al., 2016) etc. For melon, CM-23 (Zhang et al., 2016), Charentais (Hooghvorst et al., 2019), Vedrantais (Liu et al., 2022; Giordano et al., 2022) etc. As it can be observed from literature there is no clear preference for a specific genetic background regarding transformation and the selection is made based on the local varieties available each time according to the research group area Contrary to literature evidence, Charentais melon was used for transformation in the current

research however it did not result into transgenic plants even though transformed calli carried the expected CRISPR/Cas9 edits. Given the previous it is quite possible that the genotype plays a minimal role in the production of full transgenic plants compared to other treatments of the transformation and regeneration procedure.

Conclusion

CRISPR/Cas9 genome editing is a cutting edge technology that is used for research and commercial purposes in plant breeding. By inducing targeted mutations in loci associated with susceptibility to plant pathogens it is possible to generate crops resilient to multiple environmental stressors whether biotic or abiotic. Cucurbits suffer from various pathogens including the well-known mildews as well as newly emerged ToLCNDV. For this purpose, the current research attempted to knock out several candidate S- genes in these crops in order to confer them resistant to the aforementioned diseases. Due to the known problematic and inefficient nature of cucurbits stable agrobacterium transformation which is the most straight forward technique for CRIPSR/Cas9 delivery *in planta* it was not possible to obtain fully transgenic/edited plants from transgenic/edited calli. Optimization of the transformation procedure was achieved by a) altering the hormonal fine-tuning of regeneration medium by removing ABA b) increasing the temperature of regeneration to increase the growth rates of calli, c) addition of a washing step with high antibiotic concentrations to improve selection of transgenic tissue. Despite all these alterations regeneration of transgenic shoot primordia still did not result into fully transgenic plants as they were unable to grow further. These results together indicate that more steps regarding treatments of the material should be examined in order to produce fully grown transgenic plants.

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Chapter 8

General Discussion

General Discussion

Plant disease resistance, evolution and crop germplasm

Plants engage in complex interactions with a multitude of biotic factors, which can function in opposition or in synergy with their growth and development (Delaux et al., 2021). Plant pathogenic agents whether they are fungal, bacterial, or viral, exert a negative pressure over the plants which hampers their development and fitness. This pressure imposed by plant pathogenic agents exhibits a selective nature, characterized by varying levels of susceptibility among different plant species and even specific cultivars within a species. Furthermore, within a particular crop, certain pathogenic strains or races may exert a more pronounced impact on specific varieties or genotypes. This selectivity in pathogen pressure plays an important role in driving the continuous co-evolutionary arms-race between plants and pathogens, eventually influencing the genetic diversity and adaptivity observed in both populations (Kumara et al., 2022; Karasov et al., 2014).

Evolutionary processes in plants including gaining resistance or losing susceptibility traits can vary in their natural occurrence speed depending on numerous factors, including, the amount of applied selective pressures (Ueda et al., 2017), mutation rates of species (Bronham et al., 2009) or population sizes (Vandati et al., 2017). Gaining new mechanisms to defend against a pathogen is mainly dependent on the development of the respective resistance (R)- genes that recognize pathogen effectors. This can happen by gene duplication, diversifying selection, and genetic recombination (Jones et al., 2006). If there is existing genetic diversity or the presence of pre-existing R gene variants the acquisition of novel R genes may occur relatively quickly (Shen et al., 2006). However, if the required genetic variation is low or absent, the process of gaining novel R genes may be slower and depend on the occurrence of rare mutations or the introduction of new genetic material through gene flow (Yang et al., 2013). Losing susceptibility to a pathogen can occur through negative selection of susceptibility (S)- genes which includes gene deletions and mutations (van Schie & Takken., 2014). Individuals carrying a particular genetic trait that renders a plant susceptible to a pathogen are more likely to exhibit reduction in fitness and consequently lower probability of survival. Loss of susceptibility, which is recessively inherited, is often considered more durable (van Schie et al., 2014) as typically regards essential plant functions that are less likely to be altered by mutations. On the contrary, specific effector recognition can be disrupted by pathogens more easily by mutations in their corresponding effector.

Loss of susceptibility can have negative consequences for plant fitness under certain conditions due to the crucial functional roles of S-genes in plant physiology (Hückelhoven et al., 2013). An example of a susceptibility gene that, when knocked out, impairs plant growth is the eukaryotic translation initiation factor 4E (*eIF4E*). eIF4E is a host protein that is hijacked by certain plant viruses (Nicaise et al., 2003; Xu et al., 2017; Zlobin et al., 2023) to facilitate their replication and spread within the plant. When the virus binds to eIF4E, it enhances viral translation and replication processes. However, *eIF4E* also plays essential roles in the host plant's normal growth and development (Raabe et al., 2019), independent of viral infection. Knocking out or downregulating *eIF4E* expression can result in impaired plant growth and development due to disruptions in the translation of host mRNAs involved in critical cellular processes (Zafirov et al., 2021). Evolutionary processes, such as natural selection and adaptation, can favour the preservation of gene isoforms that provide advantages in different biological or environmental conditions. Alternative splicing can give rise to mRNA isoforms that encode different protein products or exhibit variations solely in untranslated regions. These isoforms have the potential to influence mRNA stability, localization, or translation, thereby contributing to functional diversity and regulatory complexity within the cell (Stamm et al., 2005). Isoforms may have varying expression patterns, tissue specificity, or interactions with other molecules, allowing them to fulfil specialized roles in specific cellular processes or developmental stages. The presence of multiple gene isoforms provides organisms with increased complexity and flexibility in their molecular repertoire, enabling them to fine-tune biological processes, respond to environmental cues, and adapt to changing conditions (Lopez, 1995). The rich diversity of *eIF4E* isoforms contributes to the complementarity of their roles allowing normal plant development and survival. Consequently, detection of natural mutations in the germplasm of plant species that are associated with resistance against certain viral infections has been previously reported (Ruffel et al., 2002; Wang et al., 2012; Robalia & Caranta, 2006). Due to the previous, the exploration of non-functional susceptibility genes in the germplasm of multiple crops is a promising way to identify durable broad-spectrum resistance that can be incorporated into commercial varieties (Pavan et al., 2010; Gawenhs et al., 2013).

Cucurbit germplasm is a potential treasury of lost susceptibility

The first melon genome was successfully sequenced and annotated in 2012 (Garcia-Mas et al., 2012), marking a significant milestone in melon genomic research. This achievement provided an overview of the genetic composition, organization, and functional elements of the melon genome, enabling researchers to unravel the molecular mechanisms underlying important traits and develop more targeted breeding strategies for melon improvement. In 2020, the publication by Castanera et al. introduced an

improved reference genome for melon, providing an enhanced foundation for melon genomic studies. Subsequently, in 2021, Demirci et al., conducted the re- sequencing of 100 melon genome accessions, forming the basis of current research findings concerning the loss of susceptibility in this crop. These advancements in melon genomic research have contributed to our understanding of melon susceptibility traits, facilitating the development of strategies for generation of disease resistance.

As shown in **chapters 2** and **3** melon possesses various recessive QTLs for resistance against the three most important pathogens, powdery mildew (PM), downy mildew (DM), and *tomato leaf curl New Delhi virus* (ToLCNDV). (Cui et al., 2022, Siskos et al., 2022). Many of these QTLs mapped on a large region of each chromosome. Narrowing down the genomic region associated with the target trait could reduce the list of the candidate genes that are responsible for the traits and therefore lead to a more targeted breeding process.

High impact mutations (frameshift) e.g insertions/deletions in the coding sequence of candidate susceptibility genes can lead to a gene knock-out and a loss of function protein. Moderate impact mutations e.g non-synonymous SNPs, can lead to amino acid changes that can alter the properties of the coded protein rendering it possibly non-functional for the pathogens. The analysis of melon germplasm can provide insights into the identification of causal genes responsible for resistance to certain pathogens. By examining re-sequencing data from 100 CGN melons, we were able to identify numerous mutations in various candidate susceptibility genes associated with PM, DM, and ToLCNDV. Notably, specific mutations that were correlated with resistance against these pathogens shed light on the involvement of these genes in susceptibility (**Chapters 4 & 6**).

From a large number of genes that were examined through SNPeff analysis only a few were found to carry frameshift mutations (e.g *MLO1, TCP14*) while for most genes the moderate impact mutations were abundant across several melon accessions (*PriL, PELOTA, PMR4 etc.*). Moderate impact mutations exhibit a decreased likelihood of leading to the production of non- functional proteins, which suggests that these mutations are less likely to result in the loss of their functional role, either for the pathogen or the plant. As mentioned previously, numerous S-genes play crucial roles in normal plant development and viability. As a result, natural knockouts, or mutations that completely disrupt the function of these genes, are not frequently observed in the germplasm. Moreover, various changes that can occur in *cis*-elements (e.g gene promoters) (Chu et al., 2006; Zaka et al., 2018) of candidate S-genes are also overlooked by focusing exclusively on the coding gene sequences. The prediction

on the effect of these ‘’obscure’’ mutations is of crucial importance for a complete investigation of susceptibility loss in melon germplasm. A representative example considers our findings regarding *PriL* (**Chapter 3**) which although identified only as a moderate impact mutation in multiple melon accessions it had lost its functional role for ToLCNDV. The detection of this loss of susceptibility occurred by examining only a small QTL with a limited number of top candidate genes. However, when a candidate S-gene does not participate in an already mapped QTL region or when the region is too large, detection of key moderate mutations in the germplasm could be quite time consuming and cumbersome.

Nowadays, with the newly emerging advancements on artificial intelligence and machine learning protein functional prediction could be an efficient method to tackle such limitations. AI algorithms can help predict the three-dimensional structure of a protein based on its amino acid sequence (Jumper et al., 2021, Gomes et al., 2022). This is crucial because the structure of a protein determines its function and interactions with other molecules. Accurate prediction of protein structure using AI methodologies can yield valuable insights into the potential effects of moderate mutations on protein folding, stability, and overall functionality. Leveraging machine learning techniques, AI can develop predictive models by capitalizing on existing knowledge regarding protein mutations and their corresponding impacts. These models are trained on comprehensive datasets encompassing information on mutations, protein structures, and associated phenotypic outcomes. Through this training process, AI models can acquire the ability to forecast the influence of specific mutations on protein function. Consequently, these models can be instrumental in evaluating newly identified mutations and offering valuable insights into their potential consequences (Ben-Cohen et al., 2022; Sun et al., 2021).

Finally, in addition to CGN melon lines, multiple watermelon (Guo et al., 2019) and cucumber (Wang et al., 2018) lines part of the U.S. National Plant Germplasm System (NPGS) as well as others (Qi et al., 2013) were fully re-sequenced hereby transforming cucurbits germplasm into a potential treasure trove of susceptibility loci. By exploiting these resources in an efficient way taking into advantage the latest tools of technology and science is pivotal for a thorough exploration of recessive resistance in these crops.

Shedding light onto unknown territories of geminiviral replication

DNA Primase Large Subunit (*PriL*) participates in a major QTL associated with the recessive resistance to ToLCNDV on chromosome 11 of melon (Saez et al., 2017). By delving into the CGN melon germplasm, it has been discovered that *PriL* harbors multiple non-synonymous single nucleotide polymorphisms (SNPs) across multiple accessions. Intriguingly, two mutations were associated with ToLCNDV resistant

phenotypes in melon. The mutation at position 4 (Tyr4His) of the PriL protein, which regards a tyrosine to histidine substitution (Y- H), has been significantly correlated with the most robust resistant phenotypes to ToLCNDV and is the leading candidate as the causal factor of resistance. The second mutation that was associated with an intermediate resistance level was the one at position 125 (Pro125Leu) of the protein and considered a proline to leucine (P-L) change (Siskos et al., 2023).

PriL is a universal primer making tool present in all living organisms (Kuchta et al., 2010). The fundamental role of DNA primase in synthesizing primers for initiating double-stranded DNA synthesis was first elucidated by Scherzinger et al. in 1977, using a bacteriophage model. Subsequently, in the early 1980s, the presence of DNA Primase involved in primer synthesis was observed in various organisms, including the insect *Drosophila* (Conaway et al., 1982), humans (Tseng et al., 1982), and yeast (Plevani et al., 1985). In a study published in 1992, Saunders et al. elucidated a key mechanism underlying the replication of the *African cassava mosaic virus* (ACMV), a member of the begomovirus family. Specifically, it was demonstrated that the process of synthesizing double-stranded DNA from the single-stranded virion initiates with the generation of an RNA primer. By establishing the requirement of an RNA primer for the initiation of double-stranded DNA synthesis, their study unveiled an essential step in the viral replication cycle. Notably, they identified the presence of an RNA primer in close proximity to the hairpin-loop region of the geminiviral genome. However, despite their meticulous investigation, the exact sequence, size, and precise positioning of the RNA primer as well as the origin remained elusive. In spite of the discovery of the RNA primer, Saunders et al. did not suggest a role for DNA Primase, including PriL, for the production of this primer. In a recent study, Wu et al. (2021) made significant advancements in our understanding of geminiviruses, including begomoviruses, by elucidating the roles of different polymerases in their replication processes. Notably, they demonstrated that polymerase α is indispensable for the conversion of the single-stranded circular genome of geminiviruses into a double-stranded form. Today, 30 years after Sanders et al., highlighted the requirement of RNA primer for viral replication, based on our functional and genetic analyses it is proposed that since the ss-DNA genome of the virus lacks a component that makes primers for its DNA replication, geminiviruses utilize host's *PriL*. Furthermore, we suggest that *PriL*, participates in a wider replication complex formed along with DNA Primase Small Subunit (*PriS*) and polymerase α (Pol α). The complex is responsible for the creation of an RNA primer and its subsequent extension from 3' to the 5' of the circular ss-DNA molecule of the virus creating the complementary strand necessary for viral replication (Fig.1). ToLCNDV is sharing similar ways of replicating its genome with other geminiviruses. Therefore *PriL* should have a conserved functional role for these ss-DNA viruses. Indeed, reduced *PriL*

expression led to significant lower titers to ToLCNDV and to two other geminiviruses, TYLCV and BCTV, in *Nicotiana benthamiana* (Siskos *et al*, 2023).

Comprehending the mechanism of *PriL* mediated resistance

Considering the conservation and fundamental role of PriL in DNA replication it is not surprising that any factor hampering PriL functionality or bioavailability has adverse consequences for plant fitness and viability. The proof to the previous is the lack of frameshift mutations in melon germplasm as well as the severe developmental phenotypes upon silencing melon *PriL* specifically impede its functionality against the virus while at the same time securing normal plant development remain unclear. Further investigation is required to elucidate the molecular mechanisms by which the detected alterations affect the interaction between melon PriL and the virus, leading to a potential inhibition of its antiviral activity. The difference between the native plant DNA templates utilizing the replication complex for their replication and geminiviruses lies in the presence of a conserved 11 base pair (bp) long stem- loop structure within the circular viral ss-DNA. As we have shown (Siskos et al., 2023) the stem loop of geminiviruses resembles the replication fork that is generated during the DNA replication process in eukaryotes therefore it is probably the starting point of primer formation initiation. By constructing a 3D model of melon primase initiation we show that the space at the junction of PriS and PriLNTD is a potential site for the docking of the stem-loop upon initiation of primer synthesis on the geminivirus circular DNA. This is because, based on our model, this space is occupied by the 3' of melon's DNA template which is the starting point of primase activity during native DNA replication.

DNA replication in plants as in any other double stranded DNA organism starts by unwinding of the double stranded helix with the help of helicase proteins in the DNA replication origin. In geminiviruses this unwinding during the early geminiviral replication is not necessary since the DNA is already single stranded.. Additionally, during DNA unwinding helicases are known to be followed by topoisomerases which secure structural integrity of the unwound DNA strand on the replication fork (hairpin loop resembles to replication fork) of ds-DNA organisms (Alberts et al., 2002). It is unknown whether factors that are unnecessary for Geminiviruses but still participate in close proximity to the mutated primase can affect indirectly the early replication of the latter.

Based on the previous we consequently hypothesized that either a) the docking of the viral stem loop structure is hampered or limited by the mutations (since they are near to the putative stem loop docking site), or b) other viral or melon protein(s) may participate in the recruitment and holding of geminivirus stem-loop, and the mutations disrupt the interaction of primase with this accessory protein which is a less likely scenario.

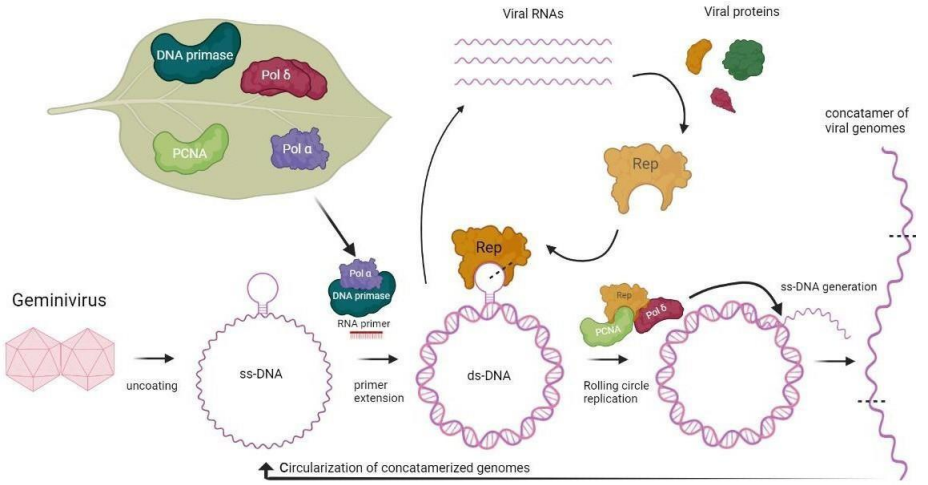

Figure 1. Proposed model for geminiviral replication incorporating current insights on the role of DNA primase. The reproductive cycle of begomoviruses involves a series of orchestrated steps. Upon entering the plant host, the virus uncoats, allowing the single-stranded DNA to enter the nucleus. Inside the nucleus, the single-stranded DNA is converted into double-stranded DNA with the assistance of the plant's Polymerase α (Wu et al., 2021), in conjunction with DNA primase (Siskos et al., 2023). The viral genes present on the double-stranded DNA are then transcribed into mRNA by the host's cellular machinery, resulting in the production of a limited set of six to eight viral proteins (Gong et al., 2021). Viral protein REP protein makes a nick in the double-stranded DNA at the hairpin loop, and starts the so-called rolling circle replication, by recruitment of the host's Polymerase d (Wu et al., 2021). This leads to a concatemer of single- stranded viral genomes. This concatenation of genomes is fragmented into individual single-stranded DNA genomes and circularized. (Figure designed using Biorender.com)

Initially, it is imperative to functionally assess whether the mutation affects the primase activity of melon *PriL* on specific templates. To investigate this, we are currently performing primer synthesis assays (Baranovskiy et al., 2016) which allow the detection and quantification of newly synthesized primers by the primosome complex. By *in vitro* synthesis and purification of melon's wild-type (WT) and (Tyr4His)/(Pro125Leu)-mutant PriL proteins along with the Pol *α* (POLA1 and POLA2) and PriS proteins we will evaluate their respective primase activities on two templates: a) the original template, which contains the hairpin loop structure simulating the interaction, and a b) modified template where the hairpin structure has been deliberately disrupted. This comparative analysis will provide valuable insights into how the mutations influence the primase functionality of melon PriL in the presence and absence of the hairpin structure. In addition to this, it is interesting to know which part of the viral genome is primed by the primase. Sequencing of the total RNA from a plant that has been infected with ToLCNDV at different time points and mapping the reads to viral genome by focusing on detection of small reads that are close to the hairpin could be useful. An

ideal plant for this approach would be *Nicotiana benthamiana* since it is a quite good host for the virus and is a model plant for transient assays. However, sequencing RNA from the mentioned *in vitro* assay looks even more promising, also when using whole, circular ss-DNA viral genomes as templates.

Arabidopsis is also a model plant that is widely utilized for gene functional studies. Moreover, it has also been at the forefront of utilizing CRISPR-Cas9 technology for precise genome editing and could be an ideal system for studying the impact of *PriL* mutations (Puchta & Fauser, 2014). CRISPR-Cas constructs designed to induce targeted disruptions in the native *PriL* gene (AT1G67320) of *A. thaliana*. Each construct could simultaneously carry a homologue version of *PriL* from melon, with the specific mutations. The transgenic Arabidopsis plants can be later subjected to inoculation with an infectious clone of the *cabbage leaf curl virus* (CaLCuV), a begomovirus known to infect Arabidopsis (Trejo-Saavedra et al., 2009). Subsequently, the viral titers can be assessed using quantitative polymerase chain reaction (qPCR) analysis at the DNA level, comparing the levels of viral DNA in both inoculated and non-inoculated seedlings of both wildtype and transgenic lines.

An alternative and potentially more expedient strategy would involve complementing melon CGN accessions harboring one or both identified mutations with the wildtype (WT) allele. Subsequently, these transgenic plants could be subjected to ToLCNDV inoculation, allowing for the assessment of potential restoration of susceptibility and measurement of viral titers. However, it is important to note that the challenges associated with stable transformation of melon limit the efficiency of this approach.

Profound understanding of the fundamental structural and functional consequences of these changes will provide valuable insights into the intricate molecular interplay between *PriL* and geminiviruses, ultimately contributing to a comprehensive understanding of plant-virus interactions.

Most of the above mentioned experiments are currently conducted in a new TKI project, called PRIMASE.

Nature shows the way: Prospects for cross species plant resistance

The DNA primase is a ubiquitous component involved in DNA replication across various organisms, including plants (Frick et al., 2001). Given its essential role in fundamental cellular processes, complete functional inhibition of this subunit for conferring resistance is probably not feasible as it leads to lethality. However, it is possible to modify the subunit in a manner that allows it to retain its functional role in

plant cells while rendering it ineffective for the virus, as demonstrated in melon. Natural variability is one of the best sources of information regarding the effect of mutations for the host and the pathogen. Natural mutations found in plant germplasm represent the optimal version of each gene for plant fitness in a certain environment at a certain time, as all other versions were lost though negative selection. Consequently, melon *PriL* was naturally altered in such a way that is not exploitable by ToLCNDV but still functional for host cellular processes. Melon is a host for geminiviruses and subject to their selective pressure for thousands of years. Interestingly, the geographical origin of melon aligns with the region where geminiviruses, including those of the begomovirus genus, are believed to have originated. This region is Southeast Asia (Nawaz-ul-Rehman et al., 2009; Qi et al., 2013, Chomicki et al., 2020). The overlapping geographic origins of melon and geminiviruses in Southeast Asia suggest a potential long-standing coexistence and coevolutionary relationship between the crop and the viral pathogens in this particular geographical region. Therefore, it is logical that beneficial mutations especially in very basic host factors that are most commonly hijacked by the pathogen are modified overtime until there is a sufficient balance on this selective pressure. In cases where susceptibility factors are highly conserved and essential for basic cellular functions, the options for modifications are further constrained. The more fundamental the susceptibility factor, the fewer changes can be made without compromising the plant's overall fitness and functionality. Any alterations must strike a delicate balance between reducing susceptibility to the pathogen and maintaining the functionality of the susceptibility factor within the plant, however the large co-evolutionary time between plant and virus has probably allowed melon a significant evolutionary advantage.

The investigation of analogous changes in other crops is of great interest. To facilitate this exploration, it is important to initially identify plant species that have co-evolved with geminiviruses and share a common place of origin. Subsequently, the germplasm of these species should be thoroughly examined for mutations in the DNA primase gene. This assessment should be complemented with functional studies focusing on the protein domains and disease assays to discern the mutations that effectively contribute to disease resistance. Documenting these changes and their impact on DNA primase is valuable for predicting the potential effects of genome editing techniques, such as single base pair editing. This information can be used to guide the generation of resistance against geminiviruses in crops that have not undergone sufficient evolutionary processes to naturally develop a resistant primase allele.

This comprehensive approach, which integrates insights from evolutionary biology, germplasm analysis, functional studies, and predictive genomics, holds promise for enhancing disease resistance in various crops beyond cucurbits.

DNA primase and CRESS viruses: from single cell organisms to higher eukaryotes

The CRESS (Circular Rep-Encoding Single-Stranded) virus group represents a diverse assemblage of viruses characterized by several distinctive features. These include circular single-stranded DNA (ssDNA) genomes, the presence of Rep-encoding genes, and replication via rolling circle replication (RCR) (Zhao et al., 2021; Rosario et al., 2015). Within this taxonomic group, notable members include geminiviruses and nanoviruses, which encompass known plant pathogenic species with significant economic importance (Zhao et al., 2019). While circular ssDNA viruses have been associated primarily with plant viruses, it is noteworthy that they possess the ability to infect a wide range of organisms beyond plants (Malathi & Devi, 2019). This expanded host range includes diatoms, archaea, fungi, birds, and mammals (Zhao et al., 2019). Of particular relevance to mammals are circoviruses, which have been implicated in globally occurring diseases such as porcine circovirus-associated disease (PCVAD) in pigs (Gillepsie et al., 2009) and psittacine beak and feather disease (PBFD) in birds (Raidal et al., 2015). It is important to highlight that CRESS viruses themselves do not encode DNA primases, the enzymes responsible for initiating DNA synthesis during replication. Instead, it is hypothesized that CRESS viruses rely on host primases to facilitate the conversion of their circular ssDNA genomes into double-stranded replicative intermediates. In-depth exploration of the functional role of DNA primases in geminiviral replication can therefore yield valuable insights applicable to a broader understanding of viral replication, including the replication strategies employed by other CRESS viruses. Such knowledge not only expands our comprehension of viral replication mechanisms but also informs the development of antiviral strategies and the management of viral infections across diverse organisms.

The universal potential of *PELOTA* in resistance against geminiviruses

Geminiviruses, like other viral plant pathogens, have the ability to exploit host factors in a manner that aligns with the requirements of different infection stages. Throughout the course of infection, these viruses may interact with and manipulate various host factors to facilitate their own replication, movement, and evasion of host defense mechanisms (Hanley-Bowdoin et al., 2013). The adaptability of geminiviruses to harness host factors in a stage-specific manner enables them to effectively exploit and subvert host cellular processes, contributing to their successful establishment and dissemination within plant populations (Ferreira et al., 2021). Host factors that determine viral susceptibility can be categorized based on their roles in various aspects of the viral lifecycle. These factors encompass: a) facilitation of viral translation (Machado et al., 2017), b) promotion of viral replication (Mäkinen, & Hafrén, 2014; Mine et al., 2012; Pogany et al., 2008; Wang et al., 2008), c) facilitation of viral movement (Carluccio

et al., 2014), d) modulation of host antiviral gene silencing mechanisms (Cheng et al., 2016), e) involvement in virion assembly and disassembly as well as others less direct mechanisms (Garcia Ruiz, 2018).

PELOTA emerged as a crucial plant component in the intricate interplay between solanaceous crops and *Tomato yellow leaf curl virus* (TYLCV) as well as other geminiviruses. Specifically, the discovery of a single amino acid mutation in the tomato germplasm (Lapidot et al., 2015), leading to resistance against the virus, marked the initiation of investigations into the functional role of the PELOTA gene in plant virus susceptibility as well as its potential beyond the tomato- TYLCV pathosystem. TYLCV is a highly detrimental geminiviral pathogen with a global impact on solanaceous plants such as tomato and pepper (Morilla et al., 2005; Kil et al., 2016). The precise mechanism by which the pelota gene affects viral susceptibility is still unclear, but it is hypothesized to involve the regulation of viral RNA transcription and replication processes given the role of the protein in mRNA surveillance (Koeda et a., 2021; Ren et al., 2022).

Currently it is known that the functional role of PELOTA in geminiviral susceptibility is not limited only to tomato-TYLCV pathosystem, but it considers a wider range of geminiviruses that affect solanaceous crops (Ren et al., 2022). In addition to viruses, interestingly, PELOTA mutants have also been shown to indirectly confer bacterial blight resistance in rice by differential activation of immune responses (Zhang et al., 2017).

Until recently, geminiviruses were not regarded as major pathogens for cucurbit crops in Europe. However, the emergence of *tomato leaf curl New Delhi virus* (ToLCNDV) in the Mediterranean region (Moriones et al., 2017; Desbiez et al., 2021; Fortes et al.,2016; Juarez et al., 2014; Orfanidou et al., 2019) has led to a growing concern, particularly in melon cultivation. ToLCNDV epidemics in Spain have specifically posed a considerable threat to melon production. In addition to reducing plant fitness, ToLCNDV infection causes fruit cracking, rendering them unsuitable for market and more susceptible to secondary infections. The involvement of PELOTA, a protein associated with geminiviral resistance, in the proliferation of ToLCNDV is currently unknown. Nevertheless, considering the broad-spectrum resistance conferred by *PELOTA*, particularly the *ty-5* resistance gene, against this group of viruses (Ren et al., 2022), it is plausible that ToLCNDV utilizes *PELOTA* for its propagation. Another intriguing aspect to consider is whether the melon *PELOTA* gene is among the host factors that contribute to geminivirus susceptibility, as previously observed for solanaceous crops like tomato and pepper. Therefore, we hypothesize that if melon's *PELOTA* promotes susceptibility to geminiviruses, including ToLCNDV, it would be an interesting target gene for developing resistant cucurbit varieties through breeding

strategies. An additional significant aspect of *PELOTA*-based resistance lies in its relevance to mixed infections involving TYLCV and ToLCNDV in tomato. TYLCV, a major tomato pathogen, is a geminivirus that relies on the functional role of PELOTA for its efficient proliferation and is often found in mixed infections with ToLCNDV which promote the proliferation of the latter (Vo et al., 2022). The above underscores the importance of this gene as a potential target for generating resistance in cucurbits. Alterations in *PELOTA* can disrupt the interactions between TYLCV and the host, thus limiting the proliferation also of ToLCNDV.

In **Chapter 4** of this thesis, we present functional evidence demonstrating that the *PELOTA* homologue derived from a non-solanaceous species, specifically melon, indeed exerts a notable influence in promoting susceptibility to TYLCV in tomato. This implies a wider role of this gene in susceptibility to geminiviruses beyond Solanaceae. The recessive resistance locus *ty-5* in tomato, which confers resistance against TYLCV, is attributed to a specific mutation involving the substitution of Valine with Glycine. This amino acid substitution occurs within a highly conserved region of the tomato PELOTA protein. Our research demonstrates that the Valine residue participates inside a conserved motif known as GSVK, which is located in close proximity to the *N*-terminus of the tomato PELOTA protein. Remarkably, the GSVK motif is not confined to PELOTA proteins found exclusively in Solanaceous crops. Instead, it is situated in the same position across protein homologues of various plant families. This high degree of conservation suggests that the motif has been maintained throughout evolutionary history due to its functional significance and potential contributions to plant fitness. Moreover, the evolutionary implications of such widespread conservation are intriguing, as they hint at the possibility of the virus having developed an established adaptation to this host factor over an extended period of time. It is interesting to know how mutations in such a conserved domain of the protein like the GSVK do not impact plant fitness maintaining the protein functionality for the plant.

The functional significance of the GSVK motif in PELOTA for the plant remains elusive, necessitating further investigation to unravel its role in protein functionality. While it is true that the GSVK motif has been associated with resistance against TYLCV in tomato, it is important to note that not all geminivirus PELOTA-based susceptibility can be solely attributed to this specific motif. Recent studies, such as the work conducted by Koeda et al. (2021), have reported the presence of natural mutations in the PELOTA protein (in areas not including the motif) of other plant species, such as pepper, which were associated with resistance against geminiviruses like *pepper yellow leaf curl Indonesia virus* PepYLCIV and *pepper yellow leaf curl Aceh virus* PepYLCAV. These findings imply that geminivirus susceptibility involves mechanisms beyond the GSVK motif alone and there could be

a broader adaptation of these viruses to this host factor. Given the fact that PELOTA might be a crucial gene for plant development and viability functional studies involving knocking out of the gene could result into severe developmental defects similar to other species (Adham et al., 2003; Ding et al., 2018) It is also important to highlight that the indirect impact of these mutations on the virus, mediated through other factors, cannot be disregarded. PELOTA's involvement in various physiological pathways, including defense responses, suggests that mutations in this protein have the potential to influence pathogen susceptibility through indirect mechanisms or through interaction with other host factors. A notable example comes from research in rice (Zhang et al., 2018), where a single substitution mutation in the native PELOTA has been demonstrated to confer resistance against bacterial blight. This resistance is achieved through the activation of the salicylic acid pathway, highlighting the interconnected nature of *PELOTA's* role in distinct plant physiological processes, including defense signaling.

PELOTA has undergone modifications in tomato and pepper germplasm, resulting in conferred resistance to geminiviruses while maintaining its plant functionality. In the CGN melon germplasm, no frameshift mutations were detected in PELOTA. However, a single moderate impact mutation was identified in multiple accessions, with an unknown effect on resistance. Notably, the GSVK motif remained unaltered in all melon accessions. Considering the predicted implications of the V-G amino acid change in the interaction between the host and geminiviruses, CRISPR/Cas9 single base genome editing holds promise as a valuable tool for investigating the effects of this change in melon and for generating resistant cucurbit varieties. Nonetheless, the arduous agrobacterium-mediated transformation procedure specific to cucurbits as seen in **chapter 7** poses challenges and constitutes this approach less appealing. As a second approach, it would be interesting to examine the status of the motif in various cucurbit germplasms and even more melon collections in order to detect potential natural resistance to geminiviruses similar to the *ty-5* locus. Eventually, based on the above, *PELOTA* is a promising candidate for ToLCNDV susceptibility in melon and possesses immense potential for generating durable resistance against the virus in cucurbits.

Could *PELOTA* determine host specificity for geminiviruses?

Viral host specificity refers to the ability of a particular virus to infect and replicate within specific host plant species or plant families. Broad host ranges in viruses may be maintained through selection in environments that are diverse. In such environments, viruses encounter a variety of host plants, each with different genetic backgrounds and defense mechanisms (McLeish et al., 2018). This diversity provides an opportunity for viruses to evolve and adapt to infect multiple host species. Viruses often exhibit a narrow host range, infecting only a limited number of closely related plant species,

e.g. tobacco mosaic virus (TMV) (Knapp et al., 2011), however others can infect a broader range of plants, e.g. cucumber mosaic virus (CMV) (Bhat & Rhao, 2020). Host specificity is determined by numerous factors, including the interaction between viral proteins and host plant receptors, as well as the compatibility of the viral replication machinery with the host cellular machinery (Garcia-Ruiz, 2018).

The Geminiviridae family comprises a diverse group of plant viruses that infect a wide range of host plants. Geminiviruses are characterized by their small, circular, single-stranded DNA genomes (Hanley-Bowdoin et al., 2013). Geminiviruses are classified into several genera with the most diverse one being *Begomovirus*. The *Begomovirus* genus includes viruses transmitted by whiteflies and primarily infects dicotyledonous plants. Within the *Begomovirus* genus, there are numerous viral species such as TYLCV, ToLCNDV and others (Fiallo-Olive & Castillo, 2023). The *Curtovirus* genus, also belonging to *Geminiviridae* includes on the other hand, viruses transmitted by leafhoppers with the most well-known species in this genus being BCTV, which causes considerable damage to sugar beets and other related crops (Luna et al., 2020). Previous studies have demonstrated that the *ty-5* locus is capable of providing complete resistance against multiple begomoviral species. However, when it comes to the *Curtovirus* BCTV, the resistance conferred by the *ty-5* locus is only partial (Ren et al., 2022). The previous could imply a differential mediation of *PELOTA* in susceptibility among different geminiviral genera. Interestingly, the melon *PELOTA* was not able to restore susceptibility to BCTV in *ty- 5* background tomato. There is speculation that the inability of BCTV to effectively multiply in *ty-5* tomato plants, which express a functional *PELOTA* homologue derived from melon, could be linked to the virus's poor adaptation in cucurbits in comparison to solanaceous plants. Consequently, this limited adaptation may indicate a lack of compatibility with native host susceptibility factors such as *PELOTA* and therefore an inability to utilize them. Investigating the potential role of *PELOTA* in determining host specificity of *Beet curly top virus* (BCTV) in cucurbits would be interesting for studying the evolutionary role of this gene in geminiviral host adaptability. Finally, to address this, an experimental approach could involve the overexpression of the *PELOTA* gene from a primary BCTV host in melon plants, with the goal of restoring compatibility between the virus and melon.

The first report of *MLO1* natural loss of function in cucurbits confers partial resistance to PM in melon

Powdery mildew (PM) susceptibility is tightly linked to the functional presence of *MLO* genes (Acevedo-Garcia et al., 2014; Jacot et al., 2021). Mutations in *MLO* genes have been identified in various plant species (Kusch et al., 2017), including specific cucurbit crops (Tek et al., 2022; Berg et al., 2015), providing resistance against powdery mildew

pathogens. *MLO* genes are typically abundant in plants, with each species possessing 10-15 homologues that encode proteins characterized by seven hydrophobic transmembrane domains, three extracellular loops and *N*-terminus, and three intracellular loops and *C*-terminus containing a calmodulin binding domain. In addition to their role in disease susceptibility, *MLO* genes serve essential developmental functions in plants, such as root thigmomorphogenesis (Bitzinski et al., 2014), fertilization (Kessler et al., 2010), colonization by arbuscular mycorrhizal fungi (Jacot et al., 2020; Hilbert et al., 2020), and responses to biotic stress through the regulation of calcium signaling (Kim et al., 2002). Phylogenetic analysis has classified MLO proteins into six distinct clades (Devoto et al., 2003). Clades IV and V, associated with PM susceptibility in monocot and dicot species, respectively (Ioveno et al., 2015), exhibit distinct molecular traits (Appiano et al., 2015) due to their evolutionary divergence.

In cucurbits, the study of *MLO* genes has been carried out primarily in cucumber where three Clade V homologues are reported to contribute to PM susceptibility, *MLO1*, *MLO8* and *MLO11* (Berg et al., 2017). Specifically, *MLO8* is the major gene providing susceptibility in cucumber and has been found naturally mutated in cucumber and melon PM resistant germplasm (Berg et al., 2015; Zhang et al., 2023). Functional studies utilizing genome editing have indicated that *MLO8* participates in cucumber's pre-invasive defenses against PM, while *MLO1* and *MLO11* are involved in post-invasive defense (Tek et al., 2022). In our study (**chapter 6**), *MLO1* homologue was found naturally mutated in CGN melon germplasm carrying a two-nucleotide insertion that leads to a frameshift. Subsequent disease assays with PM race 3.5+ showed a partial PM resistance that co-segregated with the mutation in the F2 population. Intriguingly, resistance detected was indeed associated with a putatively post-invasive effect as the colonization of the mildew initiated relatively normal in the mutants. However, the colonies were unable to develop, and they were fading overtime leaving a chlorotic spot on the leaf. An intriguing approach would involve the implementation of quantitative polymerase chain reaction (qPCR) assays to accurately quantify powdery mildew (PM) infection and assess resistance in a more reliable manner. By using qPCR, the abundance of PM pathogens can be measured, providing quantitative data on the level of resistance exhibited by the host plants. This method offers a robust and precise means of evaluating resistance against PM. In addition to qPCR, the incorporation of histological assays would provide valuable insights into the underlying mechanisms of resistance during the infection process. Histological techniques, such as microscopy and staining methods, enable the visualization of cellular and tissue-level responses to PM infection. These assays allow for the examination of structural changes, cellular defense reactions, and the localization of PM pathogens within the host tissues. Consequently, histological

analysis provides a deeper understanding of the specific interactions between the host plant and the PM pathogen, shedding light on the mechanisms underlying resistance.

This discovery marks the first identification of a natural loss-of-function mutation in the *MLO1* gene in cucurbits, following the previously reported natural PM-resistant mutants in cucumber (Nie et al., 2015; Berg et al., 2015) and melon (Hong et al., 2015) associated with the *MLO8* gene. The presence of this mutated locus, along with the associated mutated *MLO* gene variants such as *mlo8*, holds significant potential as valuable genetic resources for crop improvement strategies. A promising avenue for future research would involve conducting extensive screenings of melon germplasm to identify natural mutations in the third clade V homologue, *MLO11*. Identifying additional natural mutations in MLO genes could contribute to the development of a triple mutant hybrid with enhanced and durable resistance against PM. These genetic resources could be effectively utilized in breeding programs to introgress the desired traits into elite cultivars, thereby improving their resistance to PM. By leveraging multiple sources of resistance, including the mutated locus and other known resistance genes, breeders can achieve a broader and more durable resistance against PM in melon.

Agrobacterium mediated transformation is limiting high-throughput CRISPR/Cas9 genome editing in cucurbit crops

CRISPR/Cas9 genome editing has emerged as a powerful tool for conducting gene functional studies and generating disease resistant crops by targeting plant susceptibility genes since its widespread adoption in the scientific community over the past decade (Jiang et al., 2013; Jiang et al., 2014). This revolutionary technique allows precise modifications to the DNA sequence of plants, facilitating targeted investigations into the functions of specific genes and their involvement in plant susceptibility to various pathogens (van Schie & Takken, 2014). The most prevalent method utilized in crop genome editing for the aforementioned objectives involves gene knockouts. This technique aims to disrupt the function of a specific gene by introducing targeted mutations into its DNA sequence. With CRISPR/Cas9, the Cas9 protein, complexed with a guide RNA (gRNA), scans the genome until it identifies a DNA sequence that matches the gRNA's protospacer sequence. Once the target site is recognized, the Cas9 protein induces a double strand break at that precise location. Subsequently, the cell's innate DNA repair mechanisms are activated in response to the double-strand break. The primary repair pathways involved are non-homologous end joining (NHEJ) and homology-directed repair (HDR). NHEJ is the more commonly employed pathway, frequently resulting in the insertion or deletion of nucleotides at the break site. These modifications can cause frameshift mutations, disrupting the gene's reading frame and rendering it non-functional (Jaganathan et al., 2018; Borelli et al., 2018). Alternatively,

genome re-writing through homology-directed repair (HDR) or base editing techniques can offer an alternative strategy to mitigate host manipulation by modifying the targets of pathogen-derived effectors or molecules in a manner that evades recognition. (Schenke & Cai, 2020). *Agrobacterium*-mediated transformation serves as the primary approach for integrating the CRISPR/Cas9 editing machinery into plant cells to induce heritable mutations (Xu et al., 2014; Zhang et al., 2018; Pan et al., 2017; Yu et al., 2017). This technique involves the use of a vector containing the CRISPR/Cas9 components, transformation of *Agrobacterium tumefaciens* with theses vectors, co-cultivation of *A. tumefaciens* with plant tissues, selection, and regeneration of transformed plants as well as subsequent analysis and validation of the edited plants.

The research presented in **chapter 7** focused on the utilization of Agrobacterium-mediated transformation in cucurbits to achieve heritable loss of susceptibility in cucumber and melon. The study initiated by selecting several candidate gene homologues from melon and cucumber, which were previously identified as contributors to susceptibility to PM, DM, and geminiviral infections in other plant species. The CRISPR/Cas9 system was employed to target specific exonic regions in order to knock out candidate susceptibility loci. The selected protocol for this study (Nasanato et al., 2013) was chosen due to its notable transformation efficiency of 12% in cucumber. This is particularly appealing considering that the transformation efficiency in cucumber is generally low, typically ranging from 0.1% to 5% (Liu et al., 2023). Similarly, in melon, the efficiency of agrobacterium-mediated transformation follows similar trends, with reported efficiencies ranging between 0.7% and 3% in various studies (Don et al., 1991; Bordas et al., 1997; Guis et al., Yalcin-Mendi et al., 2004). However, in certain cases, the transformation efficiency in melon can reach up to 12% (Nunez-Palenius et al., 2006). The protocol of Nasanato et al., included vacuum infiltration of agrobacteria which could potentially maximize the amount of agrobacteria entering the cotyledons and therefore increase the amount of transformed tissue in the cotyledon. Despite multiple efforts which included hundreds of cotyledon explants from cucumber and melon, obtaining fully transgenic plants was not possible in our hands.

Three main inhibiting factors contributed to the inability of obtaining transgenic plants:
a) the initial production of shoots instead of roots from transgenic calli and the limited callus regeneration; b) the large amounts of regenerating tissue escaping antibiotic selection; and c) the inability of the transgenic tissue to regenerate shoots that would end up in full transgenic plants. The first factor was overcome by removing the ABA from the regeneration medium while maintaining the BAP concentrations intact. ABA removal altered the hormonal fine- tuning of our melon and cucumber cotyledons and promoted shooting instead of rooting in the calli. At the same time increasing

the temperature of regeneration to 28°C increased dramatically the regeneration of shoots and calli. The next problem that we faced was the large outgrowth of non-transgenic tissue escaping kanamycin which was partly solved by implementing a high dosage antibiotic treatment prior to transferring the tissue into regeneration medium. Additionally, using GFP fluorescence incorporated in our CRISPR/CAS9 constructs we removed the non-fluorescing parts of the calli. After the optimization of the aforementioned steps, we managed to regenerate transgenic shoots from fluorescing calli, however these shoots were never able to grow into full plants. By sequencing the fluorescing calli we detected various CRISPR/Cas9 induced deletions in the exonic region of the targeted susceptibility genes in melon showing a good efficacy of our generated constructs to mutate the plant's genome.

Our study is not unique in observing the challenges associated with regenerating fully transgenic melon plants from transgenic calli. Chovelon et al. (2011) also acknowledged the same difficulties encountered during this stage. The researchers connected the recalcitrance to the disruption of melon's meristematic structures caused by the exposure of explants to Agrobacterium during the stable cotyledon transformation process. Moreover, Chovelon et al. proposed that the aggressiveness of the Agrobacterium strain used is a crucial factor influencing this phenomenon. They suggested that utilizing new strains with reduced aggressiveness towards melon explants could improve the regeneration from transgenic calli. Confirming the findings of Chovelon et al., our study also observed that the regenerated transgenic shoots lacked apical meristems, which renders it inherently impossible to regenerate full plants from them. This emphasizes the need to address the disruption of meristematic structures caused by Agrobacterium and highlights the importance of improving transformation techniques. Specifically, the successful transformation of cucumber and melon has been reported by several research groups. However, these studies often employ unique and not reproducible materials and methods. To enhance transformation success, various practical treatments and specific combinations of Agrobacterium-melon/cucumber lines have been explored. For instance, Nasanato et al. (2013) demonstrated that certain practical factors, such as the number of filter papers used in the co-cultivation medium, significantly influence the humidity maintained inside the petri dish during co-cultivation. This, in turn, affects the aggressiveness of the bacteria on melon cotyledons and transformation efficiency. Overall, achieving an efficient method for producing transgenic plants requires careful fine-tuning of multiple factor interplay. These include optimizing the interaction between Agrobacterium strains and melon explants, as well as implementing precise practical treatments during the transformation process. By addressing these challenges, researchers could enhance the efficiency and reproducibility of melon transformation and enable the production of transgenic plants.

In conclusion, there is an increasing emerging literature on CRISPR/Cas9 published over the last years utilizing agrobacterium mediated transformation in cucurbits to confer resistance against pathogens despite the low efficiencies of the method (Chandrasekaran et al 2016; Fidan et al., 2023; Zhang et al., 2020). In our opinion, optimization of this method could lead to similar results in our hands. However, the sustainability of utilizing this method for high throughput functional screening of multiple candidate susceptibility genes in cucurbits, considering reported transformation and regeneration efficiencies is discouraging.

Concluding remarks

Currently, the European melon and cucumber cultivation faces major challenges due to prevalent diseases such as PM, DM, and ToLCNDV. To combat these issues effectively, it is imperative to implement novel, durable, and broad-spectrum resistance strategies in commercial varieties. The co-evolutionary relationship between cucurbits and the aforementioned pathogens has contributed to the shaping of their genomes, resulting in a combination of valuable resistance traits and susceptibility-related traits. Our research aimed to elucidate the specific genetic factors that render cucurbits susceptible to these diseases. Through investigations of the available melon germplasm, we aimed to identify loss-of-function susceptibility gene mutants.

By examining natural variability valuable insights could be extracted considering how geminiviruses, an important group of plant pathogenic viruses including ToLCNDV, use host plant cell machinery to replicate their genome. After fine mapping of a known melon recessive resistant QTL for ToLCNDV and sequencing of parental genomes we concluded that *PriL* is the top candidate gene for the resistance. Moreover, considering that multiple ToLCNDV resistant melon accessions were carrying mutations on *PriL* gene which were correlated with the resistance, the role of this gene in viral infection was further investigated. *PriL* is universal DNA primer making tool for the replication of DNA in all living organisms. This gene is utilized by ToLCNDV which lacks such a gene for replicating its DNA. Interestingly, it was hypothesized that the mutations found in *PriL* negatively affect the priming of the viral ss-DNA replication of ToLCNDV and potentially of all geminiviruses. The potential of these findings on the CRESS virus replication, a larger viral group that apart from geminiviruses includes farm animal pathogenic viruses too, is worth exploring.

Another significant insight on geminivuses considers *PELOTA* which was previously shown to contribute to TYLCV and other geminiviruses susceptibility in Solanaceae. Using functional assays we propose a broader participation of this gene in susceptibility, beyond Solanaceae and for the first time, in cucurbit crops. Moreover, by examining the

PELOTA protein sequences of multiple plant families we show a universally conserved motif, GSVK, that holds promise for generating recessive resistance against multiple geminiviruses in any plant species without plant fitness costs.

Apart from viral pathogen resistance, powdery mildew recessive resistance was identified in melon germplasm. A Clade V MLO gene, *MLO1*, which was previously shown to contribute to PM susceptibility in cucumber carried a frameshift mutation in a CGN melon accession. The mutation co-segregated with recessive partial resistance to PM race 3.5+ in a way that supports the speculated post-invasive role of this gene in susceptibility. The resistance could be used in combination with the major *mlo8*-based resistance to improve its overall spectrum and durability.

Finally, we propose ways to improve the notoriously cumbersome agrobacterium mediated transformation of melon and cucumber for CRISPR-Cas9 genome editing in order to knockout susceptibility genes. We acknowledge the need of measures during transformation that will prevent the meristematic damage of transformed tissue in order to obtain full transgenic/edited melon plants and we underline CRISPR/Cas9 limitations for a high throughput research in cucurbits.

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Appendices

Summary

Cucurbit crops are cultivated worldwide primarily for their fruits, which serve as a rich source of essential vitamins and nutrients, making them integral components of a well-balanced diet. Cucurbits are susceptible to various diseases, which can significantly impact their growth, yield, and overall quality. Given the current epidemiological situation in European melon and cucumber cultivation, there is a pressing need to develop novel, broad-spectrum, and durable resistance against three major diseases, namely powdery mildew (PM), downy mildew (DM), and *tomato leaf curl New Delhi virus* (ToLCNDV). Since dominant resistance is often specific and quickly overcome by pathogens the efforts were focused on investigating the potential of recessive resistance in these crops which is known to be less pathogen specific and more long lasting under pathogen pressure. By combining genomic and functional approaches we present new insights on generating resilient crops to the aforementioned pathogens.

In **chapter 1,** we present a brief historical description of the impact of agricultural research and plant breeding on human civilization. Furthermore, by acknowledging the latest progress in research and technology we highlight promising directions of agricultural research based on the increasing needs for food production. Eventually, the emphasis is centred on disease resistance breeding in cucurbit crops as they constitute an important asset of agriculture and human nutrition that suffers from old and newly emerging diseases which have a negative impact on quality and yield.

In **chapter 2**, a thorough exploration of available knowledge on the classic cucurbit pathogens PM and DM, is carried out, in an effort to comprehend certain ecological and molecular aspects of these diseases as well as to report all currently available mapped resistances in wild and cultivated melon. In this chapter we have summarized the outcomes of mildew resistance identification over the past 50 years or more, highlighting key findings from various publications. More specifically, we show that PM resistance has been identified in 239 melon accessions and downy mildew resistance in 452 accessions of both *Cucumis melo* and its wild relative, *C. figarei*. Through genetic mapping studies conducted on several resistant sources, 25 dominant genes, two recessive genes, and seven QTLs conferring PM resistance have been identified over the years. Similarly, eight dominant genes and 11 QTLs associated with DM resistance have also been reported. By aligning reported marker sequences with the chromosomes of the melon cv. DHL92 genome, we have successfully established a physical anchoring of a considerable number of these resistant loci on their respective chromosomes. Finally, we propose strategies to breed melon varieties with durable and broad-spectrum PM and/or DM resistance.

In **chapter 3**, we present an overview regarding all available knowledge on ToLCNDV, a new viral pathogen that threatens European melon cultivation. Since its initial introduction in the Mediterranean region, the virus has caused substantial economic losses, including reduced yields and the production of non-marketable fruits, often displaying cracks. Given the absence of chemical treatments against viral pathogens, the primary focus has shifted towards resistance breeding. We provide an overview of the most recent advancements in melon breeding for ToLCNDV resistance and summarize all relevant loci documented thus far in cucurbits. To pave the way for future developments, we propose an alternative approach to traditional breeding methods by leveraging knowledge on genes that confer susceptibility to the virus in melon and other cucurbits.

In **chapter 4**, we conducted sequencing of the melon accession K18, which carries the quantitative trait locus (QTL) for recessive resistance to ToLCNDV on chromosome 11. Furthermore, we analysed DNA sequence data from 100 CGN melon genomes. Our findings revealed a conserved mutation in the *DNA Primase Large subunit* (*PriL*) of all resistant accessions challenged with ToLCNDV. Additionally, through silencing experiments involving the native *Nicotiana benthamiana PriL* gene and subsequent challenges with three different geminiviruses, we observed a significant reduction in viral titers for all three viruses, suggesting a crucial role of *PriL* in geminiviral replication. Geminiviruses replicate their genetic material within the nucleus of host plant cells by exploiting the DNA replication machinery of the host. Although they rely on host DNA polymerases for the conversion of their DNA into double- stranded form and subsequent replication, the precise mechanism underlying the initial step of this process—the conversion of incoming circular ssDNA into double-stranded DNA—has remained elusive for nearly three decades. In this chapter, we propose a model elucidating the putative role of *PriL* during the initiation of geminiviral DNA replication, wherein it serves as a regulatory subunit of the primase complex, generating an RNA primer at the onset of DNA replication, analogous to the DNA Primase- mediated initiation observed in other organisms.

In **chapter 5,** we investigated whether the melon *PELOTA* gene, a homolog of the pelota gene of tomato which has been shown to contribute to geminiviral susceptibility, serves a respective functional role. It is known that a certain single nucleotide polymorphism (SNP) in *PELOTA* of tomato (*ty-5* recessive resistance locus) can confer plants resistant against multiple geminiviruses . In this research, we demonstrate that the introduction of the functional melon *PELOTA* in a *ty-5* background tomato restores susceptibility to the geminiviruses TYLCV, as evidenced by viral titers and symptom development.

These findings highlight the broad significance of the *PELOTA* gene in geminiviral susceptibility, independent of the host plant species.

In **chapter 6,** by utilizing re-sequencing data obtained from a comprehensive collection of 100 melon genomes, we conducted allele-mining analyses focused on the identification of known S genes associated with PM and DM resistance. Through this approach, we successfully identified specific accessions harboring loss-of-function mutations in a melon homologue of *TCP14*, a susceptibility gene known to confer DM resistance in Arabidopsis. Additionally, we discovered mutations in a Clade V MLO gene, namely Cm*MLO1*, which has previously been linked to PM susceptibility in cucumber. In-depth investigation using a segregating population provided compelling evidence that a two-nucleotide insertion within the coding sequence of the Cm*MLO1* gene co-segregated with partial resistance to *Podosphaera xanthii*, the causative agent of PM in cucurbits. These findings not only validate earlier hypotheses concerning the involvement of this specific gene in PM susceptibility but also suggest that the mutated allele could serve as an advantageous asset in melon breeding programs.

In **chapter 7**, we employed the standard and widely used agrobacterium-mediated transformation method to deliver CRISPR/Cas9 constructs into cucumber and melon plants. Our aim was to introduce targeted mutations in the genomes of transgenic melon and cucumber calli in order to knock out candidate susceptibility genes. We report significant challenges in the subsequent regeneration of complete plants from transgenic calli, which aligns with the previously acknowledged difficulties associated with stable transformation and regeneration in these particular crop species. To address this issue, we implemented various optimization strategies to improve existing transformation protocols.

Finally, in **chapter 8,** we discuss on the mechanisms of the detected resistances, and we propose an implementation of the extracted insights on breeding strategies aiming for durable-broad spectrum resistance for cucurbit crops and beyond.

Samenvatting

Cucurbitaceae-gewassen worden wereldwijd verbouwd vanwege hun vruchten, die een rijke bron zijn van essentiële vitamines en voedingsstoffen en daarmee een belangrijk onderdeel vormen van een uitgebalanceerd dieet. Deze gewassen zijn vatbaar voor verschillende ziekten die een aanzienlijke invloed kunnen hebben op hun groei, opbrengst en algehele kwaliteit. Gezien de huidige epidemiologische situatie in de Europese teelt van meloen en komkommer, is er een dringende behoefte aan de ontwikkeling van nieuwe, breed-spectrum en duurzame resistentie tegen drie belangrijke ziekten, namelijk echte meeldauw (powdery mildew, PM), valse meeldauw (downy mildew, DM) en *tomato leaf curl New Delhi virus* (ToLCNDV). Aangezien dominante resistentie vaak specifiek is en snel wordt doorbroken door pathogenen, is de focus gericht op het onderzoeken van de potentiële rol van recessieve resistentie in deze gewassen, die bekend staat als minder specifiek voor pathogenen en duurzamer. Door het combineren van genomische en functionele benaderingen presenteren we nieuwe inzichten in het genereren van weerbare gewassen tegen de genoemde pathogenen.

In **hoofdstuk 1** geven we een beknopt overzicht van de impact van landbouwkundig onderzoek en plantenveredeling op de menselijke beschaving. Bovendien stellen we, met erkenning van de laatste vooruitgang in onderzoek en technologie, richtingen voor om te voldoen aan de voedingsbehoeften van een groeiende wereldbevolking. Uiteindelijk ligt de nadruk op ziekteresistentieveredeling in cucurbitaceae-gewassen, omdat deze een belangrijke troef zijn in de landbouw en menselijke voeding, maar te kampen hebben met oude en nieuwe opkomende ziekten die de kwaliteit en opbrengst verlagen.

In **hoofdstuk 2** wordt een grondige verkenning van de beschikbare kennis over de klassieke cucurbitaceae-pathogenen echte en valse meeldauw (PM en DM) uitgevoerd, met als doel bepaalde ecologische en moleculaire aspecten van deze ziekten te begrijpen, evenals alle beschikbare in kaart gebrachte resistenties in wilde en gekweekte meloen. In dit hoofdstuk hebben we de resultaten van de identificatie van resistenties in de afgelopen 50 jaar of langer samengevat, waarbij belangrijke bevindingen uit verschillende publicaties zijn benadrukt. Meer specifiek tonen we aan dat er 239 meloen accessies met PM-resistentie zijn geïdentificeerd en 452 accessies met DM-resistentie, zowel in *Cucumis melo* als in de wilde verwante soort *C. figarei*. Door genetische kartering in verschillende resistente bronnen zijn in de loop der jaren 25 dominante genen, twee recessieve genen en zeven QTL's geïdentificeerd die PM-resistentie verschaffen. Evenzo noemen we acht dominante genen en elf QTL's geassocieerd met DM- resistentie. Door gemelde merkervolgordes te koppelen aan de chromosomen

van het meloenras DHL92-genoom, hebben we met succes een fysieke verankering verkregen van een aanzienlijk aantal van deze resistente loci op hun respectieve chromosomen. Ten slotte stellen we strategieën voor om meloenrassen te kweken met duurzame en breed-spectrum PM/DM- resistentie.

In **hoofdstuk 3** geven we een overzicht van alle beschikbare kennis over ToLCNDV, een nieuw viraal pathogeen dat de Europese meloenteelt bedreigt. Sinds de eerste introductie in het Middellandse Zeegebied heeft het virus aanzienlijke economische verliezen veroorzaakt, waaronder verminderde opbrengsten en de productie van onverkoopbare vruchten die vaak scheuren vertonen. Gezien het ontbreken van chemische behandelingen tegen virale pathogenen, ligt de primaire focus op resistentieveredeling. We bieden een overzicht van de meest recente ontwikkelingen in meloenveredeling voor ToLCNDV-resistentie en vatten alle relevante loci die tot nu toe zijn gedocumenteerd in cucurbitaceae samen. Om de weg te effenen voor toekomstige ontwikkelingen, stellen we een alternatieve benadering voor traditionele veredelingsmethoden voor door kennis te benutten over vatbaarheidsgenen in meloen en andere cucurbitaceae-gewassen.

In **hoofdstuk 4** hebben we de sequentiebepaling uitgevoerd van de meloen accessie K18, die het kwantitatieve kenmerklocus (QTL) draagt voor recessieve resistentie tegen ToLCNDV op chromosoom 11. Bovendien hebben we DNA-sequentiegegevens geanalyseerd van 100 meloen genomen afkomstig van het Centrum voor Genetische Bronnen (CGN-WUR). Onze bevindingen onthulden een geconserveerde mutatie in de DNA Primase Large subunit (PriL) van alle resistente accessies die zijn blootgesteld aan ToLCNDV. Bovendien hebben we via experimenten waarbij het endogene *Nicotiana benthamiana PriL*-gen is stilgelegd en vervolgens geïnoculeerd met drie verschillende geminivirussen, een significante vermindering van de virale titers waargenomen voor alle drie de virussen, wat een aanwijzing is voor het cruciale belang van *PriL* bij geminivirale replicatie. Geminivirussen repliceren hun genetisch materiaal binnen de kern van gastheercellen door gebruik te maken van de DNA- replicatiemachine van de gastheer. Hoewel ze afhankelijk zijn van gastheer-DNA-polymerasen voor de omzetting van hun DNA in dubbelstrengs vorm en daaropvolgende replicatie, is het precieze mechanisme dat ten grondslag ligt aan de initiële stap van dit proces - de omzetting van binnenkomend circulair enkelstrengs-DNA in dubbelstrengs-DNA - bijna drie decennia lang onduidelijk gebleven. In dit hoofdstuk stellen we een model voor dat de rol van PriL verduidelijkt tijdens het initiëren van geminivirale DNA-replicatie, waarbij het fungeert als een regulerende subeenheid van het primase-complex, dat een RNA-primer genereert bij de aanvang van DNA-replicatie, analoog aan de door DNA Primase gemedieerde initiëring die in andere organismen wordt waargenomen.

In **hoofdstuk 5** hebben we onderzocht of het meloen *PELOTA* gen, een homoloog van het pelota-gen in tomaat dat bekend staat om bij te dragen aan geminivirale gevoeligheid, een vergelijkbare functionele rol speelt. In dit onderzoek werkten we met een tomatenlijn waarin *PELOTA* zodanig gemuteerd is dat het gen niet functioneel meer is, waardoor de tomatenlijn resistent is geworden tegen het geminivirus TYLCV. We introduceerden het functionele *PELOTA*-gen uit meloen in deze tomatenlijn. Dat herstelde de gevoeligheid TYLCV herstelt, zoals bleek uit virale titers en symptoomontwikkeling. Deze bevindingen benadrukken het brede belang van het PELOTA-gen bij geminivirale gevoeligheid, onafhankelijk van de gastheerplantensoort.

In **hoofdstuk 6** hebben we met behulp van re-sequentiegegevens, verkregen uit een uitgebreide verzameling van 100 meloen genomen, allel-analyses uitgevoerd gericht op de identificatie van bekende S-genen die geassocieerd zijn met PM- en DM-resistentie. Met behulp van deze benadering hebben we succesvol specifieke accessies geïdentificeerd die verlies van functie (loss-of-function) mutaties bevatten in een meloen-homoloog van *TCP14*, een gevoeligheidsgen dat bekend staat om DM-resistentie in Arabidopsis. Bovendien hebben we mutaties ontdekt in een klasse V *MLO*-gen, namelijk *CmMLO1*, waarvan een homoloog in komkommer eerder was gelinked aan PM-gevoeligheid. Diepgaand onderzoek met een splitsende populatie leverde overtuigend bewijs dat een insertiesequentie van twee nucleotiden binnen de coderende sequentie van het *CmMLO1*-gen cosegregeert met gedeeltelijke resistentie tegen *Podosphaera xanthii*, de veroorzaker van PM bij cucurbitaceae-gewassen. Deze bevindingen bevestigen niet alleen eerdere hypothesen over de betrokkenheid van dit specifieke gen bij PM-gevoeligheid, maar suggereren ook dat het gemuteerde allel een bijdrage kan leveren in meloenveredelingsprogramma's.

In **hoofdstuk 7** hebben we de standaard en veelgebruikte agrobacterium-gemedieerde transformatiemethode gebruikt om het CRISPR/Cas9-vector in komkommer- en meloenplanten te introduceren. Ons doel was om gerichte mutaties in het genoom van transgene meloen-calli te introduceren om kandidaat-gevoeligheidsgenen uit te schakelen. We rapporteren significante uitdagingen bij de daaropvolgende regeneratie van volledige planten uit transgene calli, wat overeenkomt met de eerder erkende moeilijkheden die gepaard gaan met het regenereren van transgene komkommerplanten. We identificeerden echter succesvolle transformatie- gebeurtenissen in beide soorten, en de volledige details van de gebruikte protocollen en resultaten worden beschreven.

In **hoofdstuk 8** bespreken we de belangrijkste bevindingen en conclusies van dit proefschrift en bieden we een toekomstperspectief voor de ontwikkeling van resistentie tegen de drie bestudeerde en belangrijke ziekten in cucurbitaceae-gewassen. We

benadrukken het potentieel van recessieve resistentie en het belang van het begrijpen van de functionele mechanismen van gevoeligheidsgenen om duurzame resistentie te ontwikkelen. Daarnaast wijzen we op de mogelijkheid van het gebruik van nieuwe veredelingstechnologieën, zoals genome editing met CRISPR/Cas9, om gerichte mutaties te introduceren in gevoeligheidsgenen en zo resistentie te creëren. Ten slotte benadrukken we de noodzaak van nauwe samenwerking tussen onderzoekers, veredelaars en telers om deze nieuwe inzichten in de praktijk te brengen en de cucurbitaceae-teelt te beschermen tegen ziekten

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Moving deeper, to the hidden forces that drove this endeavour successfully to an end, I salute with love and gratitude my friends and my family.

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Θεόφιλος, Χρήστος, Σπύρος και Anne, my Wageningen brothers (and sister) with whom we shared things that cannot be put in paper. Φιλιά, να είστε πάντα καλά.

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Lampros Siskos,

Wageningen 24/11/2023

About the author

Lampros Siskos was born and raised in Heraklion of Crete, Hellas. In 2010 he was admitted to the Agricultural University of Athens, School of Plant Science, from where he graduated in 2015. In 2018 he obtained an MSc degree in Plant Science with a specialization in Plant Pathology & Entomology from Wageningen University and Research, the Netherlands. In 2019 he started a PhD in the Laboratory of Plant Breeding of Wageningen University and Research where he performed research on the susceptibility genes of cucurbit crops for three economically important diseases: powdery mildew, downy mildew and *tomato leaf curl New Delhi virus*.

Education statement

Education Statement of the Graduate School Experimental Plant Sciences

Issued to: Lampros Siskos
Date: 24 November 2023
Group: Plant Breeding
University: Wageningen University

	1) Start-Up Phase	date	cp
►	**First presentation of your project**		
	Disease resistance in cucurbits by knocking out susceptibility genes	15 Apr 2019	1.5
►	**Writing or rewriting a project proposal**		
	Disease resistance in cucurbits by knocking out susceptibility genes	01 Jun 2019	6.0
►	**MSc courses**		
		Subtotal Start-Up Phase	***7.5***

	2) Scientific Exposure	date	cp
►	**EPS PhD days**		
	EPS PhD days 'Get2Gether', Soest (NL)	10-11 Feb 2020	0.6
	EPS PhD days 'Get2Gether', online	01-02 Feb 2021	0.4
►	**EPS theme symposia**		
	EPS Theme 2 Symposium & Willie Commelin Scholten Day 'Interactions between plants and biotic agents', online	09 Feb 2021	0.2
	EPS Theme 3 Symposium 'Metabolism and adaptation', Wageningen (NL)	05 Nov 2021	0.3
	EPS Theme 2 Symposium & Willie Commelin Scholten Day 'Interactions between plants and biotic agents' online	08 Feb 2022	0.2
►	**National platform meetings**		
	Annual Meeting 'Experimental Plant Sciences', online	12-13 Apr 2021	0.5
	Annual Meeting 'Experimental Plant Sciences', Lunteren (NL)	11-12 Apr 2022	0.6
	Annual Meeting 'Experimental Plant Sciences', Lunteren (NL)	17-18 Apr 2023	0.6
►	**Seminars (series), workshops and symposia**		
	Monday seminar WUR Plant Breeding: Jack Vossen, Jaap Wolters	21 Jan 2019	0.2
	Monday seminar WUR Plant Breeding: Eric van de Weg, Dong Zhang	13 May 2019	0.2

2) Scientific Exposure	**date**	**cp**
Monday seminar WUR Plant Breeding: Charlotte Prodhomme, Zhe Yan	27 May 2019	0.2
Monday seminar WUR Plant Breeding: Chris Maliepaard, Tatiana Martí Ferrando	20 May 2019	0.2
Monday seminar WUR Plant Breeding: Chengcheng Cai, Pauline van Haperen	03 Jun 2019	0.2
Monday seminar WUR Plant Breeding: Xuexue Shen, Alejandro Thérèse Navarro	17 Jun 2019	0.2
Monday seminar WUR Plant Breeding: Huayi Li, Yanlin Liao	18 Jun 2019	0.2
Monday seminar WUR Plant Breeding: Francesco Pancaldi, Yerisf Torres Ascurra	08 Jul 2019	0.2
Monday seminar WUR Plant Breeding: Ernst-Jan Eggers, Xulan Wang	26 Aug 2019	0.2
Monday seminar WUR Plant Breeding: Mark Sterken & Wim van den Berg	02 Sep 2019	0.2
Monday seminar WUR Plant Breeding: Katharina Hanika, Johan Baars	23 Sep 2019	0.2
Monday seminar WUR Plant Breeding: Yury Tikunov, Annemarie Dechesne	30 Sep 2019	0.2
Monday seminar WUR Plant Breeding: Olga Scholten, Paul Arens	07 Oct 2019	0.2
Monday seminar WUR Plant Breeding: Marieke Jeuken, Miguel Ramirez Gaona	11 Nov 2019	0.2
Monday seminar WUR Plant Breeding: Raul Masteling (NIOO-KNAW), Eleni Koseoglou	25 Nov 2019	0.2
► **Seminar plus**		
► **International symposia and congresses**		
CRISPRcon 2019, Wageningen (NL)	20-21 Jun 2019	0.6
VI International Symposium on Cucurbits, Ghent (BE)	30 Jun - 04 Jul 2019	1.5
XIIth Eucarpia Meeting on Cucurbit Genetics and Breeding, online	24-28 May 2021	1.2
XVII SOLANACEAE 2022 International Conference on the Plant Family of Solanaceae, Thessaloniki (GR)	01-05 Nov 2022	1.3
► **Presentations**		
Presentation at first consortium meeting: Investigating recessive resistance against three economically important diseases	28 May 2019	1.0
Presentation at Annual Meeting 'Experimental Plant Sciences': Shedding light onto unknown territories of geminiviral replication: New prospects for cross-species plant resistance	11 Apr 2022	1.0

	2) Scientific Exposure	date	cp
	Poster presentation at XVII SOLANACEAE 2022: Shedding light onto unknown territories of geminiviral replication: New prospects for cross-species plant resistance	01 Nov 2022	1.0
	Presentation at final consortium meeting: Insights on recessive resistance on Cucurbits	23 May 2023	1.0
►	**Interviews**		
	Annual meeting with external advisor	10 Oct 2019	0.1
	Annual meeting with external advisor	23 Jul 2020	0.1
	Annual meeting with external advisor	28 Jan 2021	0.1
	Annual meeting with external advisor	28 Mar 2023	0.1
►	**Excursions**		
		Subtotal Scientific Exposure	***15.4***

	3) In-Depth Studies	date	cp
►	**Advanced scientific courses & workshops**		
	Course Introduction to RNA-SEQ by Marc Galland, Amsterdam (NL)	01-05 Mar 2021	1.5
	EPS Bioinformatic Introduction Course, online	05-09 Jul 2021	1.5
►	**Journal club**		
	Member of the Plant Breeding PhD and Postdoc literature discussion group	2019-2021	1.0
►	**Individual research training**		
	Work in cucurbits experimental greenhouse and laboratory, SAKATA B.V, Almeria (ES)	11-15 Oct 2021	1.5
		Subtotal In-Depth Studies	***5.5***

	4) Personal Development	date	cp
►	General skill training courses		
	Participation in the Plant Breeding PhD Discussion Club (peer consultation), Wageningen (NL)	2019-2020	0.6
	EPS Introduction Course, Wageningen (NL)	29 Oct 2019	0.3
	WGS course Supervising BSc & MSc Thesis Students, Wageningen (NL)	17-18 Jun 2021	0.6
	WGS PhD Workshop Carousel, Wageningen (NL)	12 May 2023	0.3
	WGS course Searching and Organising Literature for PhD candidates, Wageningen (NL)	16-17 May 2023	0.6
	WGS Career Assessment, Wageningen (NL)	26 May 2023	0.3
	WGS workshop Reviewing a Scientific Manuscript, Wageningen (NL)	08 Jun 2023	0.1

4) Personal Development		**date**	**cp**
	WGS course Research Data Management, Wageningen (NL)	15-29 Jun 2023	0.5
►	**Organisation of scientific meetings, PhD courses or outreach activities**		
►	**Membership of EPS PhD Council**		
		Subtotal Personal Development	***3.3***

5) Teaching & Supervision Duties		**date**	**cp**
►	Courses		
►	**Supervision of BSc/MSc projects**		
	Msc thesis: Recessive resistance in melon for Downy mildew, Powdery mildew & Tomato leaf curl New Delhi virus	01 Apr 2021	1.0
	Msc thesis: Investigating recessive resistance to ToLCNDV in melon	22 Sep 2022	1.0
	MSc Research Practice: Examining the role of PELO in geminiviral susceptiblility in melon	15 Feb 2023	1.0
	Subtotal Teaching & Supervision Duties		3.0
		TOTAL NUMBER OF CREDIT POINTS*	**34.7**

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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