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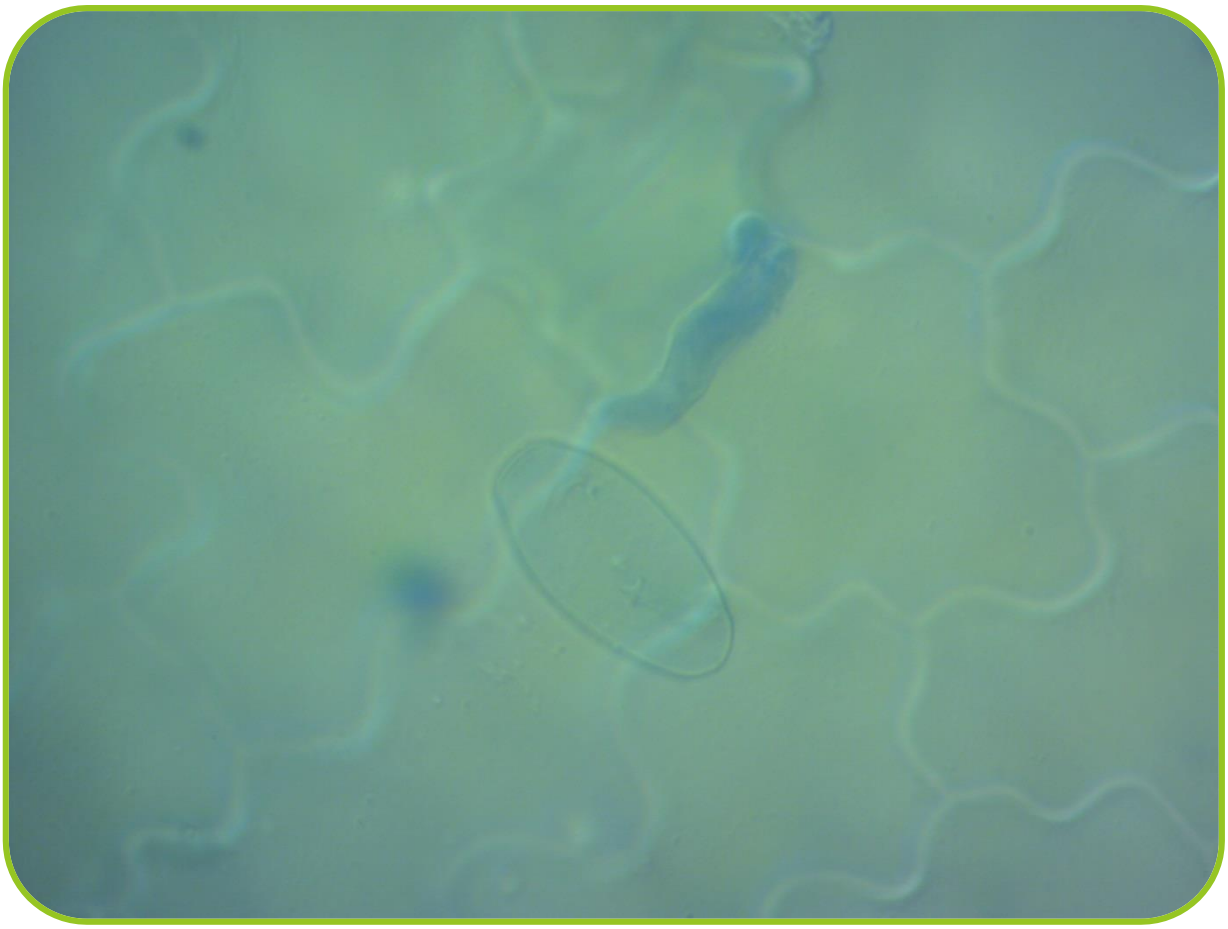

Insights in resistance and non-host interactions of powdery mildews

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MSc. Thesis Report

CONFIDENTIAL

Image: a barley powdery mildew (*Blumeria graminis* f. sp. *hordei*) spore forming an appressorium germination tube in a tomato epidermal cell.

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MSc. Thesis Report

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Abstract

Powdery mildew is a widely-spread plant disease caused by a highly diverse group of obligate biotrophic fungi of the *Erysiphales* order. This disease affects the production of several important crops around the world. Breeding strategies to develop resistant plants against powdery mildew are an ongoing effort. An important new alternative to the use of dominant *resistance* (*R*) genes in plant breeding is based on the silencing of *susceptibility* (*S*)-genes. *S*-genes are present in plants and their impairment results in a recessively-inherited resistance. Possibly the most studied example of such genes is the *MILDEW RESISTANCE LOCUS O* (*MLO*) gene. Several monocot and dicot crop species carrying loss-of-function alleles of the *MLO* gene are resistant against powdery mildew pathogens. In the first experiment of this thesis, three candidate *MLO* homologues in cucumber were cloned, setting the basis for their functional characterization. The second experiment concerned the elucidation of the effect of the heterologous expression of barley *HvMLO* in a tomato background in the light of its non-host interaction with barley powdery mildew. Additionally to the *S*-gene strategy, in tomato, resistance to powdery mildew could also be exploited from quantitative resistance loci (QRLs) found in wild species. *Ol-qtl2* is a QRL that confers partial resistance to powdery mildew. A candidate gene responsible for the resistance in this QRL has been identified to code for a receptor-like protein (RLP). Recent findings point that RLPs interact with receptor-like kinases (RLKs) to trigger defence responses. The RLKs *SOBIR1* and *SOBIR1-like* in tomato have been found to interact with several RLPs. The third experiment of this thesis concerned the virus-induced gene silencing (VIGS) targeting *SOBIR1* and *SOBIR1-like* in tomato plants carrying *Ol-qtl2* to verify the involvement of the candidate RLP gene in quantitative resistance.

Contents

1. General introduction

1.1 Powdery mildew

Powdery mildew is a plant disease caused by a complex group of highly diverse, obligate biotrophic fungal pathogens that affect over 650 monocot species and over 9000 dicot species (Schulze-Lefert&Vogel, 2000) including several economically important crops like barley, tomato, cucumber, pea, pepper and eggplant. These fungal pathogens belong to the order *Erysiphales* (Braun *et al.*, 2002). The characteristic symptoms of powdery mildew are the result of the abundant production of conidia that macroscopically can be recognized as whitish pustules that can extend along the leaves (Figure 1-1A), stems, flowers and fruits of the host plant (Glawe, 2008).

In tomato, powdery mildew is caused by *Oidium lycopersici*, *O. neolycopersici* and *Leveiullua taurica*. *Arabidopsis* is a host for *Golovinomyces cichoracearum*, *G. orontii*, *G. cruciferarum* and *Oidium neolycopersici.* Barley powdery mildew is caused by *Blumeria graminis f. sp. hordei (Bgh)* and in cucumber, this disease is caused by *Podosphera fusca* (syn. *Podosphera xanthii)* and *Golovinomyces cihoracearum* (syn. *Erysiphe cichoracearum*).

The life cycle of the pathogens causing powdery mildew may involve either or both a sexual phase (teleopmorph) and an asexual phase (anamorph) (Glawe, 2008). Infection of powdery mildew starts when an ascospore or conidiospore lands on a host. A germination tube is formed, and it later elongates to form a hyphae and an appressorium (Schulze-Lefert&Vogel, 2000), which are nipple-shaped to lobed outgrowths that allow the spore to attach to the host surface (Braun *et al.*, 2002). Barley powdery mildew, *Bgh*, forms a primary germination tube that attaches to the cell surface before forming a second germination tube that elongates into an appressorium (Figure 1-1B, 1-1D). After this, a penetration peg and, later, a haustorium are formed. A haustorium is a feeding structure responsible of maintaining the parasitic relationship with the host, it is an extension of the penetration peg and is formed inside of the host cell (Fotopoulos *et al.*, 2003, Schulze-Lefert&Vogel, 2000). When a successful infection occurs, hyphae of powdery mildew pathogens generally grow externally, branching outside the host's cells. However, pathogens belonging to the *Phyllactinieae* tribe, including the tomato pathogen *L. taurica*, can grow inside the tissue of the host (Glawe, 2008). In incompatible and non-host interactions with powdery mildew, papillae formation in the plant cell wall is a common mechanism to halt the infection (Underwood&Somerville, 2008) (Figure 1-1C; Chapter 3). In general, high humidity conditions and tempered weather favour the incidence of powdery mildew. However, the causing pathogens are widely distributed around the world and swift expansion appears to be occurring as a result of factors like climate change and longer growing seasons (Glawe, 2008).

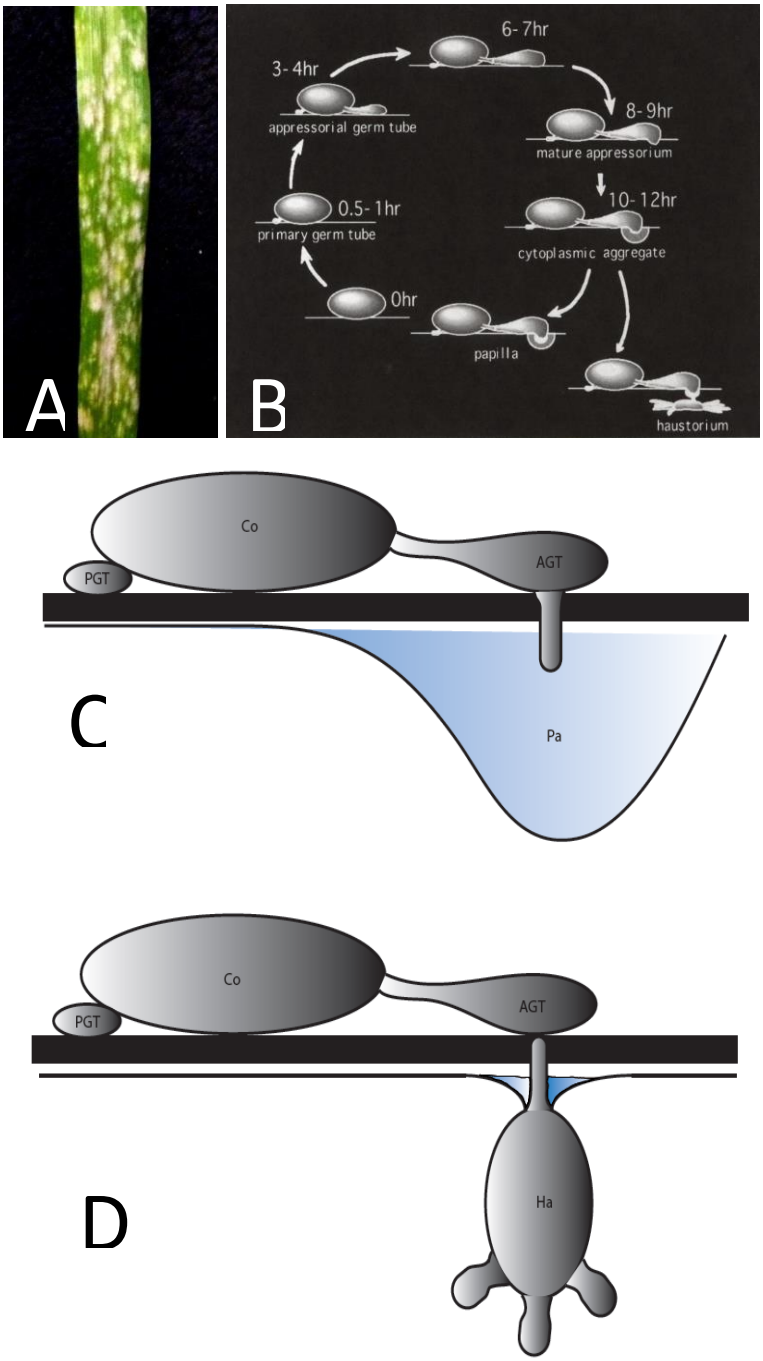

Figure 1-1. Symptoms and infection process of *Blumeria graminis* f. sp. *hordei* (Bgh) on barley. A) Common symptoms of a barley leaf infected with *Bgh*. White pustules are the result of abundant production of conidia in the upper side of the leaf. B) Infection process of *Bgh* starting at 0hr with the landing of the conidia in a host. A functional haustorium is formed when it affects a susceptible host. A papilla is a common mechanism to halt the infection in non-hosts and incompatible hosts (Kunoh *et al.*, 2002). C) Scheme of an infection halted by the formation of a cell wall apposition. Papilla is formed arround the penetration peg, making impossible to the pathogen to establish a functional haustorium. D)Scheme of the common structures of powdery mildew; the germintion tube elongates into an appressorium and later a feeding structure (haustorium) is formed. PGT: primary germination tube; Co: conidiospore; AGT; Appressorium germinitaion tube; Ha: haustorium (based on Kunoh *et al.,* 2002).

1.2 Plant-pathogen interactions

Plants are exposed to a vast amount of biotic stresses and thus have developed numerous mechanisms of protection. Most plants are susceptible only to a relatively small number of adapted pathogens and are resistant to the majority of potential pathogens, a phenomenon described as non-host resistance (Jones&Takemoto, 2004, Nuernberger&Lipka, 2005). A pathogen can establish an infection only when it is able to overcome all the defence layers of a plant's immune system.

The plant immune system is composed by two main layers: a passive (basal or preformed) defence and an active (or inducible) defence. The former constitutes the initial layer of defence and is comprised by waxy cuticular layers and preformed antimicrobial compounds, also called phytoanticipins (Jones&Dangl, 2006). The inducible defence, on the other hand, has been described using the zigzag model (Jones&Dangl, 2006). Two inducible response mechanisms are identified in this model, namely PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) (Figure 1-2).

The plant immune system is able to recognise some conserved molecules present in pathogens known as pathogen-associated molecular patters (PAMPs) by its pattern recognition receptors (PRRs). Receptor like kinases (RLKs) constitute a well-known example of PRRs. RLKs normally contain an N-terminal extracellular domain that defines its specificity to recognize PAMPS and an intracellular kinase domain that activates downstream signalling (Greeff *et al.*, 2012). Common extracellular domains of RLKs include the leucine-rich repeat (LRR) and LysM domains. A second major class of receptors present in the cell-surface are the receptor-like proteins (RLPs). RLPs share structural similarities with RLKs but lack a cytoplasmic kinase domain. Activation of downstream signalling of some RLPs has recently been proven to depend on their interaction with RLKs (Liebrand *et al.*, 2013). In tomato, the gene product of *SUPRESSOR OF BIR1-1*(*SOBIR1*) and *SOBIR1-like* ortologues of *Arabidopsis* are known to interact with several important RLPs including Cf, Ve1 an Eix2 (Liebrand *et al.*, 2013). Once the plant's PRRs are able to recognise PAMPS and start a downstream cascade, the PTI is activated. Induction of cell wall appositions and production of reactive oxygen species, phytoalexins, hydrolytic enzymes and pathogenesis-related proteins are commonly triggered by PTI (Nuernberger&Lipka, 2005).

Some pathogens are able to subdue PTI by delivering effectors that interfere with the defence mechanisms, leading to effector-triggered susceptibility (ETS). When the plant's resistance (R) proteins are able to specifically-recognize such effectors an immune response called effector-triggered immunity (ETI) is initiated, which is generally associated with programmed cell death, referred as hypersensitive response (HR), and the activation of defence responses. In the case of biotrophic pathogens, the defence responses are regulated by the salicylic-acid dependent pathway and normally

lead to HR. In the case of necrotrophic pathogens, jasmonic acid and ethylene signalling activate a different set of immune responses (Glazebrook, 2005).

Recently, it has been argued that the use of the zigzag model to explain the inducible response in plants could lead to generalizations such as that PTI is a weaker and slower-responding type of ETI. However, both mechanisms share numerous signalling components and the different cell events that they trigger may only differ in a quantitative manner (Thomma *et al.*, 2011) .

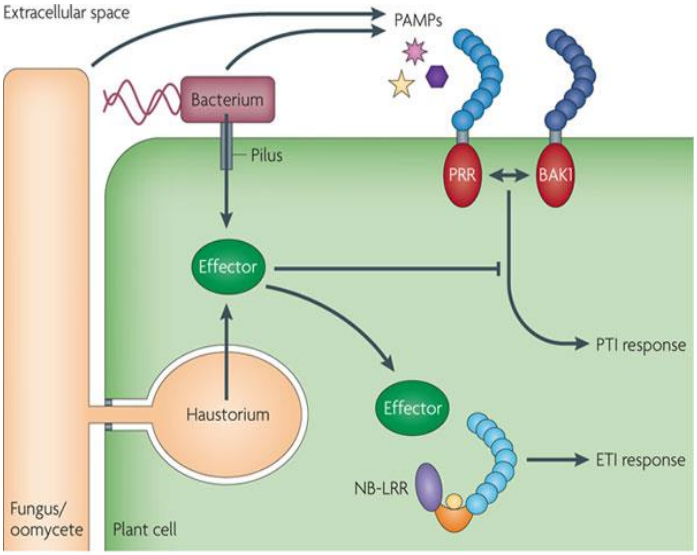

Figure 1-2. Scheme showing the two possible immune responses of a plant upon a the attack of a pathogen (Dodds&Rathjen, 2010). Extracellular recognition of pathogen-associated molecular patterns (PAMPs) by the plant's pattern recognition receptors (PRRs) leads to a PAMP triggered immunity (PTI) response. Fungal pathogens are able to secrete effectors into the plant by the mean of haustorium, these effectors enhance their virulence and disrupt PTI. The effectors can be recognized by the plant's resistance (R) proteins. Most of these R proteins have a characteristic NB-LRR structure. Intracellular recognition of the pathogen's effectors leads to a highly specific response called effector-triggered immunity (ETI) response.

1.3 Non-host resistance

Non-host resistance is exhibited in plants when an entire species is resistant to all genetic variants of a pathogen. It is the most common type of resistance in nature (Lipka *et al.*, 2008). The mechanisms through which a plant is able to counteract the attack of an unadapted pathogen consist of several successive layers of defences and involve both constitutive and induced defence mechanisms (da Cunha *et al.*, 2006, Ham *et al.*, 2007, Thordal-Christensen, 2003). Efforts to dissect the non-host resistance mechanisms in *Arabidopsis* have provided evidence that a set of functionally redundant but operationally distinct pre- and post-invasion immune responses are accountable for this phenomenon (Lipka *et al.*, 2008).

Non-host resistance is considered in the zigzag model as a result of at least two possible mechanisms (Jones&Dangl, 2006). The first being a lack of functional effectors in the pathogen that leads to an uncompromised PTI. This durable resistance is also called basal resistance in host-systems (Schweizer,

2007). The second one is the presence of functional *R* genes encoding NB-LRR type of proteins that recognize one or several *Avr* genes from the pathogen, leading to ETI. The timing and amplitude of the responses of each scenario differ and exert different evolutionary pressure on the host and the pathogen (Jones&Dangl, 2006). Schulze-Lefert and Panstruga proposed a unifying model for non-host, pathogen host range and pathogen speciation (Schulze-Lefert&Panstruga, 2011). According to this model, both NB-LRR- and PRR-triggered immunity contribute to non-host resistance in a way that when the evolutionary distance between host and non-host plant species becomes bigger, the contribution of NB-LRR protein-triggered immunity to non-host resistance reduces and the contribution of PRR-triggered immunity rises.

Non-host resistance involving fungal pathogens is considered pre-haustorial or pre-penetration when a haustorium-forming pathogen is unable to establish a fully functional haustorium and thus is unable to obtain nutrients from the cell (Niks&Marcel, 2009). Papillae formation, also called cell wall apposition, is a common feature of this type of non-host resistance (O'Connell&Panstruga, 2006) and can also be followed by a post haustorial hypersensitive response (Lipka *et al.*, 2008, Niks&Marcel, 2009).

Furthermore, according to the occurrence of hypersensitive reaction, non-host resistance has been classified into two types, namely type-I and type-II non-host resistance (Mysore&Ryu, 2004). In type-I non-host resistance the pathogens fail to overcome preformed barriers and general elicitor-induced plant defence responses like cell wall thickening, phytoalexin accumulation and papillae formation. Type-II non-host resistance, on the other hand, is always associated with a rapid localized necrotic hypersensitive response. The occurrence of both types of non-host resistance is, however, not exclusive, as a same plant species can display both types, and a same pathogen can cause different types of resistance in different plant species (Uma *et al.*, 2011).

1.4 Resistance genes

ETI is triggered after a highly-specific recognition of the pathogen's effectors by proteins in the plants encoded by resistance (*R*) genes. Most of these proteins are intracellular receptor proteins of the nucleotide binding-leucine-rich repeat (NB-LRR) structure (Jones&Dangl, 2006). The interaction between the plant's R proteins and the pathogen's effectors can be understood following the gene-for gene model (Flor, 1971). According to this model, when the product of an *avirulence* (*Avr*) gene from the pathogen is recognized by a matching product of an *R* gene from the plant, resistance is achieved. However, this resistance can be broken when the pathogen stops producing such effectors or evolves new ones to supress ETI (Jones&Dangl, 2006). Therefore, the specificity of this interaction has implications on the genetic pressure that is exerted over both the host and the pathogen. In plant

breeding, dominant *R* genes are commonly used to confer resistance. However, pathogens can overcome this resistance when it depends on such narrow-spectrum genes.

1.5 Susceptibility genes

Most pathogens that are able to overcome resistance, especially biotrophic ones, need cooperation of the host to establish a compatible interaction. All plant genes that are able to facilitate the infection and support compatibility can be considered *S* genes (van Schie&Takken, 2014). Novel breeding strategies can be developed considering that silencing of these genes can confer recessively-inherited and durable resistance (Gust *et al.*, 2010, Pavan *et al.*, 2010) .

Van Schie and Takken (2014) have distinguished three major mechanisms by which *S* genes facilitate susceptibility and contribute to infection:

1. Genes allowing basic compatibility (prepenetration), facilitating host recognition and penetration.
2. Genes encoding negative regulators of immune signalling.
3. Genes allowing sustained compatibility (postpenetration), fulfilling metabolic or structural needs and allowing pathogen proliferation.

Of special importance for this work are the first class *S* genes, to which one of the most studied examples of *S* genes belong, the *MILDEW RESISTANCE LOCUS O* (*MLO*) genes.

1.6 *MLO* genes

The *MLO* gene was first discovered in barley (*HvMLO*), in which it was found that plants carrying a recessively-inherited loss-of-function mutation of this gene showed a complete and broad resistance to powdery mildew pathogen, *Bgh* (Jørgensen, 1992). *Bgh* spores attacking *mlo* mutant plants are unable to establish a functional haustorium (Aist *et al.*, 1988). Histological analysis showed that plants carrying the homozygous *mlo* allele are able to form larger papillae and in a faster way compared to the wild-type plants (Wolter *et al.*, 1993). The *mlo*-based resistance has been used over 40 years to confer resistance in barley against powdery mildew in the fields (Lyngkjær *et al.*, 2000) through the use of natural and induced loss-of- function alleles (Acevedo-Garcia *et al.*, 2014, Piffanelli *et al.*, 2002, Reinstädler *et al.*, 2010).

The members of the *MLO* gene family code for a polytopic membrane protein that consists of seven transmembrane domains (Figure 1-3). Although the main biochemical function of *MLO* gene product is still vague, it is known that the barley MLO protein localizes in the plasma membrane, with an extracellular N terminus and an intracellular C terminus that harbours an amphiphilic α-helix which serves as calmodulin binding domain (Devoto *et al.*, 1999, Panstruga, 2005). The MLO protein is

considered a suppressor of several basal defence reactions such as cell wall apposition, apoplastic H_2O_2 accumulation, phytoalexin production and defence-related gene expression (Reviewed by (Hückelhoven, 2007).

In barley, *mlo* resistance requires the presence of two genes, namely *ROR1* (*REQUIRED FOR mlo RESISTANCE*) and *ROR2* (Freialdenhoven et al., 1996). Until now, the isolation and characterization of *ROR1* has not been possible (Acevedo-Garcia *et al.*, 2014). However, it has been proven that *ROR2* encodes for a member of the t-SNARE (Soluble *N*-ethylmalemide-sensitive factor Attachment protein Receptor) superfamily which has been speculated to be involved in the formation of ternary SNARE protein complexes that participate in the secretion of antimicrobial compounds in a vesicle-associated defence mechanism (Collins *et al.*, 2003, Kwon *et al.*, 2008).

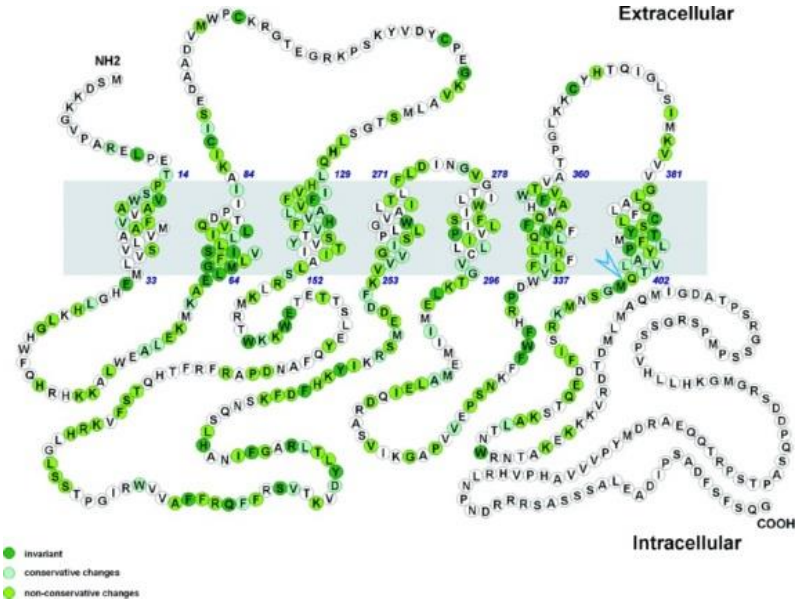

Figure 1-3. Topology of the MLO family (Devoto et al., 1999)**.** The deduced topology of seven transmembrane domains of barley MLO with an extracellular N terminus and an intracellular C terminus. The gray horizontal line represents the plasma membrane. Amino acids are represented by the letters inside the circles.

The resistance caused by the loss-of-function of *MLO* has also been characterized in *Arabidopsis thaliana*, in which triple mutant of *AtMLO2, AtMLO6* and *AtMLO12* was proven to be completely resistant against the adapted powdery mildew pathogens *Golovinomyces orontii* and *G. cichoracearum* (Consonni et al., 2006). By a combination of experimental and *in silico* studies Chen et al. (Chen *et al.*, 2006) have shown that each of the Arabidopsis *MLO* homologues is involved in diverse developmental and response processes, has unique expression patterns and its regulation is affected by a variety of biotic and abiotic stimuli, confirming that *MLO* genes play diverse roles in a diverse range of cellular processes and are not only related to susceptibility to fungal pathogens.

In *Arabidopsis*, three genes have been found to be essential for the *AtMLO2*-based resistance: *PEN1* (PENETRATION 1), *PEN2* and *PEN3* (Collins et al., 2003). PEN1 is the ortologue of barley's *ROR2*. Underwood and Somerville (Underwood&Somerville, 2008) proposed a model in which *PEN2* is believed to be involved in the enzymatic production of compounds with antifungal activity in the peroxisomes, while *PEN3* is responsible of exporting such compounds out of the plasma membrane. Through an still unknown mechanism, MLO is able to negatively regulate both PEN1 and PEN2/PEN3 pathways (Figure 1-4).

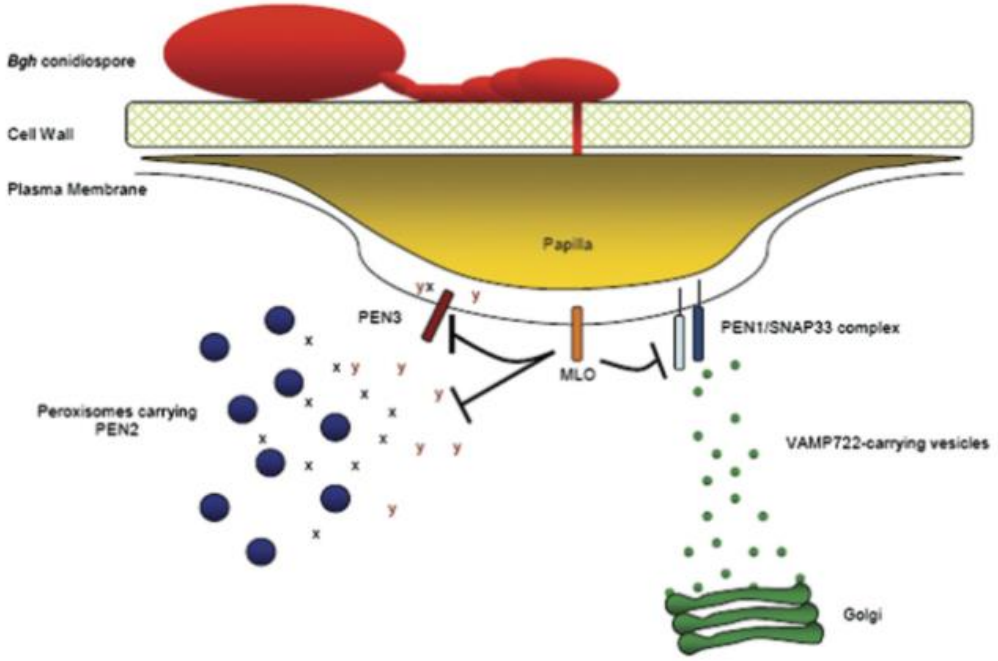

Figure 1-4. Model of the interaction between MLO and PEN proteins in barley (Underwood&Somerville, 2008). Upon the germination of a *Bgh* conidiospore, a papilla is formed below the cell wall. The MLO protein negatively regulates the activity of PEN proteins. PEN1 forms a complex with SNAP33 and is associated with the secretion of antimicrobial compounds in a vesicle-associated defence mechanism. PEN2 and PEN3 are believed to be involved in the same pathway of enzymatic production of antifungal compounds in the peroxisomes and later export of these compounds out of the plasma membrane.

In tomato, a natural occurring loss-of-function mutation in the *MLO* gene was discovered in a variety of cherry tomato (*S.lycopersicum* var *cerasiforme*) to lead to resistance against tomato powdery mildew *(Oidium neolycopersici*) (Bai *et al.*, 2008). Full powdery mildew resistance is due to a 19 base pair deletion in the coding region of the *SlMLO1* gene, mapped to the chromosomal region harbouring the *ol-2* locus. As with barley and *Arabidopsis*, in tomato plants carrying the recessive *ol-2* allele, the formation of papillae before that of a functional primary haustorium is associated with the resistance (Seifi *et al.*, 2014).

The *MLO* gene family's origin has been dated back to at least early stages of land plant evolution. *MLO* homologues have been found to be present across the plant kingdom in both monocot and dicot species (Devoto *et al.*, 2003). Additionally to barley, *Arabidopsis* and tomato, *MLO* homologues

associated with susceptibility to powdery mildew have been further functionally confirmed in pea (Humphry *et al.*, 2010), wheat (Elliott *et al.*, 2002, Várallyay *et al.*, 2012), rice (Elliott *et al.*, 2002) and pepper (Zheng *et al.*, 2013). Interestingly, these homologues cluster in two clades of the phylogenetic tree of the known MLO proteins (Figure 1-5). Monocot genes seem to be restricted to clade IV, while dicot genes cluster in clade V

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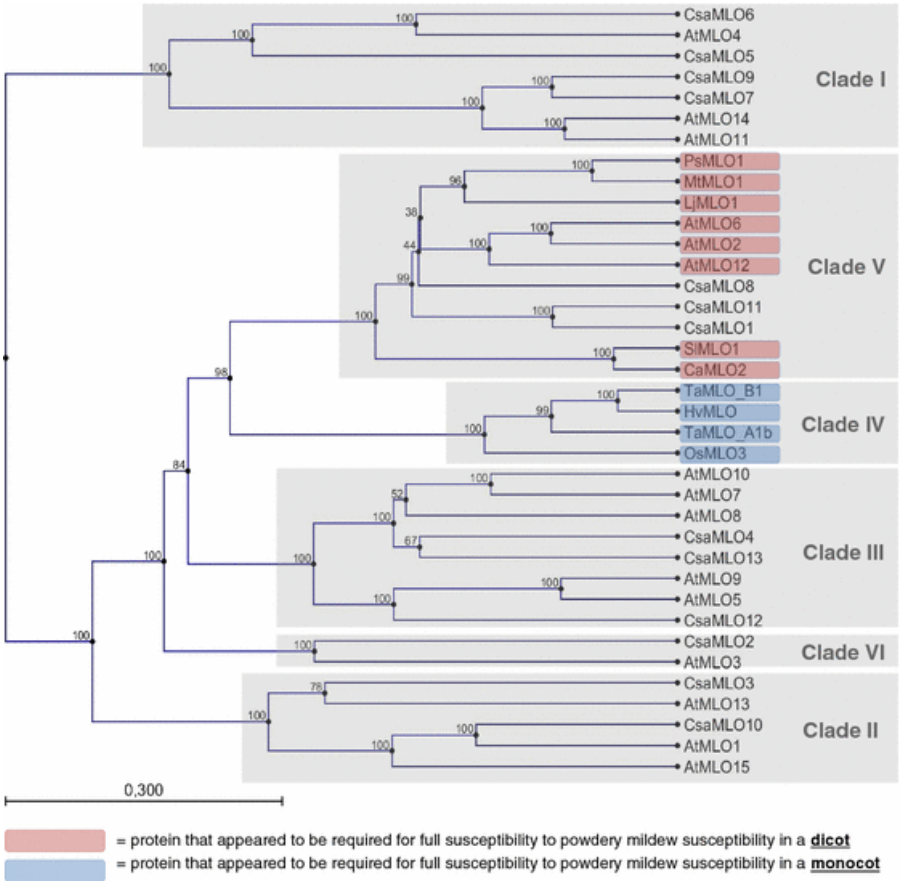

Figure 1-5 Phylogenetic tree of the MLO protein family (Schouten *et al.*, 2014). MLO proteins of *Arabidopsis*, cucumber, pea, tomato, barley and rice cluster in six different clades. MLO proteins involved in powdery mildew susceptibility cluster in clade V (dicot species) and clade IV (monocot species).

1.7 Thesis outline

This thesis is comprised by three experiments that provide insights of three different phenomena related to resistance and non-host interactions of powdery mildew.

The first experiment (Chapter 2) concerned the cloning of three candidate *mlo* homologues of cucumber and is a direct follow-up of the work made by Schouten *et al. (Schouten et al., 2014)*. The results of this experiment set the basis for the functional characterization of the *mlo* genes involved in powdery mildew susceptibility and the future exploitation of the *mlo*-based strategy to confer durable resistance to cucumber against powdery mildew.

In the second experiment (Chapter 3), a functional characterization of the heterologous expression of barley *HvMLO* in tomato was made. In this experiment, a tomato T2 family overexpressing *HvMLO* in an *ol-2* background was challenged with barley´s powdery mildew pathogen *Bgh*. Macroscopic evaluation and histological analysis was carried out to elucidate the effect of the expression of *HvMLO* in the non-host interaction.

In the third experiment (Chapter 3), virus-induced gene silencing (VIGS) was used to verify the possible candidate gene present in the quantitative resistance loci (QRL) *Ol-qtl2*, conferring partial resistance to the powdery mildew *O. neolycopersici* in tomato. According to previous studies of Faino et al (not yet published), the candidate gene is an RLP that is overexpressed upon infection with the pathogen. In this experiment, the RLK *SOBIR1* and *SOBIR-1 like* in tomato was targeted to confirm an effect on the candidate RLP in *Ol-qtl2*.

In short, the objectives of the experiments described in this thesis were:

- To clone and prepare overexpression vectors for functional characterization by complementation of three candidate *MLO* homologues in cucumber (Chapter 2).
- To assess the effect of the heterologous expression of barley's *HvMLO* in a tomato background in the light of a non-host interaction with *Bgh* (Chapter 3).
- To elucidate if the virus induced gene silencing of the kinase SOBIR1 and SOBIR1-like has an effect on the resistance conferred by *Ol-qtl2* and thus confirm that an RLP is a good candidate for the gene underlying such resistance. (Chapter 4).

A general overview of the three experiments comprising this thesis is presented in Figure 1-6.

Background: Three candidate *MLO* homologues have been identified in cucumber.

Aim: To clone and prepare an overexpression construct of the *MLO* homologues for its functional characterization.

Strategy: Produce cDNA from cucumber RNA, amplify and clone the genes and insert them in an overexpression vector.

Cloning of candidate cucumber *MLO* homologues (Chapter 2)

Background: Barley's *HvMLO* gene expressed in resistant *ol-2* tomato has been shown to compromise resistance against adapted pathogen *O. neolycopersici*.

Aim: To elucidate the effect of the expression of *HvMLO* in tomato against the non-adapted pathogen *B. graminis* f. sp. *hordei.*

Strategy: Challenge a T2 family of tomato expressing *HvMLO* with *B. graminis* f. sp. *hordei* and analyse its response macro- and microscopically.

Effect of heterologous expression of *HvMLO* in a resistant tomato background (Chapter 3)

Background: A candidate RLP-like gene has been identified in *Ol-qtl2,* a region conferring resistance against *O. neolycopersici*. Some RLPs have been found to require interaction with the RLK SOBIR1 and SOBIR1-like for downstream signalling.

Aim: To confirm the involvement of the candidate RLP-like gene and its interaction with SOBIR1 and SOBIR1-like in the resistance conferred by *Ol-qtl2*.

Strategy: Use VIGS targeting *SOBIR* and *SOBIR-1 like* in tomato plants and challenge them with powdery mildew to confirm the involvement of the candidate RLP-like gene in resistance.

VIGS for candidate gene elucidation of *Ol-qtl2* (Chapter 4)

Figure 1-6. Overview of the experiments. General background information, aim and strategy for each experiment are listed.

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2. Cloning of cucumber *MLO* homologs into a constitutive expression vector

2.1 Introduction

Cucumber (*Cucumis sativus*) is a widely cultivated plant of the *Cucubitaceae* family. Cucurbits are a family of economically important crops, including cucumber, melon, watermelon, squash and pumpkin. In the Netherlands, 410,000 tonnes of cucumber were produced in 2012 (FAOSTAT). Powdery mildew is one of the most common and widespread diseases of cucurbits. In cucumber, powdery mildew is caused by *Podosphera fusca* (syn. *Podosphera xanthii*) and *Golovinomyces cihoracearum* (syn. *Erysiphe cichoracearum*). As with other powdery mildews, the infection is easily recognizable by its clear symptoms: a whitish powdery fungal growth on both sides of the leaves, petioles and stems (Perez-Garcia *et al.*, 2009). *P. fusca* is a heterothallic fungus and an obligate biotroph that attacks epidermal cells obtaining nutrients by the means of haustorium.

Race-specific resistance against powdery mildew has been introgressed into cucumber but has, unfortunately, led to selective genetic pressure of the pathogen, resulting in the rise of virulent races (Cohen *et al.*, 2004). The publishing of the complete cucumber genome (Huang *et al.*, 2009) urged the search of new sources of resistance and the use of *S* genes to achieve durable resistance appears as a suitable strategy. Through a bioinformatic analysis, Schouten et al. (Schouten *et al.*, 2014) have identified candidate genes required for susceptibility to powdery mildew in cucumber. In this study, 13 *MLO*-like genes were described. Three of these genes, namely *CsaMLO1*, *CsaMLO8* and *CsaMLO11* were found to cluster in clade V, the clade containing the *S* genes for other dicots including *Arabidopsis*, tomato, pea and pepper (Chapter 1). These three genes harbour conserved regions of MLO-like proteins. Furthermore the expression of *CsaMLO1* was found to be upregulated 8 hours after inoculation with powdery mildew (Schouten *et al.*, 2014).

The recalcitrant nature for transformation of cucumber (Rajagopalan&Perl-Treves, 2005) makes difficult the functional studies by complementation using genetic transformation. However, complementation studies of the *mlo* gene have been done using paralogues and ortologues of members of the same phylogenetic clade in plant species that can be more easily transformed (Acevedo-Garcia *et al.*, 2014). Zheng et al. (Zheng *et al.*, 2013) demonstrated that pepper *CaMLO2* can partially restore susceptibility to the adapted powdery mildew in the otherwise resistant *ol-2* (*Slmlo1*) tomato line. As the three candidate *CsaMLO* genes cluster in clade V, complementation studies in *ol-2* tomato are a feasible option to functionally confirm the involvement of these genes in the susceptibility of cucumber towards powdery mildew.

The aim of this study was to amplify, clone and insert in a constitutive expression vector the three candidate *CsaMLO* genes clustering in clade V for further studies of complementation.

2.2 Materials and methods

2.2.1 Plant material, RNA isolation and cDNA synthesis

Leaf material for RNA extraction was provided by Henk Schouten. Leaves from a plant derived from a tilling line and from the commercial cultivar *Sheila* were used. Leaves were kept frozen at -80˚C before being grinded to a fine powder using a mortar and pestle. Powder was transferred to pre-chilled Eppendorf 2ml tubes. One ml of trizol was added to each tube and mixed for 30 seconds using a vortex. Then, 0.2ml of chloroform was added and mixed using a vortex for 15 seconds. Samples were then centrifuged at 13000 rpm at 4˚C for 20 minutes. The aqueous phase was transferred to a new Eppendorf 2ml tube. After this, 0.4ml of isopropanol was added to each tube and the samples were mixed by inversion and consequently incubated for 10 minutes at room temperature, followed by centrifugation at 13000 rpm for 15 minutes at 4˚C. At this point, a pellet was visible at the bottom of each tube. The supernatant was discarded and the pellet was washed using 0.4ml of ethanol (75%), followed by an additional centrifugation for seven minutes at room temperature. After this step, the supernatant was discarded and the pellet was let drying for 15 minutes by placing the tube upside-down on a paper wipe. Finally, the pellet was dissolved using 1x TE buffer. Once dissolved, the concentration was measured using an Isogen Nanodrop Spectrophotometer ND-1000. Isolated RNA was treated with DNAse I (Invotrogen) using the manufacturer's instructions prior to cDNA synthesis using Super Script® III 1st strand reverse transcriptase. For most cases, a 1:10 dilution of the cDNA was used in the PCR reactions.

2.2.2 Primers for amplification

Three primer pairs to amplify each of the candidate homologues *CsaMLO1*, *CsaMLO8* and *CsaMLO11* were provided by Henk Schouten for testing (Table 2-6). These primers were designed after aligning the predicted sequences of the genes with the genomic sequence of cucumber (www.icugi.org). A 200bp region upstream and downstream of each gene was selected and the complete sequence was given to Primer3Plus for primer design. The sequence CACC was added at the 5'end of each forward primer for allowing directional cloning. Three additional primers were designed: a forward primer to amplify *CsaMLO1* (Table 2-1, *CsaMLO1*-Forward) and a set of primers to amplify the middle region of *CsaMLO11* in order to confirm the sequencing data (Table 2-7). Table 2-1 shows the primers used for the actual amplification of each gene.

Table 2-1. Primers used for the amplification of *MLO* homologues in cucumber.

Gene	Primer	Sequence	Expected size
CsaMLO1	Forward	caccTTCCTTCCACACCCCTAAGA	1855bp
	Reverse	TGAATGGTGTAAACGAGATTGC	
CsaMLO8	Forward	caccCTGCCTCTCCACATGCATAA	1951bp
	Reverse	GCGCCCTGTACATGAAGAAC	
CsaMLO11	Forward	caccTTTGTTTCCCTACGCGTTCT	2151bp
	Reverse	TATACCAACCCCCAACCTCA	

2.2.3 Amplification of genes

In a first stage of the experiment, cDNA obtained from a cucumber plant from a tilling line was used for the amplification of the *MLO* genes. The amplification was carried out using the primers described in Table 2-1.

Amplification of *CsaMLO8* and *CsaMLO11* was done using *PfuUltra* II Fusion HS DNA Polymerase (Agilent Technologies) following the conditions described in Table 2-2. Amplification of *CsaMLO1* was made in two steps. Firstly using Advantage® 2 proofreading amplification kit (Clontech) at the conditions listed in Table 2-3. Secondly, a shorter (25 cycles) PCR was performed on a 1:100 (v:v) dilution of the specific product obtained with Advantage®, using Phusion high-fidelity DNA Polymerase (Thermo Scientific) at the conditions described in Table 2-4 to obtain a blunt-ended product. A set of primers to amplify the housekeeping gene *CseTip41* was used as a control for the PCR reactions.In a second stage of the experiment, cDNA obtained from a cucumber plant of the cultivar *Sheila* was used to amplify *CsaMLO8*. This was done using the primers described in Table 2-1 and *PfuUltra* II Fusion HS DNA Polymerase following the conditions described in Table 2-2.

Table 2-2. PCR conditions used for the amplification of *CsaMLO* genes using *Pfu Ultra* II Fusion HS DNA Polymerase (Agilent Technologies).

Segment	Number of cycles	Temperature	Duration
1	1	95˚C	1 minute
2	40	95˚C	20 seconds
		60˚C	20 seconds
		72˚C	1 minute
3	1	72˚C	3 minutes

Table 2-3. PCR conditions used for the amplification of *CsaMLO* genes using Advantage® 2 proofreading amplification kit (Clontech).

Segment	Number of cycles	Temperature	Duration
1	1	95˚C	1 minute
2	35	95˚C	30 seconds
		68˚C	3 minutes
3	1	68˚C	3 minutes

Table 2-4. PCR conditions used for the amplification of *CsaMLO* genes using Phusion high-fidelity DNA Polymerase (Thermo Scientific).

Segment	Number of cycles	Temperature	Duration
1	1	98˚C	30 seconds
2	25	98˚C	20 seconds
		55˚C	30 seconds
		72˚C	30 seconds
3	1	72˚C	10 minutes

2.2.4 Gel purification

PCR product purification from the agarose gel was required for *CsaMLO8* amplified from *Sheila* as only a weak band was obtained and there was accumulation of residues at the bottom of the gel (Figure 2-3E) This was done using a QIAQuick Gel Extraction Kit (Qiagen) following the manufacturer's protocol.

2.2.5 Insertion of *MLO* homologues into pENTR™/D-TOPO, cloning and confirmation

Ligation of the PCR products into a pENTR™/D-TOPO entry vector (Figure 2-1) was made using pENTR™/D-TOPO cloning kit (Invitrogen) following the manufacturer's instructions. The construct was then inserted by heat shock into XL10-Gold Ultracompetent Cells (Stratagene). After transformation, cells were plated on LB agar containing 50µg/ml kanamycin and grown overnight. Single colonies were selected from plates and each one was taken with a sterile toothpick into a 20 ml tube containing fresh liquid LB medium containing 50 µg/ml kanamycin to grow overnight. Then, 1µl of each culture was

used to perform colony PCR using DreamTaq (Table 2-5). The PCR was made using the M13 primers pairs (Forward: 5´-GTAAAACGACGGCCAG-3´; Reverse: 5´-CAGGAAACAGCTATGAC-3´). Plasmid isolation from the positive cultures was made using a QIAprep Spin Miniprep Kit. The plasmids isolated were sequenced to confirm the correct insertion of the gene in the vector.

Table 2-5 PCR conditions for colony PCR using DreamTaq.

Segment	Number of cycles	Temperature	Duration
1	1	95˚C	10 minutes
2	35	95˚C	30 seconds
		55˚C	30 seconds
		72˚C	1 minute
3	1	72˚C	10 minutes

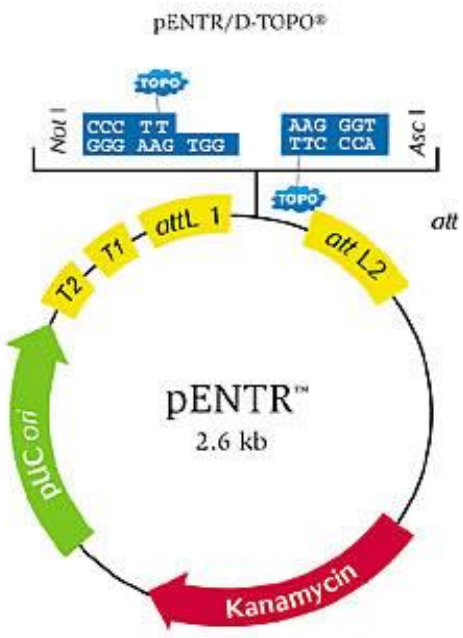

Figure 2-1. Map of the pENTR^{tm}/D-TOPO vector containing the attL1 and attL2 regions flanking the insert. The vector also contains a region that encodes for Kanamycin resistance for plasmid selection**. (**https://www.lifetechnologies.com/order/catalog/product/K240020)

2.2.6 Insertion of MLO homologues in pK7WG2 vector, cloning and confirmation

Insertion of *CsaMLO1* and *CsaMLO11* from the entry vector into the destination vector pK7WG2 (Karimi *et al.*, 2002) (Figure 2-2) was done through an LR reaction using LR Clonase Enzyme Mix (Invitrogen). After this, XL10-Gold Ultracompetent Cells (Stratagene) were transformed with the destination plasmid using heat shock. After transformation, cells were plated on LB agar containing 50 µg/ml spectinomycin and grown overnight. Single colonies were selected from plates and each one

was taken with a sterile toothpick into a 20ml tube containing fresh liquid LB medium containing 50 µg/ml spectinomycin to grow overnight. Colony PCR using DreamTaq was done using the same conditions as during the previous transformation in pENTR vector (Table 2-5). Once confirmed, plasmid isolation from each tube was made using a QIAprep Spin Miniprep Kit (Qiagen). The plasmids isolated were sequenced to confirm the correct insertion of the gene in the vector.

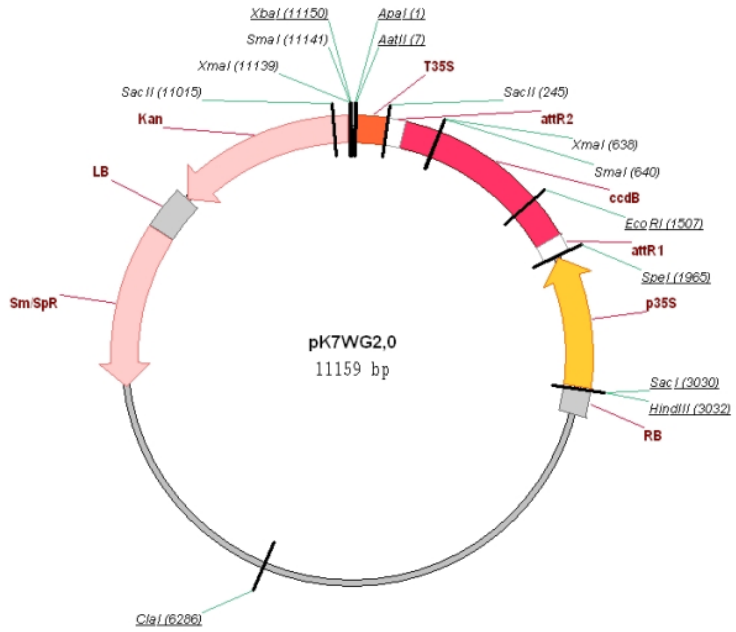

Figure 2-2 Map of the pK7WG2 vector containing the attR1 and attR2 regions flanking the region for insertion. The vector also contains the P35S promoter region for constitutive expression. The backbone of this plasmid is the pPZP200 plasmid which contains a spectinomycin resistance gene for plasmid selection (http://gateway.psb.ugent.be/vector/show/pK7WG2/search/index)

2.3 Results

From cDNA obtained from the tilling line plant, we were able to amplify and clone in pENTR^{tm}/D-TOPO entry vector *CsaMLO1* and *CsaMLO11* (Figure 2-3A, B, C). For *CsaMLO1*, amplification was first attempted using Phusion PCR kit (Thermo Scientific), however, even using different PCR conditions, we were unable to obtain a good amplification product without unspecific bands. For this reason, a two-step amplification had to be done to obtain good amplification and a blunt-ended product.

The PCR product of *CsaMLO8* appeared as two close bands when ran in an agarose gel (Figure 2-3D). Attempts to clone the upper band were unsuccessful, but cloning the lower band was possible. After sequencing, we observed that *CsaMLO1* and *CsaMLO11* corresponded to the predicted sequences, while the cloned *CsaMLO8* obtained from this plant had the entire eleventh exon missing (Supplementary material, alignment of sequences, CsaMLO8 position 2845). Later, we were able to amplify the complete *CsaMLO8* from cDNA obtained from another plant of a different cultivar, Sheila (Figure 2-3E).

All amplified products were later cloned in a pENTR^{tm}/D-TOPO vector and sequenced (Suplementary material) for confirmation. *CsaMLO1* and *CsaMLO11* were transferred and cloned in a pK7WG2 vector.

An overview of the steps achieved in this experiment for each gene is presented in Figure 2-4

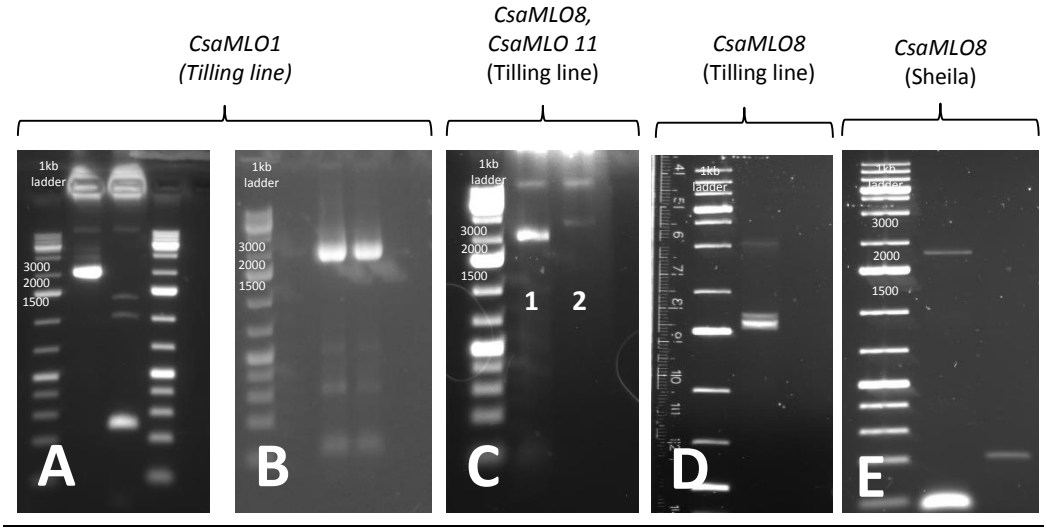

Figure 2-3 Amplification of *CsaMLO* genes. First, cDNA of a cucumber plant from a tilling line was used (A, B, C and D). Later, cDNA of a cucumber plant of the cultivar *Sheila* was used (E). To amplify *CsaMLO1* two steps were needed. A) Agarose 1% gel with the amplification of *CsaMLO1* using Advantage® 2 proofreading amplification kit, which yielded a product with sticky ends. In the image, the first well is the product using *CsaMLO1* primers and the second well is the housekeeping gene *CseTip41*, used as control. B) An additional PCR reaction was made on a 1:100 and a 1:200 dilution of the first PCR product. The product from the 1:100 was used for cloning. C) Amplification of CsaMLO8 (C1) and *CsaMLO11* (C2) from cDNA from a cucumber plant from a tilling line using *PfuUltra* II Fusion HS DNA Polymerase. D) Zoomed image of the amplification of *CsaMLO8* run in a 1% agarose gel and stained with ethidium bromide, in which two bands appear to be present. E) Amplification of *CsaMLO8* using cDNA of a cucumber plant of the cultivar Sheila.

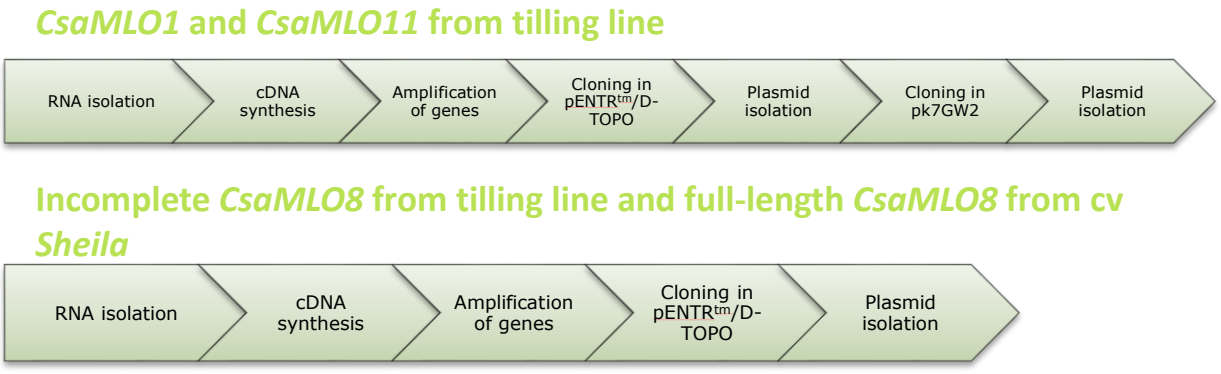

Figure 2-4. Overview of the steps achieved in this experiment. *CsaMLO1, CsaMLO11* and *CsaMLO8* with an exon missing, were successfully cloned in the pk7WG2 vector carrying a 35S constitutive promoter. These constructs are ready to be transferred to *Agrobacterium*. *CsaMLO8* was cloned and later isolated. This gene needs to be inserted and cloned in a pk7WG2 construct before transferring to *Agrobacterium*.

2.4 Discussion

The three *CsaMLO* genes clustering in clade V were successfully cloned in a pENTR^{tm}/D-TOPO vector. Sequences of each gene were confirmed to be as predicted. Additionally a sequence corresponding to *CsaMLO8* with an exon missing was cloned from the cDNA of a plant from a tilling line. The complete *CsaMLO8* was later amplified and cloned from the cDNA of a plant of the cultivar Sheila. *CsaMLO1* and *CsaMLO11* were introduced in the pK7WG2 vector, which contains a 35S promoter for constitutive expression.

Confidential information obtained from the company involved in this project indicated that the plant derived from the tilling line showed partial resistance to powdery mildew. This resistance has been mapped to a region that coincides with the position of *CsaMLO8*. This data strongly suggest that the resistance reported is due to the loss-of-function allele of *CsaMLO8.* It is suggested to continue with the transformation for functional characterization using also this fragment to confirm this is indeed a loss-of-function allele and thus confirm the gene function of *CsaMLO8* as a susceptibility gene.

When amplifying *CsaMLO11* from the tilling line plant, a weak band was obtained when the PCR product was run in an agarose gel (Figure 2-3C2). This indicates that the gene was lowly expressed. However, the amplification product was enough to clone the gene.

When amplifying *CsaMLO8* using the cDNA from *Sheila*, we were unable to obtain a PCR product for *CsaMLO8* using a 10:90 cDNA dilution, indicating that the gene was lowly expressed. However, by directly using the product from the reverse transcription, it was possible to obtain a single band of the expected size (Figure 2-3E). Due to the accumulation of PCR residues at the bottom of the gel, we decided to purify the product, which was later cloned.

While amplifying *CsaMlo8* from the tilling line plant, we identified a band above the expected band on the agarose gel (Figure 2-3D). Two possible reasons for these are speculated: that an alternative splicing could be occurring or that the plant was heterozygous for the gene. Unfortunately, an attempt to clone and sequence the upper band failed.

This work stablishes the basis for the functional characterization of the candidate *MLO* homologues and the future utilization of these *S* genes to achieve a durable cucumber resistance against powdery mildew.

The next steps to achieve a functional characterization of the genes are described in Figure 2-5.

Figure 2-5. Future steps to carry out the functional characterization of the *CsaMLO* genes. After the insertion in the pK7WG2 vector, transformation of *Agrobacterium tumefaciens* (*A.t.*), followed by plant transformation should be carried out. Functional characterization of the obtained transformants will be carried out.

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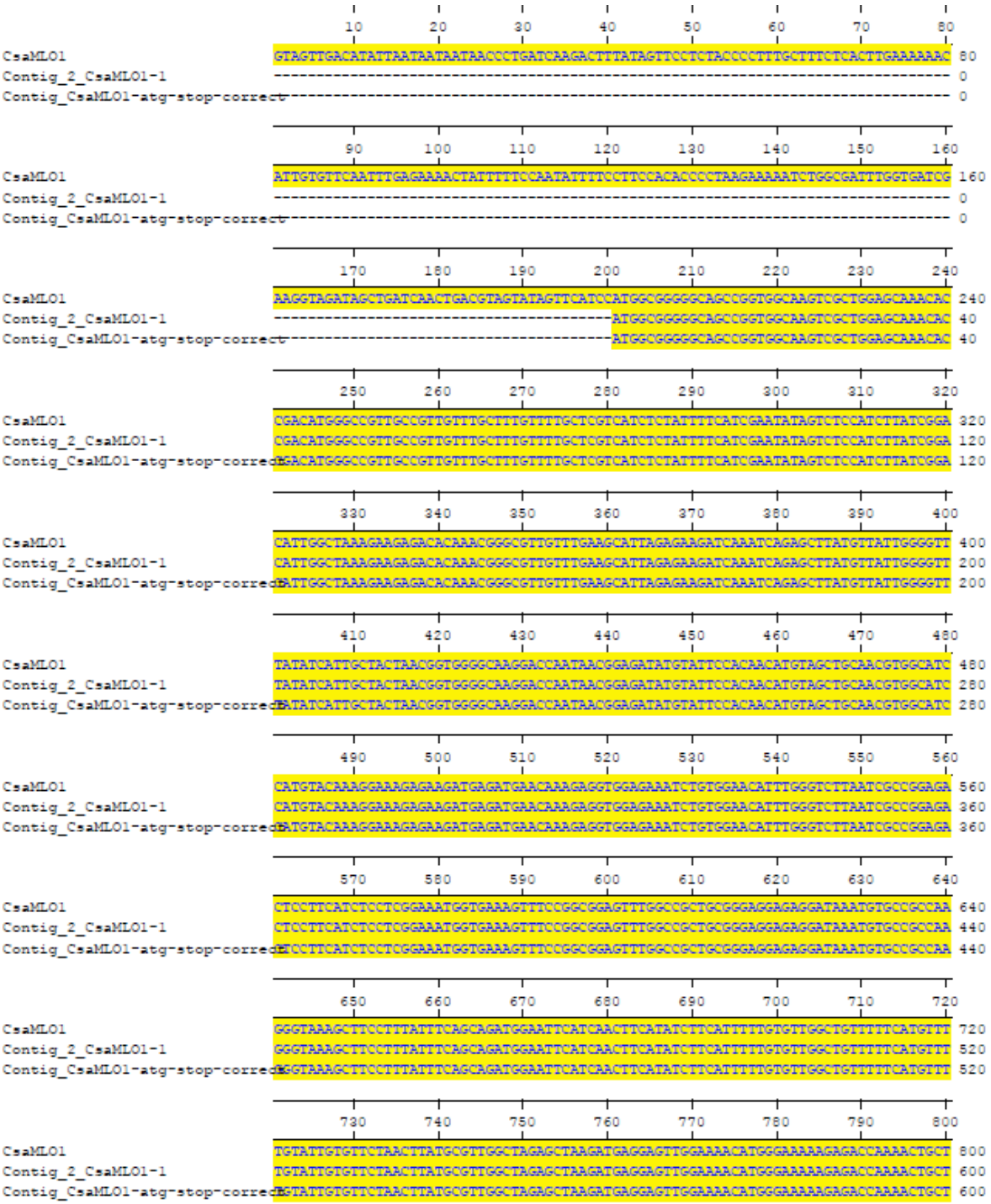
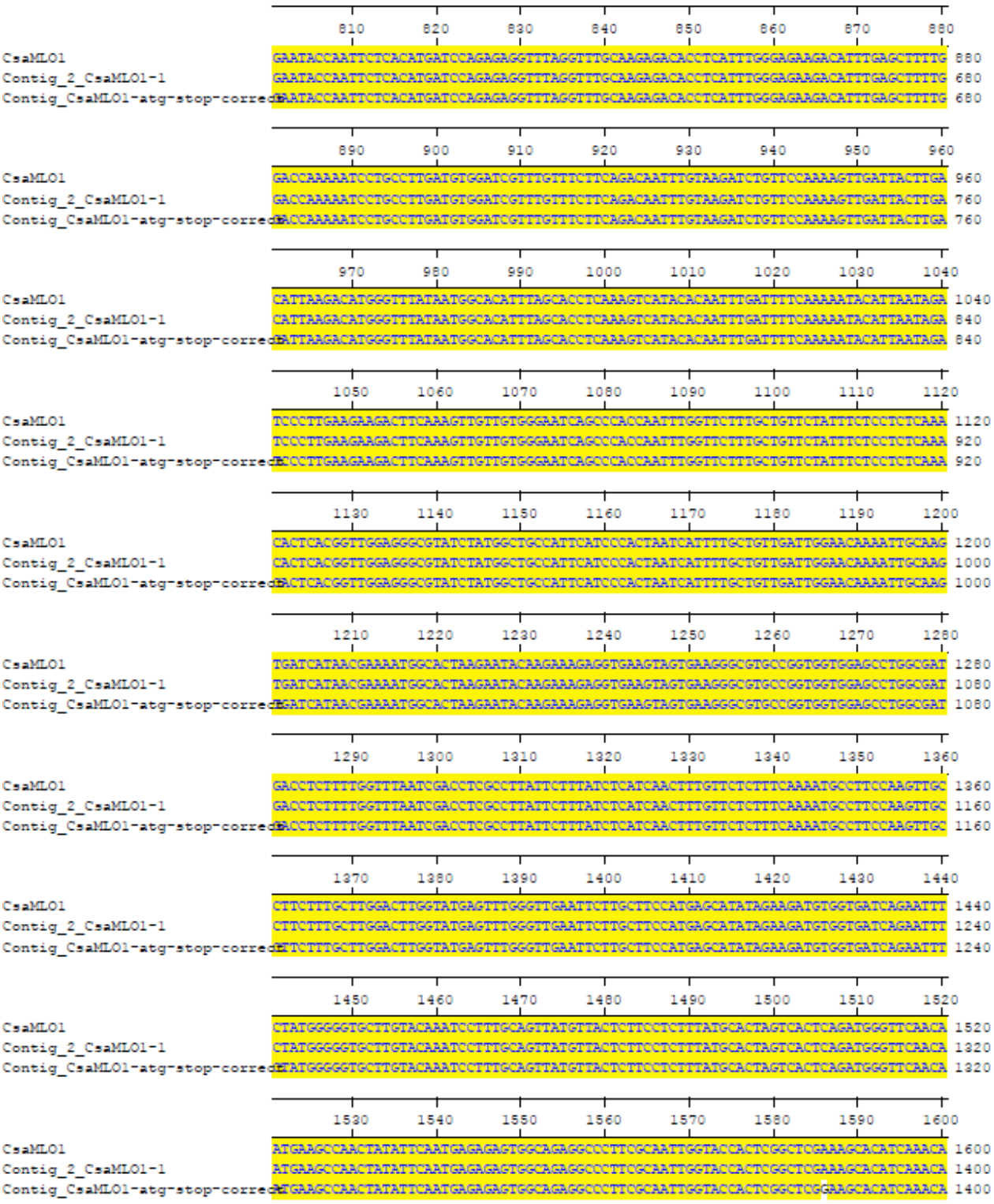
2.6 Supplementary material

2.6.1 Alignment of sequences.

CsaMLO1 alignment.

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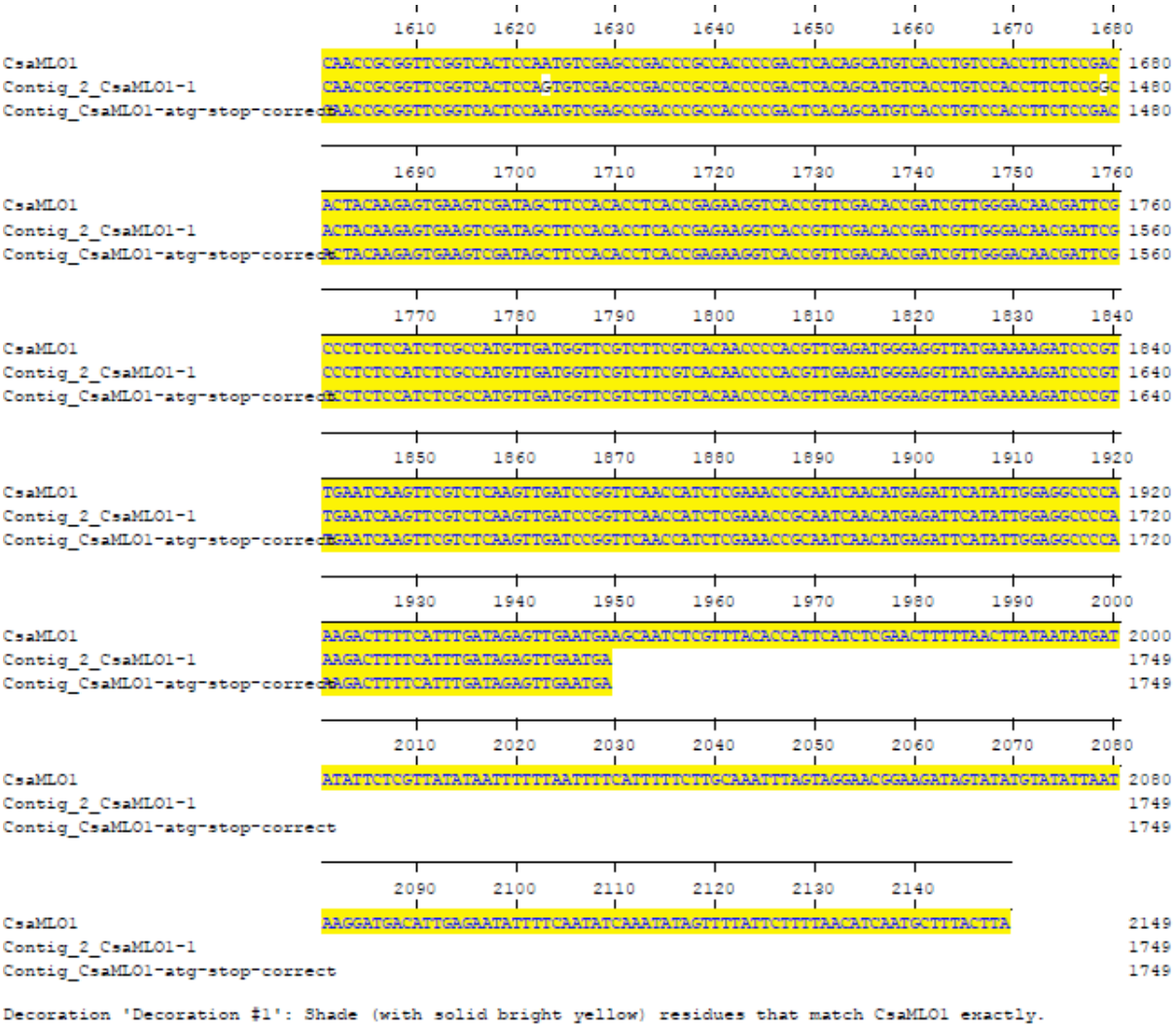
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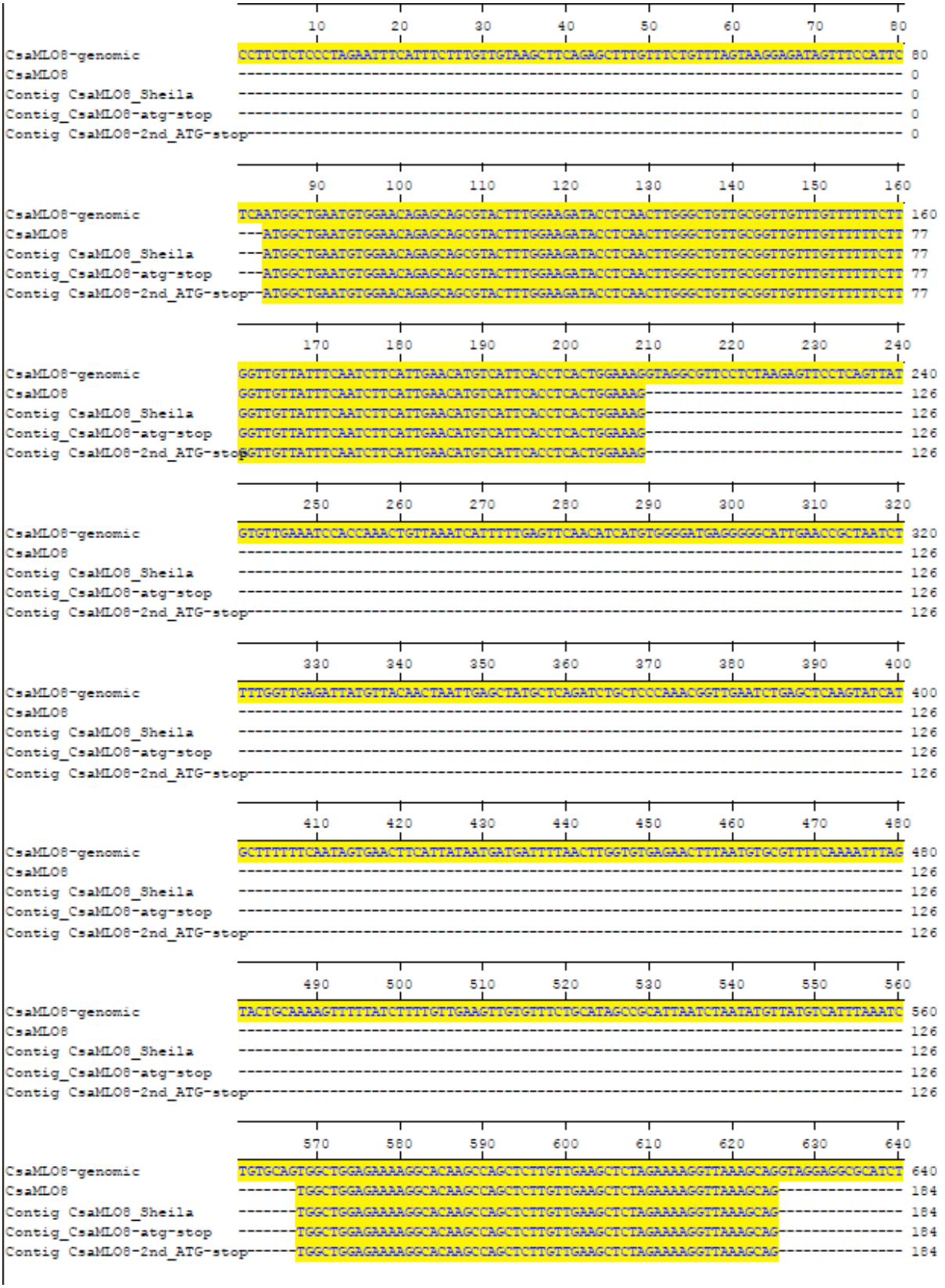
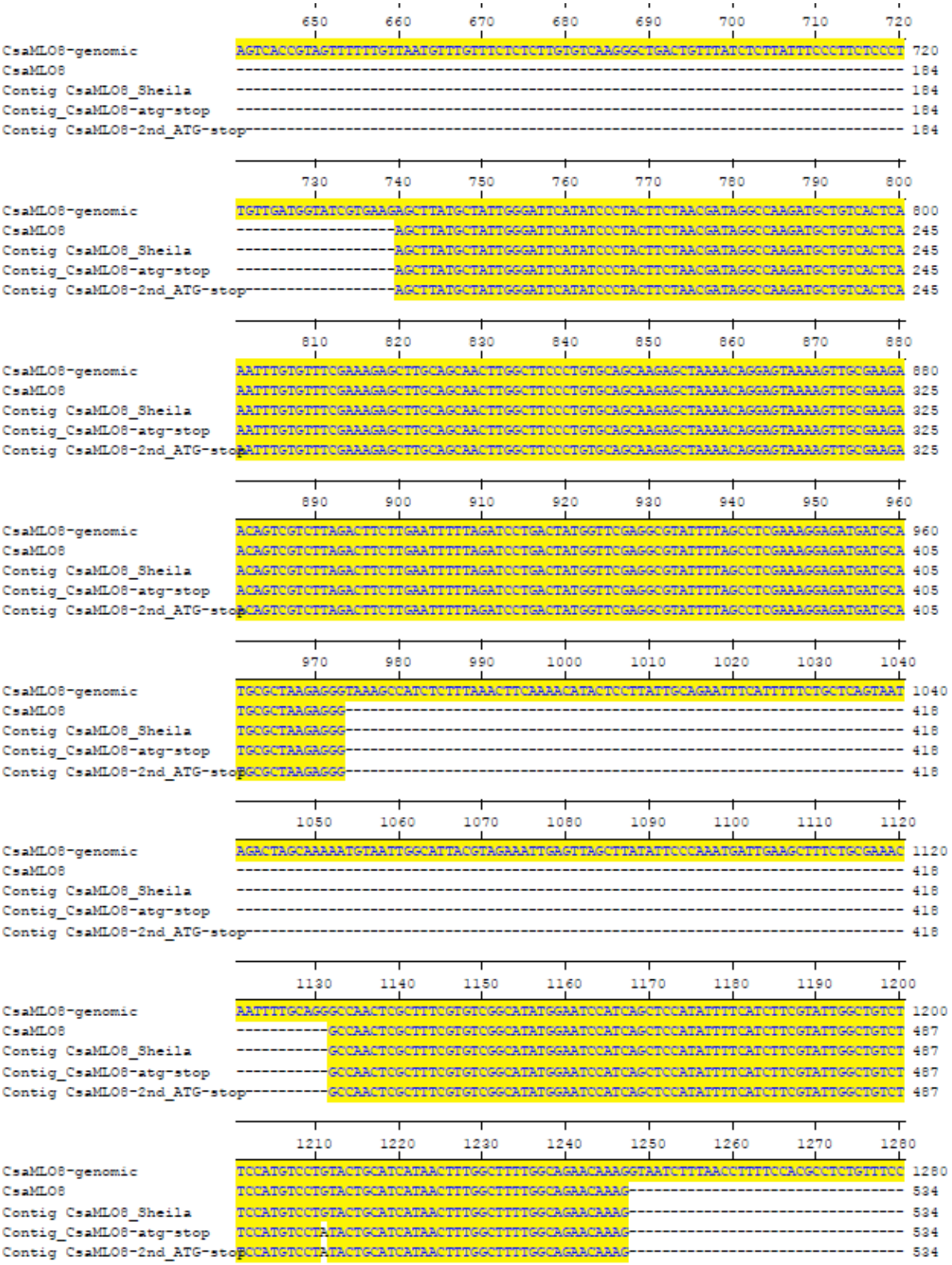
Contig_CsaMLO1-atg-stop-correct 1749
Decoration 'Decoration #1': Shade (with solid bright yellow) residues that match CsaMLO1 exactly.

CsaMLO8 alignment. Missing exon in cDNA from the tilling line at position 2845 indicated with red.

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Contig CsaMLO8_Sheila 184
Contig_CsaMLO8-atg-stop 184
Contig CsaMLO8-2nd_ATG-stop 184
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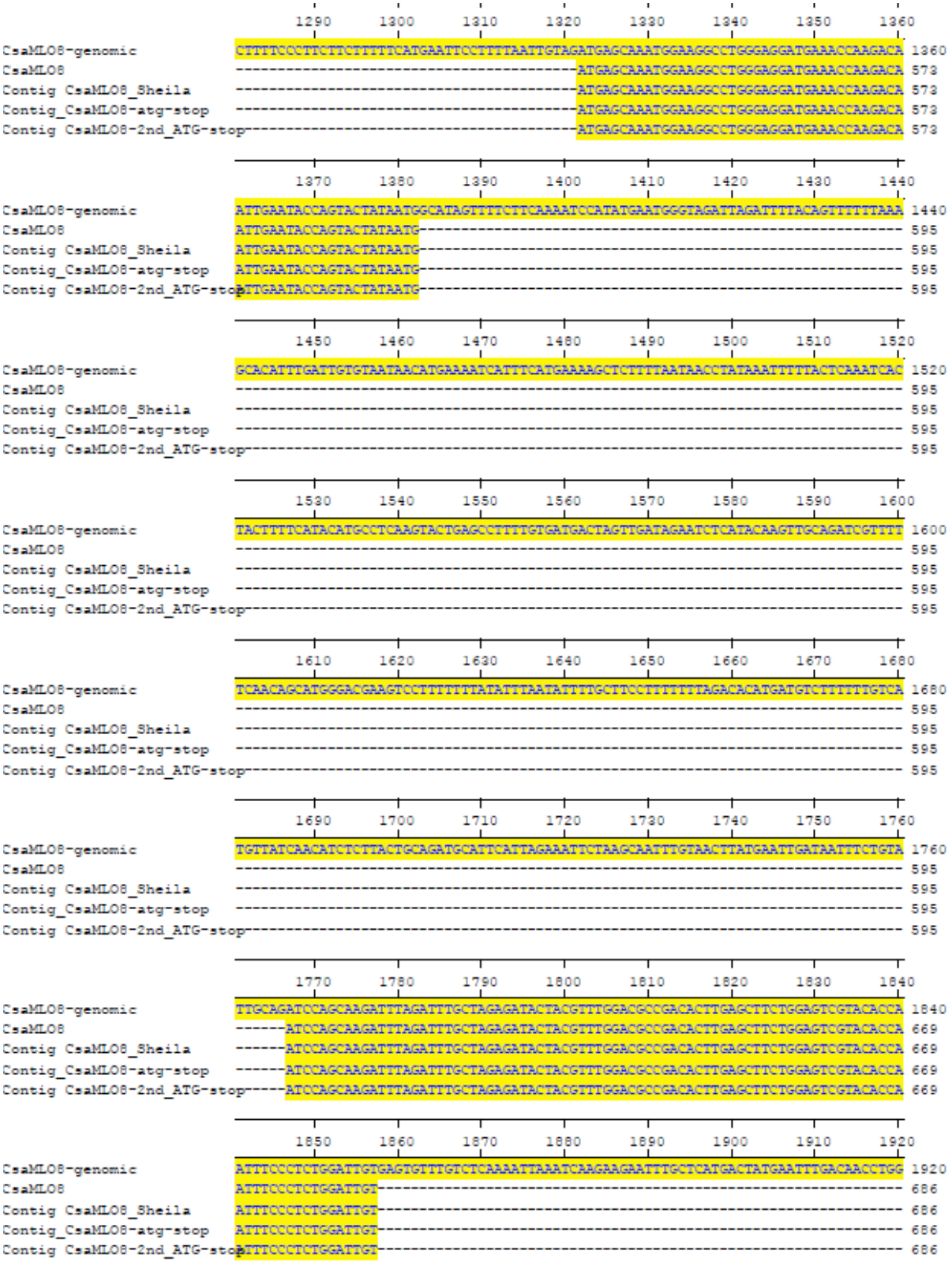
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Contig_CsaMLO8-atg-stop ATGAGCAAATGGAAGGCCTGGGAGGATGAAACCAAGACA 573
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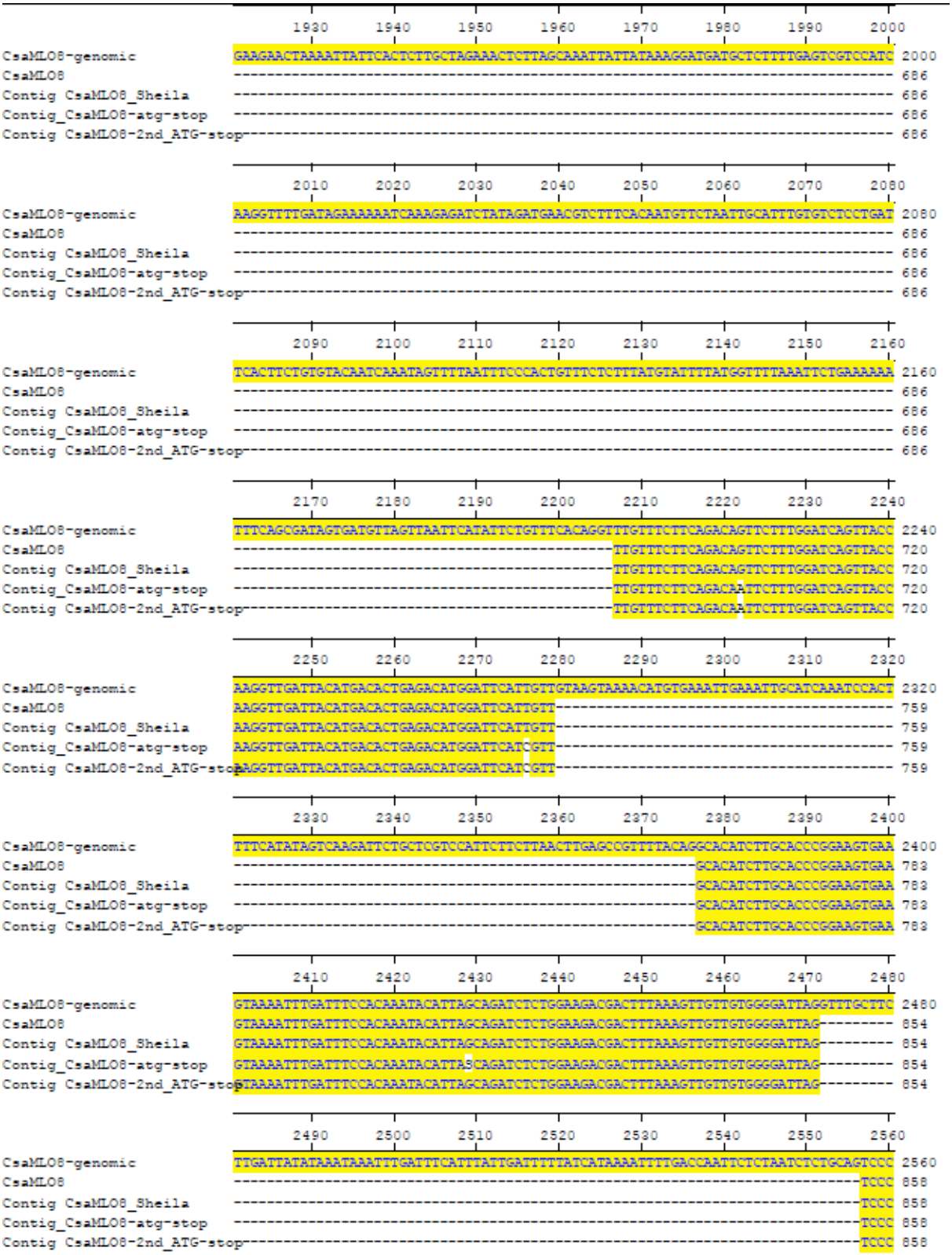
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Contig CsaMLO8_Sheila 686
Contig_CsaMLO8-atg-stop 686
Contig CsaMLO8-2nd_ATG-stop 686
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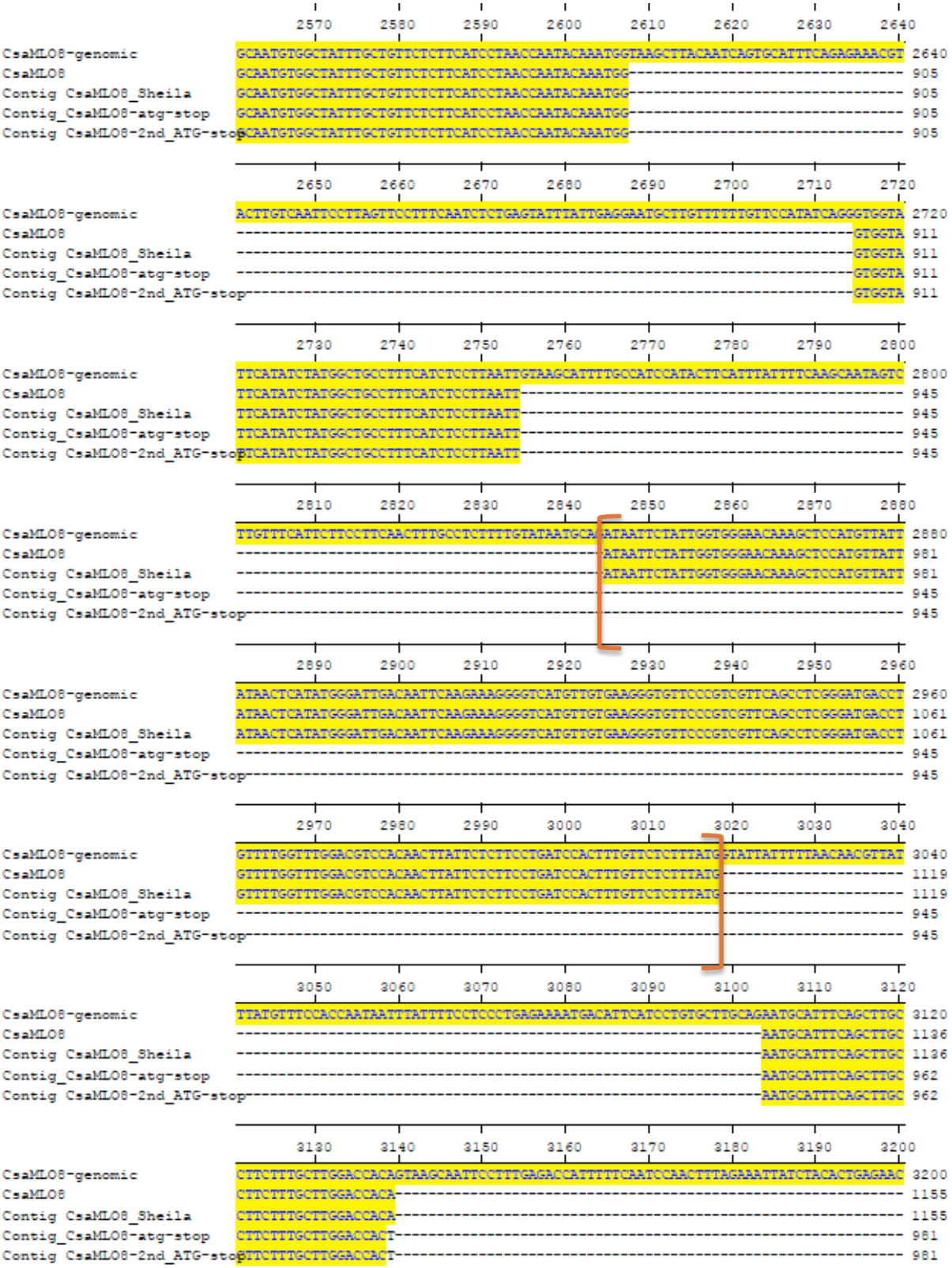
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Contig CsaMLO8-atg-stop GCAATGTGGCTATTTGCTGTTCTCTTCATCCTAACCAATACAAATGG---------- 905
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CsaMLO8 ----------GTGGTA 911
Contig CsaMLO8_Sheila ----------GTGGTA 911
Contig CsaMLO8-atg-stop ----------GTGGTA 911
Contig CsaMLO8-2nd_ATG-stop ----------GTGGTA 911
2730 2740 2750 2760 2770 2780 2790 2800
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Contig CsaMLO8-atg-stop TTCATATCTATGGCTGCCTTTCATCTCCTTAATT---------- 945
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Contig CsaMLO8_Sheila ----------ATAATTCTATTGGTGGGAACAAAGCTCCATGTTATT 981
Contig CsaMLO8-atg-stop ---------- 945
Contig CsaMLO8-2nd_ATG-stop ---------- 945
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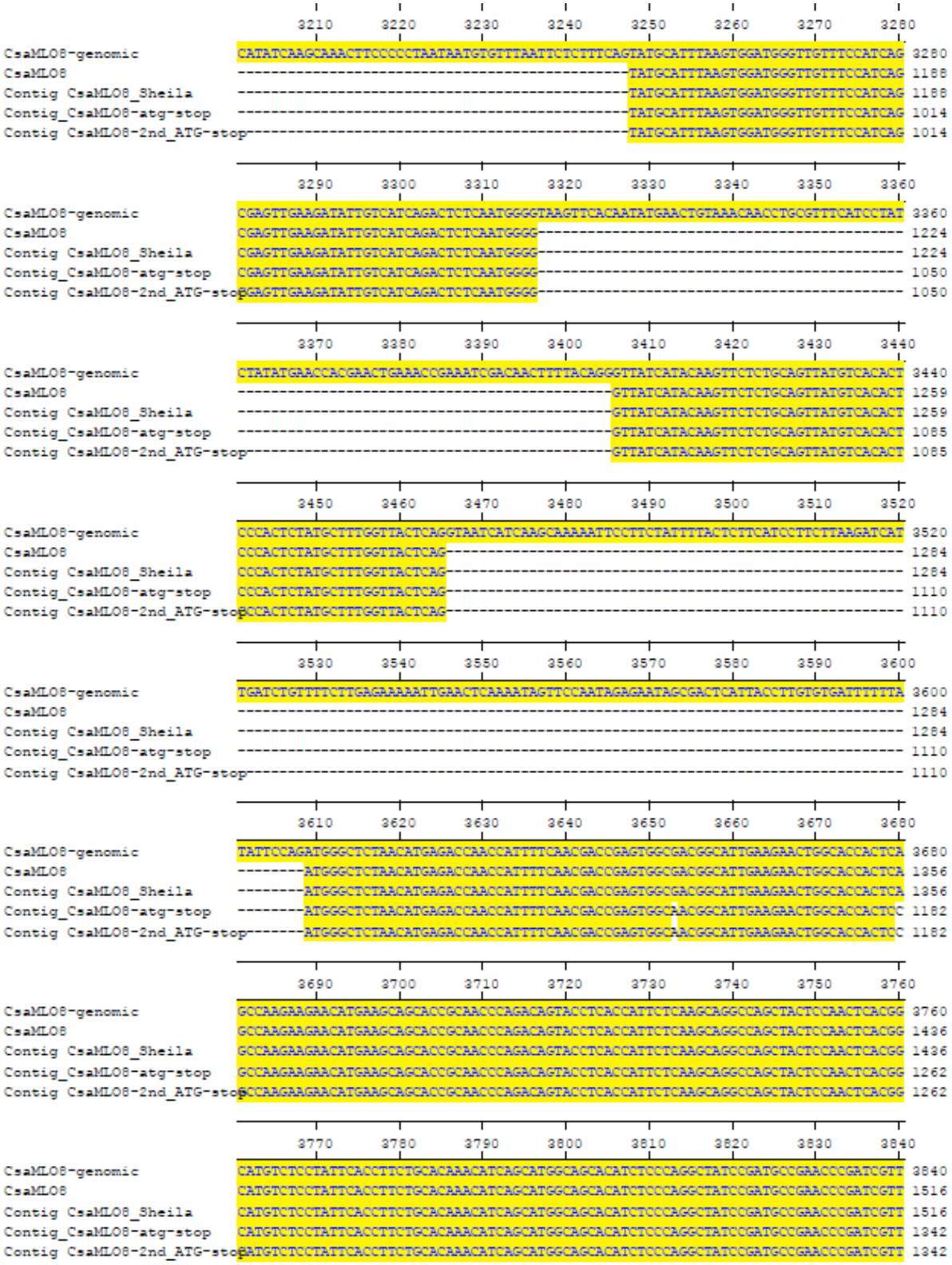
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Contig CsaMLO8_Sheila ATGGGCTCTAACATGAGACCAACCATTTTCAACGACCGAGTGGCGACGGCATTGAAGAACTGGCACCACTCA 1356
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Contig_CsaMLO8-atg-stop CATGTCTCCTATTCACCTTCTGCACAAACATCAGCATGGCAGCACATCTCCCAGGCTATCCGATGCCGAACCCGATCGTT 1342
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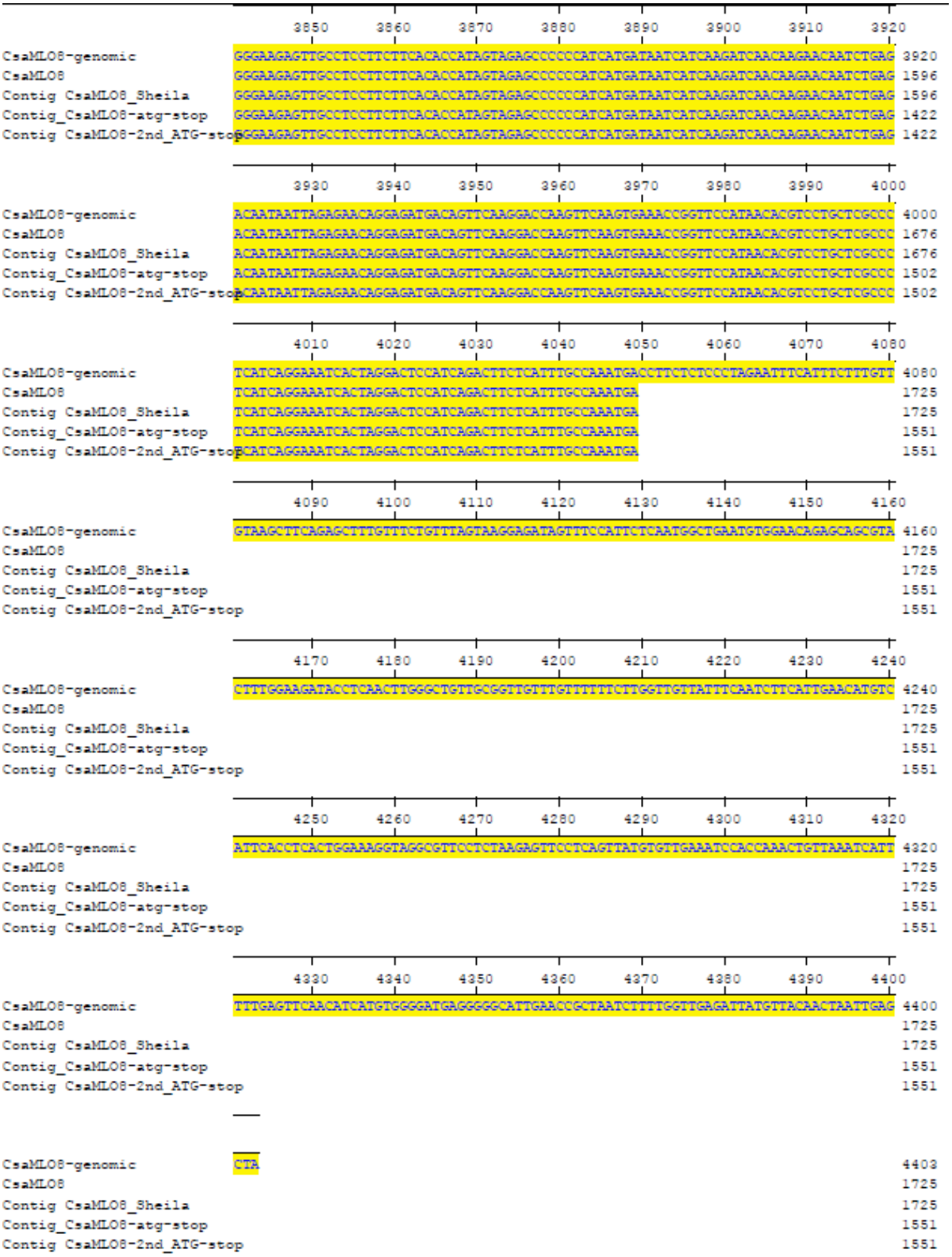
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3930 3940 3950 3960 3970 3980 3990 4000
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Contig CsaMLO8-2nd_ATG-stop ACAATAATTAGAGAACAGGAGATGACAGTTCAAGGACCAAGTTCAAGTGAAACCGGTTCCATAACACGTCCTGCTCGCCC 1502
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Contig CsaMLO8_Sheila 1725
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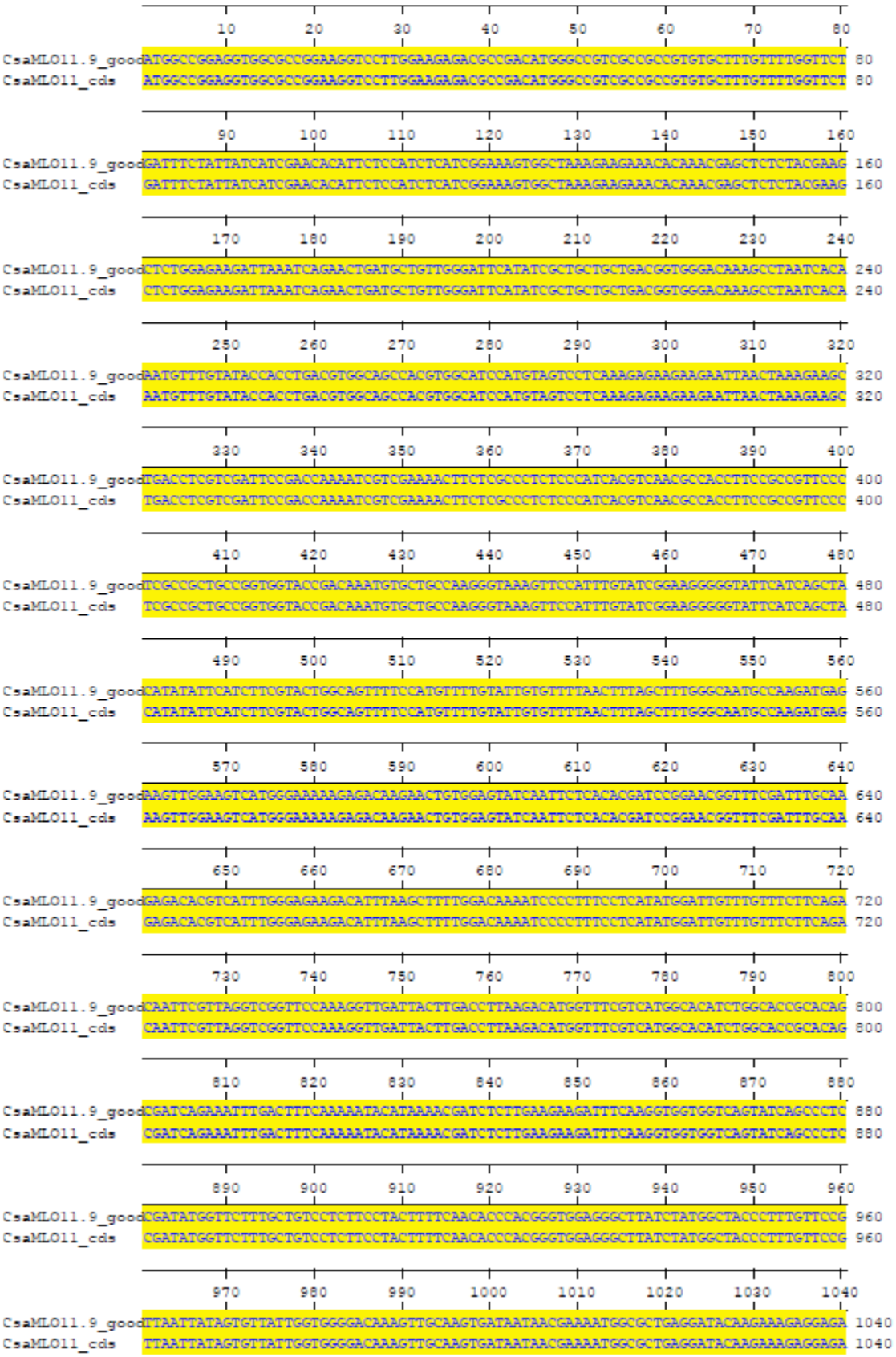
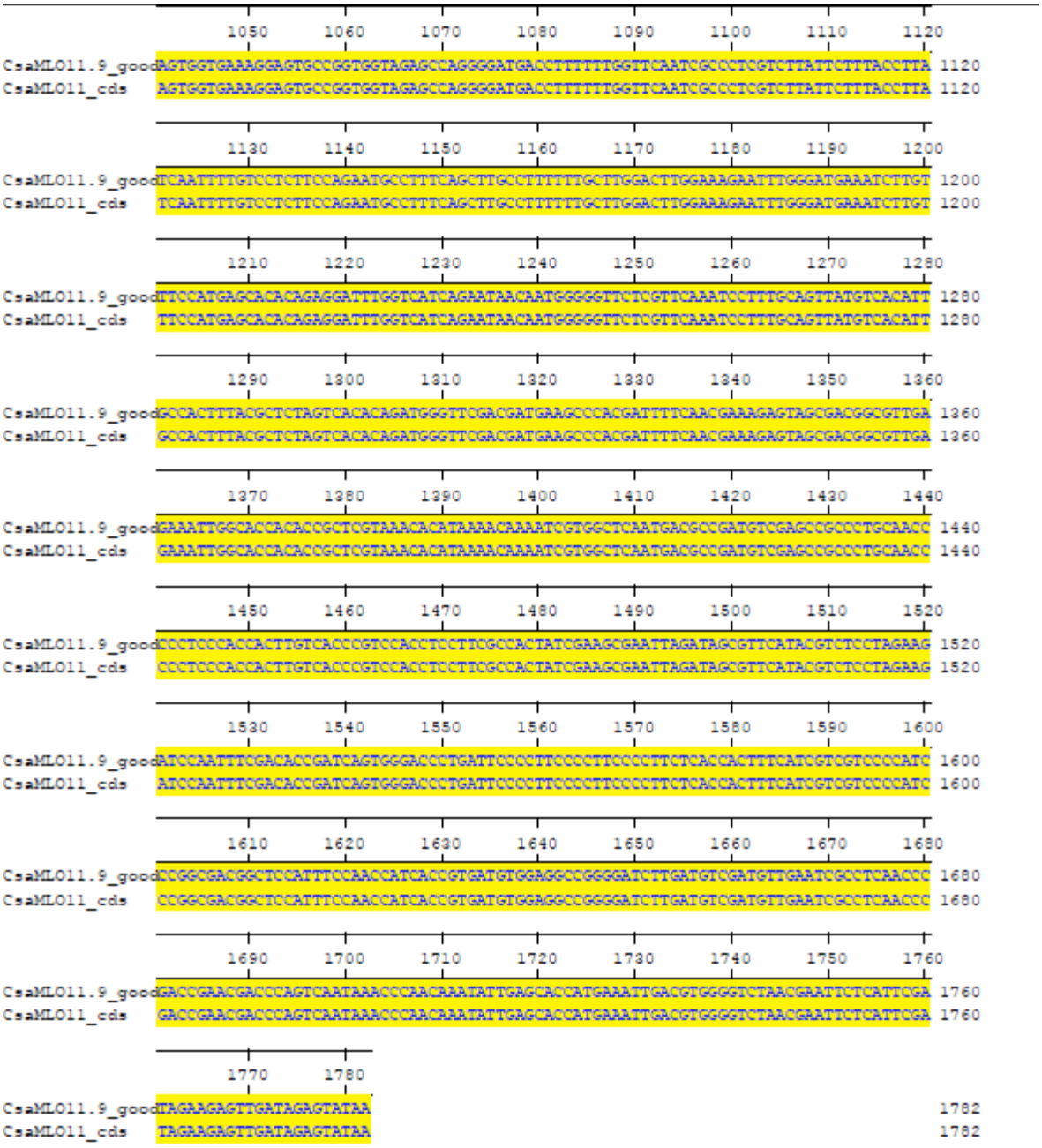
CsaMLO11 alignment.





2.6.2 Additional primer information

Table 2-6. Primer pairs tested. All primers tested for amplification of *CsaMLO* genes.

Target gene	Primer name	Forward primer	Primer name	Reverse primer	Length
CsaMLO1	FCMLO1-1	caccAAAAATCTGGCGATTTGGTG	RCMLO1-1	TGAATGGTGTAAACGAGATTGC	1836
	FCMLO1-2	caccAAAAATCTGGCGATTTGGTG	RCMLO1-2	TTCGAGATGAATGGTGTAAACG	1979
	FCMLO1-3	caccCCCCTTTGCTTTCTCACTTG	RCMLO1-3	TGAATGGTGTAAACGAGATTGC	1972
CsaMLO8	FCMLO8-1	caccCTGCCTCTCCACATGCATAA	RCMLO8-1	GCGCCCTGTACATGAAGAAC	1951
	FCMLO8-2	caccCTGCCTCTCCACATGCATAA	RCMLO8-2	CCAATCATCTCCCATGGCTA	1902
	FCMLO8-3	caccCGAAGACAGTTGTGCTTTGC	RCMLO8-3	GCGCCCTGTACATGAAGAAC	1914
CsaMLO11	FCMLO11-1	caccCTTCCAACCTTCCCCATTTT	RCMLO11-1	TATACCAACCCCCAACCTCA	2151
	FCMLO11-2	caccTTTGTTTCCCTACGCGTTCT	RCMLO11-2	TATACCAACCCCCAACCTCA	2144
	FCMLO11-3	caccTCGCCTTACACTTCCAACCT	RCMLO11-3	TATACCAACCCCCAACCTCA	2111

Table 2-7 Additional primer for confirmation of *CsaMLO11* sequence.

Primer name	Sequence
CsaMLO11-31_Fw	CGCCCTCTCCCATCACGTC
CsaMLO11-31_Rv	ATCTGGGCTTCATCGTCGAAC

2.6.3 List of plasmids sent for sequencing.

ID corresponds to the GATC cloning service identification number. Samples were labelled with the number of the gene, followed by a dot and the colony number in the plate from where they were taken. The primer used and the date in which they were sent are also described. Successful clones are indicated with a *.

pENTR/D-TOPO plasmid			
ID	**Sample (Gen.Colony)**	**Primer**	**Date**
66AI72	1.4*	M13Fw	1-7-2014
66AI73	1.4*	M13Rv	1-7-2014
66AI74	8.2	M13Fw	1-7-2014
66AI75	8.2	M13Rv	1-7-2014
66AI76	8.9	M13Fw	1-7-2014
66AI77	8.9	M13Rv	1-7-2014
66AI78	11.2	M13Fw	1-7-2014
66AI79	11.2	M13Rv	1-7-2014
66AI80	11.9	M13Fw	1-7-2014
66AI81	11.9	M13Rv	1-7-2014
66AJ01	1.27	M13Fw	2-7-2014
66AJ02	1.27	M13Rv	2-7-2014
66AJ57	11.2	M13Fw	4-7-2014
66AJ58	11.2	M13Rv	4-7-2014
66AJ59	11.8	M13Fw	4-7-2014
66AJ60	11.8	M13Rv	4-7-2014
66AJ61	11.9*	M13Fw	4-7-2014
66AJ62	11.9*	M13Rv	4-7-2014
66AJ63	8.17	M13Fw	7-7-2014
66AJ64	8.17	M13Rv	7-7-2014
66AJ65	8.20	M13Fw	7-7-2014
66AJ66	8.20	M13Rv	7-7-2014
66AJ67	8.22	M13Fw	7-7-2014
66AJ68	8.22	M13Rv	7-7-2014
66AJ69	8.28	M13Fw	7-7-2014
66AJ70	8.28	M13Rv	7-7-2014
66AJ71	1.24	M13Fw	7-7-2014
66AJ72	1.24	M13Rv	7-7-2014
92AC80	8u3	M13Fw	19-8-2014
92AC81	8u3	M13Rv	19-8-2014

92AC82	8u4	8-1Fw	19-8-2014
92AC83	8u4	8-1Rv	19-8-2014
92AC84	8u4	M13Fw	19-8-2014
92AC85	8u4	M13Rv	19-8-2014
92AC86	8L5	M13Fw	19-8-2014
92AC87	8L5	M13Rv	19-8-2014
92AC88	8L6	M13Fw	19-8-2014
92AC89	8L6	M13Rv	19-8-2014
92AC90	8s2*	M13Fw	19-8-2014
92AC91	8s2*	M13Rv	19-8-2014
92AC92	8s25	M13Fw	19-8-2014
92AC93	8s25	M13Rv	19-8-2014

pK7WG2 binary vector			
ID	**Sample (Gen.Colony)**	**Primer**	**Date**
91JH51	Lr-1.17	Pk7Fw	22-7-2014
91JH52	Lr-1.17	Pk7Rv	22-7-2014
91JH53	Lr-1.18	Pk7Fw	22-7-2014
91JH54	Lr-1.18	Pk7Rv	22-7-2014
91JH55	Lr1.19	Pk7Fw	22-7-2014
91JH56	Lr1.19	Pk7Rv	22-7-2014
91JJ96	Lr11.9	Pk7Fw	28-7-2014
91JJ97	Lr11.9	Pk7Rv	28-7-2014

3 Effect of heterologous expression of *HvMLO* in tomato in relation to non-adapted pathogen *Blumeria graminis* f. sp. *hordei*.

3.1 Introduction

Resistance against powdery mildew conferred by a loss-of-function, recessively inherited allele of the *MLO* gene has been reported in both monocot and dicot species (Chapter 1). In a recent study, functional conservation has been found to be present between a monocot and a dicot *MLO* gene in a compatible interaction (Appiano et al, in preparation). In such study, TV123701, a T2 family of transformed tomato overexpressing *HvMLO* from barley in the otherwise resistant background *ol-2*, was found to be partially susceptible against the adapted pathogen *Oidium neolycopersici* (Figure 3-1).

Non-host resistance is the result of successive layers of plant defences that leads to the inability of a non-adapted pathogen to infect a plant (Thordal-Christensen, 2003) (Chapter 1). Pre-penetration or pre-haustorial non-host resistance is present when a fungal pathogen is unable to establish a functional haustorium. This kind of resistance commonly leads to the formation of papillae and is frequently backed up by hypersensitive response (HR) (Chapter 1).

Non-host resistance and *mlo*-based resistance have been argued to rely on identical defence mechanisms (Humphry *et al.*, 2006, Thordal-Christensen, 2003). One of the most evident features shared by both types of resistance is the formation of papillae. This response has been documented to be present in the incompatible host interaction between barley carrying a loss-of-function *MLO* allele and the pathogen *Blumeria graminis* f. sp. *hordei (Bgh)*, as well as the non-host interaction of wild type *Arabidopsis* with the same pathogen (Thordal-Christensen, 2003).

In tomato, non-host resistance of cultivar Moneymaker against the barley powdery mildew pathogen *Bgh* has been reported to be dependent on HR (Sameshima *et al.*, 2004) (Figure 3-2). However, the relationship between the incidence of HR and papillae formation in response to the non-adapted pathogen *Bgh* and the heterologous expression of a functional *MLO* gene in a resistant tomato has not yet been studied.

In this experiment, Moneymaker (a tomato line naturally homozygous for the wild-type *SlMLO1*), *ol-2* (a tomato line carrying a loss-of-function mutation of *SlMLO1*) and the transgenic T2 family TV123701 (*ol-2* 35S::*HvMLO*) were challenged with *Bgh*. Macroscopic evaluation and histological analysis were carried out to elucidate the effect of the heterologous expression of the monocot *HvMLO* gene in *ol-2* tomato in a non-host interaction.

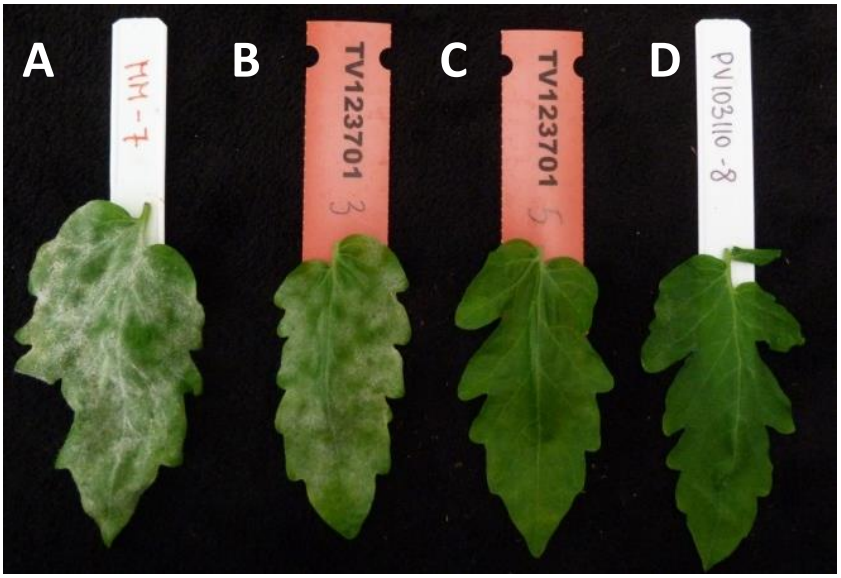


Figure 3-1 Complementation of barley *HvMLO* in a resistant tomato against powdery mildew (*Oidium neolycopersici*) (Appiano et al., in preparation)**.** A) Moneymaker leaf shows complete susceptibility to the pathogen. B) Leaf of TV123701, *ol-2* background transformed with a *35S::HvMLO* construct, displaying compromised resistance against powdery mildew. C) Leaf from a non-transgenic plant (T2 *ol-2::35S::HvMLO)* showing resistance against powdery mildew. D) *ol-2* plant showing a complete resistance against powdery mildew.

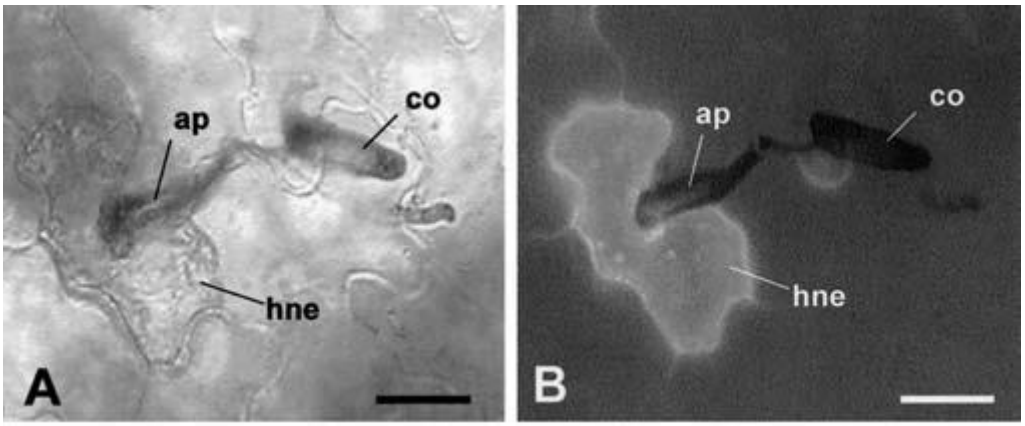


Figure 3-2 Microscopic image of the histological responses of Moneymaker tomato against Blumeria graminis f. sp. hordei (Sameshima *et al.*, 2004)**.** A) Light microscopic image and b) fluorescent microscopic image showing conidiospore (co), appressorium (ap) and hypersensitive necrosis (hne) in an infection unit 72 hpi.

3.2 Materials and methods

3.2.1 Plant material

Tomato lines Moneymaker and *ol-2* (PV103110), and transgenic T2 family TV123701 were used in this experiment. Moneymaker is susceptible to powdery mildew and naturally carries a functional *SlMLO1* gene. *ol-2* is a tomato line carrying a loss-of-function *MLO* allele, and thus resistant to powdery mildew, obtained from a cross between *Solanum lycopersium* var *cerasiforme* and *S. lycopersicum* cv *Super Marmande.* Seeds of the T2 family TV123701 (*ol-2* 35S::*HvMLO*) were provided by Michela Appiano.

Six Moneymaker and six *ol-2* plants were sown and grown in an isolated compartment in the greenhouse at Unifarm (Wageningen University). At the same time, seeds of the T2 family TV123701 were sterilized using a NaClO solution and sown on MS agar supplemented with sucrose and 50 mg/ml kanamycin for selection of transgenic plants. The sown seeds were left during two days at 4˚C to avoid dormancy effects and then transferred to a growing chamber for 12 days. Transgenic plantlets were taken to the greenhouse to be transplanted in pots. Ten plants of the transgenic T2 family TV123701 were used in this study. Additionally, two barley plants (cv. *Manchuria*), susceptible to *Bgh*, were used as controls for the pathogen inoculation.

3.2.2 Inoculation with *Bgh*

Around 18 days after transplanting, all plants were taken into an infection chamber at Unifarm (Wageningen University). This chamber was previously used only for maintaining barley plants infected with *Bgh*. An isolate of barley powdery mildew (*Bgh*), provided by Cynara Romero, was used to perform the inoculation. Fresh conidia of *Bgh* were applied using a paintbrush to the adaxial surface of barley and tomato leaves as done in a similar study by Hao et al (Hao *et al.*, 2013).

3.2.3 Staining of samples and histological analysis

At least three samples from the third or fourth leaf of the inoculated tomato plants and three barley leaves were collected at 30 and 72 hours post-inoculation (hpi). Per time point, samples from three Moneymaker plants, three *ol-2* plants and four transgenic plants were taken. Bleaching and fixation was performed by putting the leaves in a 1:3 (v/v) acetic acid-ethanol solution immediately after cutting. Only samples taken 72hpi were stained and analysed in this study. A different leaf was taken for mounting each slide. At least 48 hours after bleaching, the leaves were cut in pieces of around 3 cm^2. Leaf fragments were stained by heating them in a 1:2 (v/v) lactophenol/ethanol solution with 0.03% trypan blue for five to ten minutes at 90˚C. After staining, the leaf fragments were decoloured using 5kg:2L (w/v) saturated chloral hydrate for at least 48 hours. Once cleared, samples were mounted on glass slides with a 1:1 (v/v) glycerol-water solution and sealed using transparent nail polish. Analysis of the slides was done using a Zeiss Axiophot bright field microscope and pictures were taken with an Axiocam ERc5s and a Canon Powershot A620.

In total, three slides for barley, nine slides for Moneymaker, nine slides for *ol-2* and 12 slides for TV123701 were made. Three slides of Moneymaker and three slides *ol-2*, each of them from different plants, and nine slides of TV123701 from four different plants were analysed. For each slide, the number of non-germinated spores and the number of spores forming an appressorium germination tube were counted. The response against the non-adapted pathogen in tomato leaves was evaluated

by counting the number of infection units with HR and the number of infection units with papilla and no HR. A total of 30 germinated spores were counted on each slide. A 100x magnification was used.

In tomato, the percentage of germination was estimated relative to the total amount of spores observed. The number of infection units showing HR and papillae formation without HR are expressed as percentage of the total infection sites.

3.3 Results

3.3.1 Macroscopic evaluation

At macroscopic level, no tomato plants showed visible symptoms of powdery mildew 23 days after inoculation. Barley leaves were visibly infected at this same time point, presenting white pustules indicating sporulation on the leaves (Figure 3-3).

Figure 3-3. Macroscopic evaluation of tomato lines Moneymaker (MM) and *ol-2* (PV103110) and T2 family TV123701 (*ol-2* 35S::*HvMLO*). No powdery mildew symptoms were seen on the leaves compared to the barley control.

3.3.2 Histological analysis

Barley leaves were analysed at the microscope as a reference of normal development of *Bgh* in its interaction with a host. At 72 hpi, germinated spores and a dense hyphal growth were observed on the leaves. Functional haustoria were clearly observed in the barley samples (Figure 3-4)

In tomato, primary germination tubes and appressorium germination tubes were observed in germinated spores but no functional haustorium was present. The percentage of germination was stable across the different genotypes (above 55%). The percentage of spores that were able to form an appressorium germination tube maintained above 90% across all the samples observed (Table 3-1).

HR and papillae without HR were visible across all the samples. However, the incidence of papillae without HR was higher in *ol-2* (67.8%) than in the genotypes carrying a functional *MLO* (14.4% in Moneymaker and 30.4% in TV123701) (Table 3-1; Figure 3-6; Figure 3-5B, D and F). Contrastingly, the incidence of HR was higher in Moneymaker (80%) and TV123701 (66.7%) compared to *ol-2* (22.2%) (Table 3-1; Figure 3-7; Figure 3-5A, C and E). This leads to the indication that as *SlMLO1* in tomato allows the penetration of *Bgh* in a non-host interaction, so does *HvMLO* when overexpressed in the *ol-2* resistant tomato background and thus, *HvMLO* can functionally complement the mutant *SlMLO1* allele.

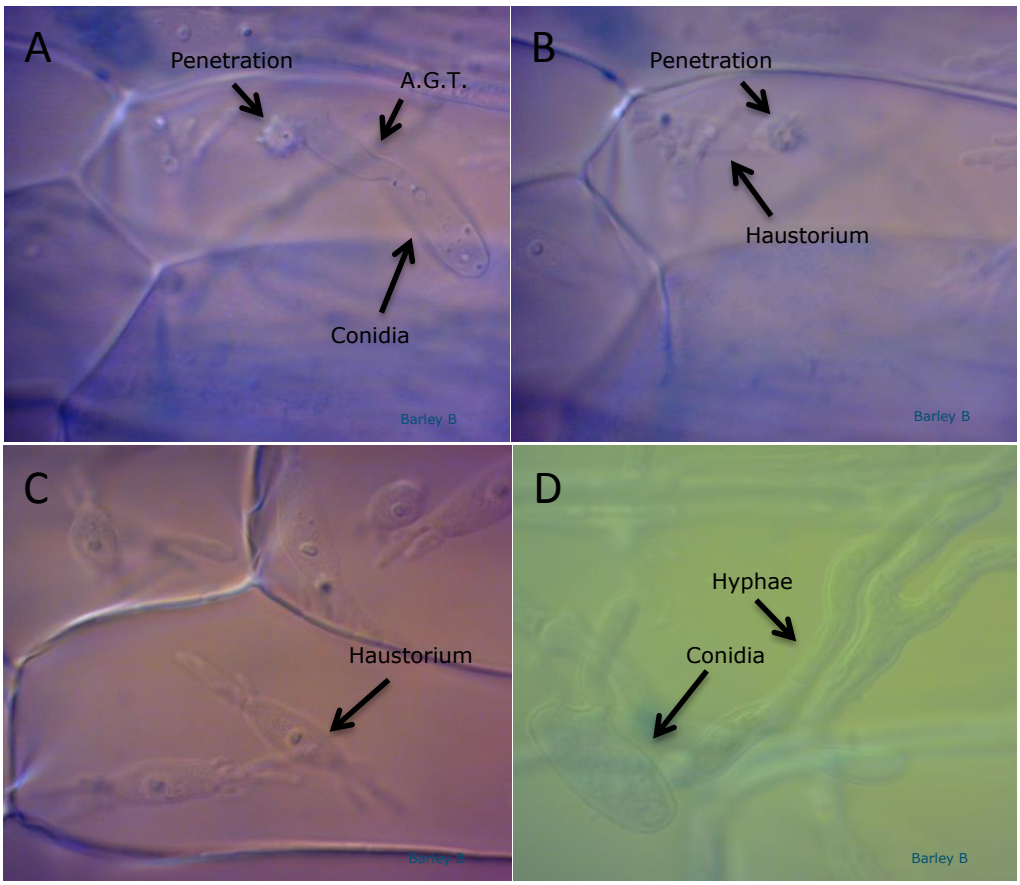


Figure 3-4. Microscopic images of the structures of *Blumeria graminis* f. sp. *hordei* (*Bgh*) infecting a susceptible barley. A) A conidia with an appressorium germination tube and the site of penetration. B) Corresponds to the same area of (A) at a deeper focal distance, showing the site of penetration and the haustorium formed inside of the plant cell. C) The characteristic shape of a functional haustorium inside of the plant cell. D) A *Bgh* conidia with an elongated hypha, showing the fungus in advanced development.

Table 3-1. Results from the histological analysis showing the average values and percentages of the different phenomena analysed. NG spores: average number of non-germinated spores. % Germ: Percentage of germinated spores relative to the total amount of spores counted. Nr AGT: average number of conidia developing an appressorium germination tube. %AGT: percentage of conidia developing an appressorium germination tube relative to the total amount of germinated spores. Nr papillae without HR: average number of infection units that presented papillae without a hypersenstive response. % papillae without HR: percentage of infection units that presented papillae without a hypersensitive response relative to the total amount of germinated spores. Nr HR: average number of infection units that triggered a hypersensitve response. %HR: percentage of infection units that triggered a hypersensitive response relative to the total amount of germinated spores. MM: Moneymaker. TV123701: T2 family expressing *HvMLO* in an *ol-2* background. Additional information on is described in Table 3-2.

Sample	NG spores	% Germ	Nr AGT	% AGT	Nr Papillae without HR	% Papillae without HR	Nr HR	% HR
MM	23.3	56.3%	28.3	94.4%	4.3	14.4%	24.0	80.0%
ol-2	21.7	58.1%	27.0	90.0%	20.3	67.8%	6.7	22.2%
TV123701	24.3	55.2%	29.1	97.0%	9.1	30.4%	20.0	66.7%

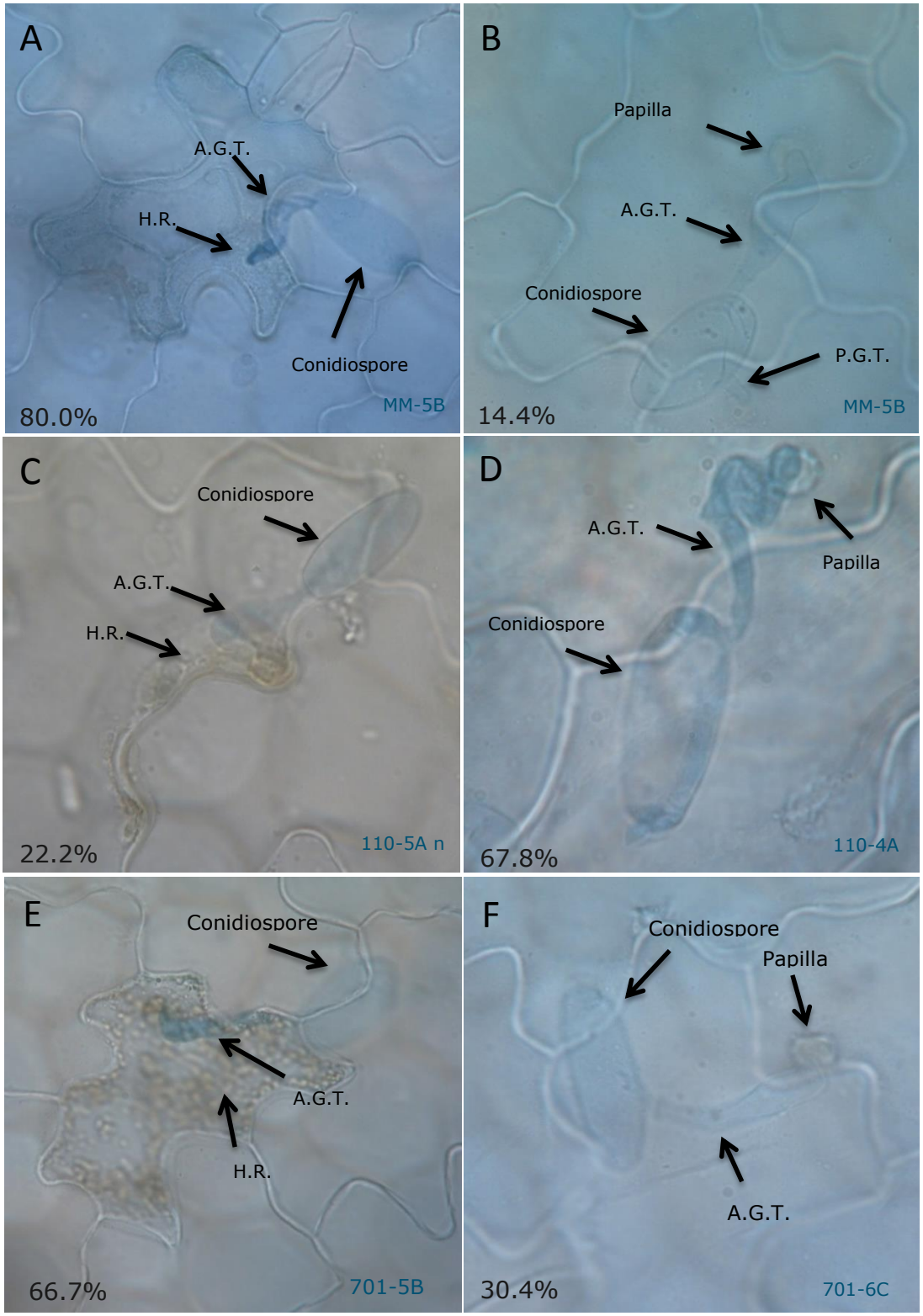


Figure 3-5 Histological responses in the non-host interaction with *Blumeria graminis* f. sp. *hordei* (*Bgh*) across the different lines and T2 family tested. Moneymaker (A and B), *ol-2* (C and D) and T2 family TV123701 (E and F) were analysed. The percentage in the left bottom corner of each picture represents the incidence of HR (A, C and E) and papillae without HR (B,D and F). A.G.T.: Appressorium germination tube. P.G.T.: Primary germination tube. H.R.: Hypersensitve response.

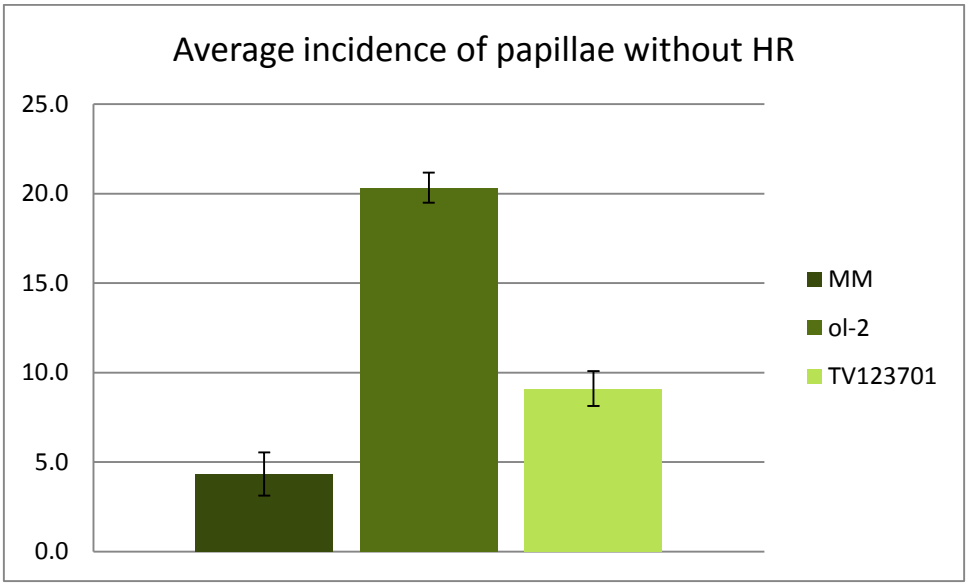


Figure 3-6. Graphic showing the average incidence of infection units that presented papillae without a hypersensitive response in Moneymaker (MM), *ol-2* and the T2 family TV123701. The highest incidence of papillae without HR was present in *ol-2*. Incidence of the same phenomenon in the T2 family TV123701 was lower than in *ol-2* but not as low as in *Moneymaker*.

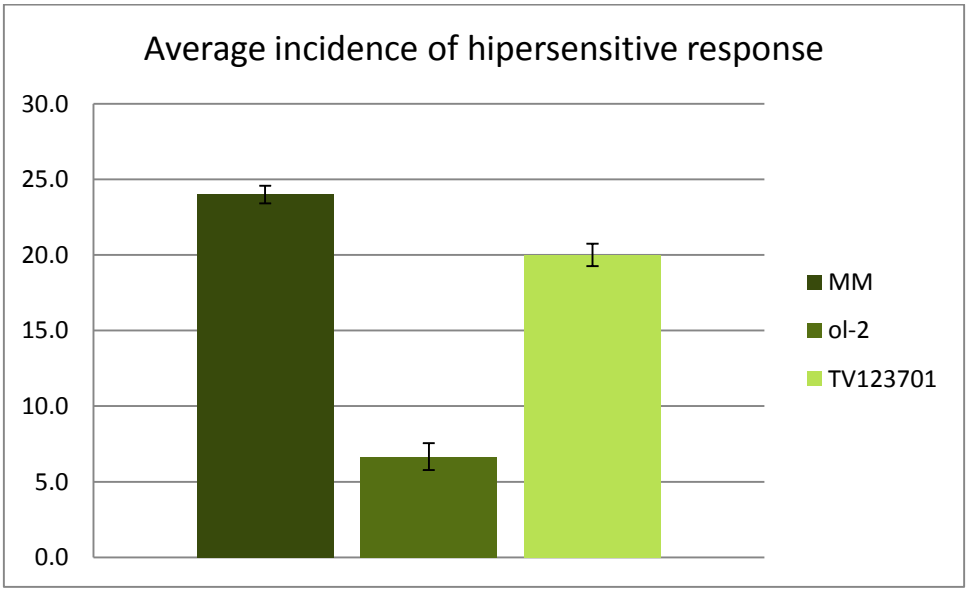


Figure 3-7. Graphic showing the average incidence of infection units that triggered a hypersensitive response in Moneymaker (MM), *ol-2* and the T2 family TV123701. The highest incidence of hypersensitive response was present in Moneymaker. The incidence of HR in the T2 family TV123701 was lower than in Moneymaker but not as low as in *ol-2*.

3.4 Discussion

The results from the macroscopic analysis showed that the heterologous expression of *HvMLO* in the *ol-2* mutant tomato background does not compromise its non-host status for *Bgh*. However, at the microscopic level, interesting variations on the cellular responses were observed. Two plant cellular events in response to *Bgh* were analysed: the formation of papillae without HR and the induction of HR.

Comparing the response of *ol-2* and Moneymaker, a non-functional *SlMLO1* in tomato appears to have a positive effect on the formation of papillae at sites of attempted penetration. The early formation of papillae at sites of fungal penetration has been associated to resistant plants carrying the loss-of-function *mlo* allele in response to adapted pathogens (Assaad *et al.*, 2004, Bai *et al.*, 2005, Lyngkjær *et al.*, 2000, Underwood&Somerville, 2008).

Appiano et al. (in preparation) showed that the expression of *HvMLO* in the *ol-2* background negatively affected the incidence of papillae, allowing growth and sporulation of *O. neolycopersici*, indicating a functional conservation with tomato *SlMLO1*. Together with the results from the present experiment, these findings suggest that the functional conservation exists also in the non-host interaction responses against *Bgh*. It is interesting to notice that the rate in which the papillae formation was affected by the expression of *HvMLO* in the present experiment is comparable with the rate in which the disease index (DI) was affected in the experiment involving *O. neolycopersici* using the same lines and T2 family. In the present experiment, papillae formation was considerably lower in the T2 family than in the background *ol-2*, while in the experiment involving *O. neolycopersici,* the DI was clearly higher in the T2 family than in *ol-2*. Interestingly, in both experiments, the levels of these parameters did not reach the same levels as in Moneymaker. Two factors may be accounted for this variation. First, that the levels of expression of *HvMLO* in the T2 family are not as high as the levels of the wild-type *MLO* gene in Moneymaker or, second, that the functional conservation between the *MLO* genes of tomato and barley is not complete.

HR post-penetration resistance in the sites of attempted fungal attack has been reported to arise as a backup defence response of the pre-penetration immune reactions in non-host interactions (Lipka *et al.*, 2008, Niks&Marcel, 2009) (Chapter 1). Additionally, the occurrence of HR provides a criterium for classification of non-host resistance (Mysore&Ryu, 2004) (Chapter 1). The cellular events analysed in this experiment can be discriminated in accordance to this classification. The cases when a papilla was formed and no HR occurred belong to non-host resistance type I, while all the cases when an HR was present belong to type II. The results of this experiment show that the presence of a functional *MLO* gene has a negative effect on the occurrence of type I non-host resistance and induces a more frequent type II reaction. However, the phenomena of papillae formation and HR are not disconnected or exclusive and important considerations should be made to account the incidence of both events over time. In this experiment, we discriminated between these two events at one time point (72hpi). However, the assessment of earlier and later time points will help to understand how the incidence of these events vary over time.

From the results of this experiment, three important conclusions can be drawn. First, a non-functional *MLO* gene can positively affect the incidence of formation of papillae as a defence response in the non-

host interaction as in host interaction. Second, the heterologous expression of the monocot gene *HvMLO* can negatively affect the incidence of papillae in tomato. And third, the non-host resistance of tomato against *Bgh* does not entirely rely on the formation of papillae.

Two clear indications that the non-host status of tomato was not compromised by the heterologous expression of *HvMLO* were found. Firstly, the macroscopic studies indicated no powdery mildew symptoms in any of the tomato plants. Secondly, the histological analysis confirmed that no functional haustorium was established by the fungus in the tomato leaves. Albeit the incidence of papillae was shown to be affected by the heterologous expression of a functional *MLO* gene, it was clear that the pathogen was still recognized by the plant immune system and HR was triggered in those cases. Indeed, non-host resistance is known to be polygenic and to rely on several immune responses (Niks&Marcel, 2009).

Other experiments to dissect non-host resistance have proven that in model plant *Arabidopsis*, resistance against *Bgh* depend on both pre- and post-invasion defences (Lipka *et al.*, 2005). The same experiment also showed that the genetic distance between a non-adapted pathogen of a certain plant and its respective adapted pathogen affects the incidence of the different defence responses triggered in the plant. It will be interesting to know if challenging with a non-adapted pathogen that is genetically closer to the adapted *O. neolycopersici* would lead to susceptibility or if the infection process would go further than the formation of an appressorium germination tube when the first layer of defence is compromised by the expression of a functional *MLO* gene.

3.5 Future research and recommendations.

Papillae formation is considered to be an early defence mechanism against adapted and non-adapted powdery mildew pathogens (Aghnoum&Niks, 2010) and is believed to provide physical and chemical barriers to stop or delay the infection process (Hückelhoven, 2007). By the outcome of the fungal penetration attempt, papillae can be classified as effective or ineffective (Chowdhury *et al.*, 2014) . In the present experiment, it was not possible to make a clear distinction between events when papillae was penetrated (ineffective papillae) and those when papillae was not present. To better assess the possible effect of *MLO* on the papillae incidence, architecture and thus its effective or ineffective characteristic, an alternative staining method using wintergreen oil (methyl salicylate) (Niks, 1986) is suggested.

Additionally, in barley, *mlo*-based resistance is reported to be associated with the formation of vesicle-like bodes containing H_2O_2 or phenolics accumulating around the papillae (Hückelhoven, 2007). These structures are believed to be multicomponent kits containing papillae components and anti-microbial compounds that could be essential to stop fungal penetration. For this reason, staining with 3,3-

diaminobenzidine (DAB) is proposed as a method to give a better insight of the effect of *MLO* over the papillae architecture and effectiveness in non-host resistance.

The MLO protein is known to negatively regulate the disease resistance associated to the effect of *PEN1*, *-2*, and *-3* in *Arabidopsis* and *ROR1* and *ROR2* in barley (Chapter 1). Lipka et al. (Lipka *et al.*, 2005) demonstrated that mutation of *PEN1* and *PEN2* genes in *Arabidopsis* had a severe effect in the fungal entry rates and the incidence of invasion associated-cell death in the non-host interaction with *Bgh*. To study the effect of the silencing of the *PEN* genes in the light of a non-host interaction in tomato would help to confirm the overlapping mechanistic defences of *mlo*-based and non-host resistance.

A first attempt of this experiment was carried out with a larger number of plants of the same lines and T2 family. However, a spontaneous infection of the adapted pathogen *O. neolycopersici* made impossible to continue with the study. The use of an infection chamber for the inoculation of *Bgh* was an effective solution for such problem, as no contamination occurred. A larger experiment with more samples would help to improve the statistical power of the results, however, the isolation of the plants from other pathogens is essential in this kind of study. Alternatively, a detached-leaf assay, as the one made by Hao et al. (Hao *et al.*, 2013) in pepper, could be a possibility for having more replicates with more control over the isolation of the samples.

The selection of the transgenic plants was made by sowing the seeds in medium with antibiotics. However, slow growth after transplanting was found on these plants. Sowing in soil and later selection through molecular markers is recommended to avoid this effect.

During the staining of the samples, boiling in the solution containing trypan blue was a critical step to produce clear slides. It is recommended to boil the samples long enough to obtain an intense blue coloration before clearing in chloral hydrate.

The histological analysis is a lengthy process that requires careful inspection of the surface of the leaves. In this analysis, three critical steps are required to be done with particular attention. First, the 40x magnification can provide a general indication of the quality of the sample and the number of infection units present. However, a 100x magnification is necessary to get accurate assessments of the cellular events. Second, the focal distance of the microscope is important for a precise analysis. An easy reference point for using a right focal distance is to maintain it at the level where the epidermal cell walls appear as a white line between the epidermal cells (Figure 3-5). Third, when inspecting the leaf surface, a hypersensitive response is a much more evident phenomenon than the formation of papillae. Special attention should be put to not oversee the infection units that have no hypersensitive response. The use of 100x magnification and an appropriate amount of light are ways to reduce mistakes in this step.

3.6 References

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3.7 Supplementary material

3.7.1 Results from the histological analysis.

Table 3-2 Results from the histological analysis showing the values and percentages of the different phenomena analysed for each sample. NG spores: number of non-germinated spores. Total IU counted: total number of infection units counted. % Germ: Percentage of germinated spores relative to the total amount of spores counted. Nr AGT: number of conidia developing an appressorium germination tube. %AGT: percentage of conidia developing an appressorium germination tube relative to the total amount of germinated spores. Nr papillae without HR: number of infection units that presented papillae without a hypersenstive response. % papillae without HR: percentage of infection units that presented papillae without a hypersensitive response relative to the total amount of germinated spores. Nr HR: number of infection units that triggered a hypersensitve response. %HR: percentage of infection units that triggered a hypersensitive response relative to the total amount of germinated spores. MM: Moneymaker. TV123701: T2 family expressing *HvMLO* in an *ol-2 background*.

Sample	Total spores counted	NG spores	Total IU counted	% Germ	Nr AGT	% AGT	Nr Papillae without HR	% Papillae without HR	Nr HR	% HR
MM-4B	56	26	30	53.6%	27	90.0%	2	6.7%	25	83.3%
MM-5B	51	21	30	58.8%	29	96.7%	5	16.7%	24	80.0%
MM-6A	53	23	30	56.6%	29	96.7%	6	20.0%	23	76.7%
110-4A	53	23	30	56.6%	27	90.0%	20	66.7%	7	23.3%
110-5A	49	19	30	61.2%	26	86.7%	18	60.0%	8	26.7%
110-6B	53	23	30	56.6%	28	93.3%	23	76.7%	5	16.7%
701-5A	55	25	30	54.5%	29	96.7%	10	33.3%	19	63.3%
701-5B	57	27	30	52.6%	30	100.0%	9	30.0%	21	70.0%
701-5C	49	19	30	61.2%	26	86.7%	9	30.0%	17	56.7%
701-6B	55	25	30	54.5%	30	100.0%	7	23.3%	23	76.7%
701-6C	57	27	30	52.6%	29	96.7%	12	40.0%	17	56.7%
701-7A	54	24	30	55.6%	30	100.0%	7	23.3%	23	76.7%
701-7B	57	27	30	52.6%	30	100.0%	11	36.7%	19	63.3%
701-7C	58	28	30	51.7%	29	96.7%	8	26.7%	21	70.0%
701-8B	47	17	30	63.8%	29	96.7%	9	30.0%	20	66.7%

4. Virus-induced gene silencing (VIGS) for candidate gene elucidation of *Ol-qtl2*

4.1 Introduction

Cultivated tomato is highly homozygous and poor genetic variability is found across the different cultivars. However, the large genetic diversity of its wild relatives is a useful genetic source for plant breeders (Bai&Lindhout, 2007). Tomato is a host for three species of powdery mildew (Chapter 1). Out of these, *Oidium neolycopersici* is a severe problem worldwide, especially in areas with high humidity. So far, nine loci have been found to confer resistance against *O. neolycopersici* in wild species of tomato (Figure 4-1). In total, six resistance genes have been characterized (*Ol-1*, *ol-2*, *Ol-3*, *Ol-4*, *Ol-5*, and *Ol-6*), all of them mapping in chromosome six, except for the recessive *ol-2*, which is found in chromosome four. Additionally, three quantitative resistance loci (QRL) have been found in the wild tomato species *Solanum neorickii* G1.1601 (Bai *et al.*, 2003) and have been fine mapped on chromosome 6 and 12 (Faino *et al.*, 2012).

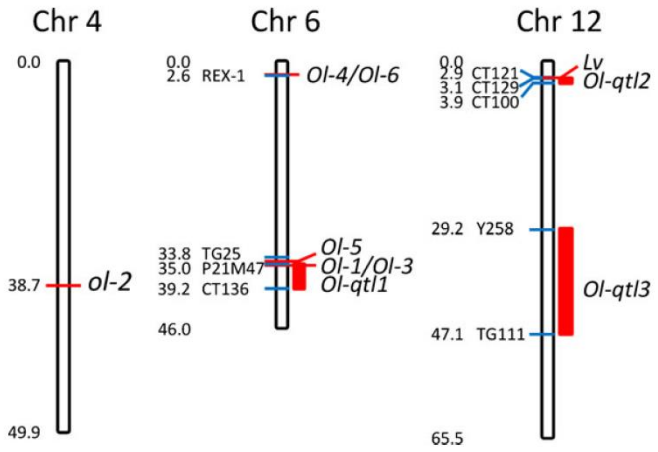

Figure 4-1. Resitance genes and quantitative trait loci conferring resistance to *Oidium neolycopersici* in tomato (Seifi *et al.*, 2014)**.**

Ol-qtl2 is a QRL that localizes on chromosome 12, adjacent to the *R* gene *Lv,* conferring resistance to another powdery mildew pathogen, *L. taurica* (Faino, unpublished results). This QRL explains about 30% of the resistance in *S. neorickii* and the response mechanisms associated with this resistance is a slow hypersensitive response (HR) under the fungal colonies *(Li et al., 2012)*. An expression analysis of the genes present in the *Ol-qtl2* region revealed the inducement of expression of an RLP-like gene upon infection with *O. neolycopersici* (Faino et al., not yet published).

Identifying the molecular mechanisms of quantitative resistance is particularly difficult, as a wide range of mechanisms can be involved in such resistance (Poland *et al.*, 2009). Compared to *R* genes, QRL-

conferred resistance is weaker and partial, but is also usually more durable (Parlevliet, 2002). It has been proposed that QRLs are mutations or different alleles of genes involved in pathogen-associated molecular patterns (PAMP) recognition (Poland *et al.*, 2009). This is the case for the *Arabidopsis* genes *BRI1* and *BRF1* which were mapped as QRLs and are proven to interact with *FLS2* for the perception of the PAMP flagellin of the bacterial pathogen *Pseudomonas syringae* pv. *phaseolicola* (Forsyth *et al.*, 2010).

PAMPS are recognized by the extracellular domains of pattern recognition receptors (PRRs; Chapter 1). Receptor-like proteins (RLPs) are a type of PRRs that lack a kinase domain for downstream signalling. Some RLPs have been found to be involved in pathogen resistance such as *Phytophthora infestans* in potato (Du, 2014), *Cladosporium fulvum* and *Verticillium* spp. in tomato (Liebrand *et al.*, 2013). Therefore, the RLP underlying *Ol-qtl2* is a good candidate resistance gene. To investigate the role of this RLP in powdery mildew resistance tomato line PV103116, carrying only the *Ol-qtl2* region from *S. neorickii*, and showing partial resistance to *O. neolycopersici*, was transformed with a silencing construct targeting the candidate RLP gene. Silencing of this RLP, or a related RLP gene, resulted in compromised resistance (Appiano, unpublished results). It has recently been found that to trigger defence responses, RLPs need to interact with receptor like kinases (RLKs). In tomato, the gene product of *SUPRESSOR OF BIR1-1*(*SOBIR1*) and *SOBIR1-like* ortologues of *Arabidopsis* are known to interact with several important RLPs including Cf, Ve1 an Eix2 (Liebrand *et al.*, 2013). Possibly, the RLP candidate gene underlying *Ol-qtl2* requires a functional *SOBIR1* or *SOBIR1-like* RLK to be able to confer resistance against powdery mildew.

Virus-induced gene silencing (VIGS) is a reverse genetics technique for gene transcript suppression that has been used for functional analysis in several plant species (Wu *et al.*, 2011). This technique relies on the post-transcriptional gene silencing machinery, that results in sequence-specific degradation of mRNAs (Burch-Smith *et al.*, 2004). This is achieved by transiently expressing a near-identical sequence to a specific gene using recombinant viruses. In tomato, several vectors for VIGS have been used, however, the construct derived from tobacco rattle virus (TRV) is particularly efficient due its capacity to spread vigorously throughout the entire plant and its relatively mild impact on the health of the plants compared to other constructs (Ratcliff *et al.*, 2001).

The aim of this experiment was to use VIGS targeting the RLK SOBIR1 and SOBIR1-like to verify the involvement of the candidate RLP in *Ol-qtl2* conferring quantitative resistance against powdery mildew in tomato.

4.2 Materials and methods

4.2.1 Plant material

Five genotypes of tomato were used to evaluate the effect of silencing *SOBIR1* and *SOBIR1-like* on their resistance against *Oidium neolycopersici* (Table 4-2). Tested genotypes consisted of a Moneymaker background introgressed with the different *Ol-qtl*s. In order to assess the effect on the genotypes PV073004 and PV043154 (containing all three *Ol*-qtls), PV043159 (containing only *Ol-qtl1*) was sown to be used as a comparative genotype.

Table 4-1. Plant material used in the experiment. Moneymaker backgrounds introgressed with the different QTLs for powdery mildew resistance.

Genotype	Characteristics
PV2002	*MM*
PV043159	*MM + QTL1*
PV083208	*MM + QTL2*
PV073004	*MM + QTL1 + QTL2/3*
PV043154	*MM + QTL1 + QTL2/3*

4.2.2 VIGS constructs

Constructs for the virus induced gene silencing (VIGS) targeting *SOBIR1* and *SOBIR1-like* of *S. lycopersicum* and *N. benthamiana* were used on this experiment (Table 4-2). The pTRV2:*SlSOBIR1*/*SlSOBIR1-like* constructs (Liebrand *et al.*, 2013) were provided by Matthieu Joosten. The pTRV2:*NbSOBIR1*/*NbSOBIR1-like* constructs were provided by Emmanouil Domazakis.

Table 4-2. TRV constructs used for transient transformation. Name of each construct and its effect on the plant are described.

Construct	Effect on the plant
pTRV2:*SlSOBIR1*/*SlSOBIR1-like*	Silencing of *SOBIR* and *SOBIR1-like* in tomato.
pTRV2:*NbSOBIR1*/*NbSOBIR1-like*	Silencing of *SOBIR* and *SOBIR1-like* in *N. benthamiana.*
pTRV2:Phytoene desaturase (PDS)	Photobleaching on the silencing tissues. Used as control for agroinfiltration.
pTRV2:*ß-glucuronidase* (GUS)	Used as a negative control.
pTRV1	RNA1 of TRV, encoding replicase and movement proteins.

4.2.3 VIGS Methodology

Two different cultures of *Agrobacterium* carrying the *TRV* constructs were made before the final culture for agroinfiltration, namely culture A and culture B. Two days before the agroinfiltration, culture A was prepared by inoculating 3 ml of LB medium containing kanamycin and rifampicin with the glycerol stocks of pTRV constructs and left to grow overnight at 28˚C in sterile tubes. One day before agroinfiltration, 100 µl of culture A were diluted in 900µl of LB to measure the OD at 600 nm. The OD measurement was used to calculate amount of culture A (X) to be added to 100 ml of YEB medium for preparing culture B containing YEB medium (Table 4-3) according to the formula:

$$X = \frac{80000/2^{dT/2}}{OD}$$

Where: dT = amount of hours between inoculation and harvest

OD = 10XOD optical density at 600 nm of culture A

Culture B was prepared adding amount X of culture A to 100 ml of YEB medium (Table 4-3) containing 10 µl of acetosyringone, 100 µl of kanamycin and 1 ml of MES (Table 4-3). This culture was left at 28˚C to grow overnight. On the day of agroinfiltration, the OD was measured again by diluting 100 µl of culture B in 900 µl of YEB medium. The concentration was used to calculate the amount of MMA (Y) (Table 4-3) to be used to resuspend the centrifuged pellet of 20 ml of culture B, according to the formula:

$$Y = 10\ (OD)$$

Where: OD = 10XOD optical density at 600 nm of culture B

The final agroinfiltration culture was prepared by centrifuging 20 ml of culture B for 8 minutes at 4000 RPM and resuspending the pellet with Y ml of MMA containing 1ml/l of acetosyringone. The final culture was left incubating for two hours before the infiltration. Agroinfiltration was performed using a syringe on the abaxial surface of the cotyledons until they were saturated with the solution.

Table 4-3. Mediums and solutions used to prepare VIGS inoculum.

Name	Composition
LB medium (1000 ml)	10 g bacteriological peptone 10 g NaCl 5 g yeast extract
YEB medium (1000 ml)	5 g beef extract

	5 g bacteriological peptone 5 g sucrose 1 g yeast extract 2 ml $MgSO_4$
MMA (1000 ml)	20 g sucrose 5 g MS salts (without vitamins) 10 ml 1M MES pH adjusted to 5.6
MES 1M	$MgSO_4$ 1M Kanamycin 1000X (50 mg/ml) Rifampicin 1000X (25 mg/ml) Acetosyringone 200mM

4.2.4 Inoculation, scoring and statistical analysis

Photobleaching present in the tomato plants agroinfiltrated with the VIGS construct targeting PDS provided an indication of the time for the inoculation of the plants. 18 days after the agroinfiltration, all plants were inoculated with an isolate of *O. neolycopersici* by spraying a spore solution (2 spores/mm^2) on the adaxial surface of the leaves. To semiquantitatively assess the infection level of tomato, a disease index (DI) scale was used (Bai *et al.*, 2005). A DI score was given to each plant with a value from 0 to 3 according to the level of infection (Table 4-4). DI was assessed 12 and 20 days after inoculation (dpi) for all the genotypes. For the genotypes carrying *Ol-qtl2*, an additional assessment was made at 14 dpi.

Table 4-4. Disease index used to assess the infection level of *O. neolycopersici* in tomato plants (Bai *et al.*, 2005)**.**

Disease index (DI)	Description
0	No fungal visible sporulation
1	Few fungal colonies
2	Up to 30% of the leaf area covered with fungal colonies
3	More than 30% of the leaf are covered with fungal colonies

Statistical analysis was done to determine if significant differences were present in the DI of the plants inoculated with the different TRV constructs. Average DI was calculated and student's t-test was used to determine significant variation.

4.2.5 Genomic DNA isolation and genotyping of plants carrying *Ol-qtl2* and *Ol-qtl1*

CAPS markers were used on genotypes PV083208, PV073004 and PV043154 to confirm the presence of *Ol-qtl2* and either the presence or absence of *Ol-qtl1*. For this purpose, genomic DNA was isolated from the plants. A DNA isolation buffer was prepared using 20% TRIS (1M, ph=7.5), 25% NaCl (1M), 5% EDTA (0.5M, PH=8.0), 5% SDS (10% v/v solution), and 45% water (all percentages v/v of the final buffer volume) according to the protocol described in Table 4-5.

Table 4-5. Protocol for genomic DNA extraction.

Step	Performed actions
1	Leaf samples of tomato were put inside a tube of a 96 tube rack along with 2 steel balls.
2	300 µl of DNA isolation buffer were added to each tube.
3	Samples were grinded using a Retch mill for 4 minutes.
4	Samples were centrifuged at 4600 rpm for 15 minutes.
5	300 µl of isopropanol were added to new tubes.
6	200 µl of the supernatant from step 4 were added to the tubes containing isopropanol.
7	Tubes from step 6 were mixed well and centrifuged at 4600 rpm for 20 minutes
8	All liquid from the tubes was poured out of the tubes and left to dry in the fume hood for two hours.
9	200 µl of MQ water were added to each tube.
10	After 15 minutes, the DNA pellets were dissolved in the water using vortex.
11	Samples were transferred to a 96-well plate.

CAPS markers *60kbF+R* and *Ol-qtl2-15kb-2F+R* were used to confirm the presence of *Ol-qtl2* in genotypes PV083208, PV073004 and PV043154. Additionally, CAPS marker *P21M47* was used to confirm the absence of *Ol-qtl1* in PV083208. Furthermore, the presence of *Ol-qtl1* was confirmed in genotypes PV073004 and PV043154 using CAPS markers 286N17 and 194N16 (Supplementary information). Table 4-6 shows the conditions used for the genotyping. Figure 4-2 shows the location of the markers used for this genotyping.

Table 4-6. Primers used for genotyping.

Marker	Qtl	Primer sequence	TM	Enzyme
60kbF+R	*Ol-Qtl2*	Fw: ATGAAACCAACACAAACGA	56˚C	*DdeI*
		Rv: ACGGCCATAACCAGACAAAG		
Ol-qtl2-15kb-2F+R	*Ol-Qtl2*	Fw: AAATTGTGATTCCGCCTCTG	55˚C	*DdeI*
		Rv: TTCAAATCCTTAACCCGGTG		
P21M47	*Ol-Qtl1*	Fw: TAACAATCTCGACCATAGTTCC	56˚C	*HaeIII*
		Rv: CCATACCCGAATTTCCTTCC		
286N17	*Ol-Qtl1*	Fw: TCCAATTGCACTCTCACCAA	56˚C	*ApoI*
		Rv: AGAAATGTGGGCTCCAACTG		
194N16	*Ol-Qtl1*	Fw: TCAGGATCCGTTTGATCTCC	56˚C	*ApoI*
		Rv: GCTTTTGCTCCATCAACACA		

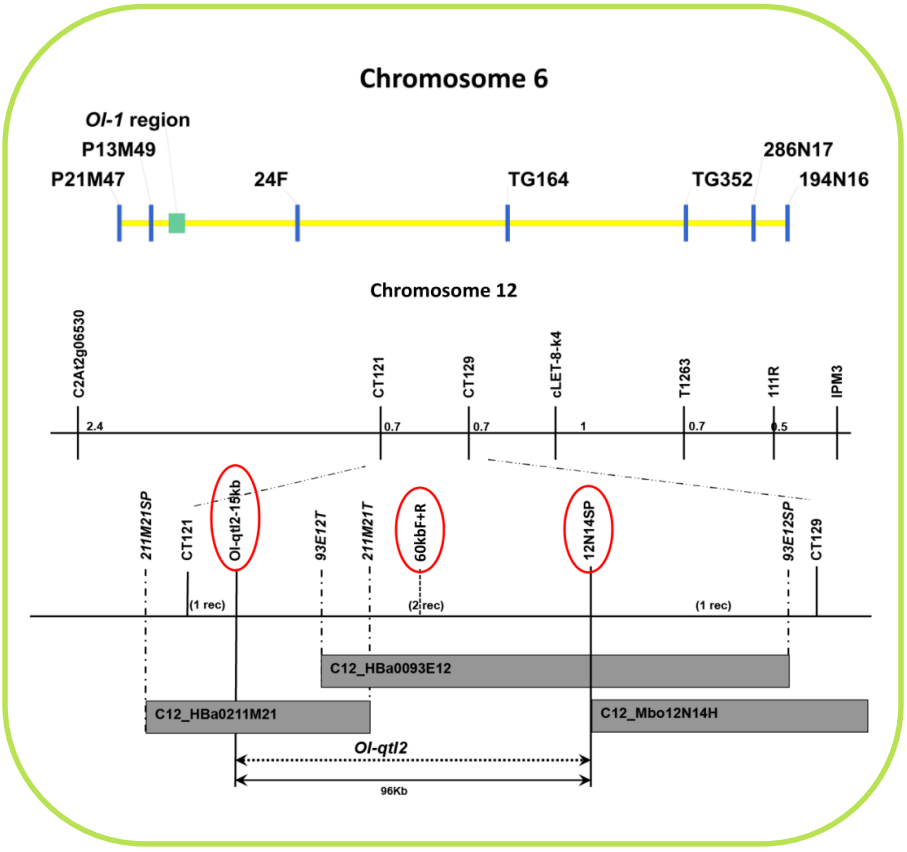

Figure 4-2. Location of molecular markers for *Ol-qtl1* and *Ol-qtl2* used for the genotyping of tomato plants.

Table 4-7. PCR conditions for genotyping using DreamTaq.

Segment	Number of cycles	Temperature	Duration
1	1	95˚C	3 minutes
2	35	95˚C	30 seconds
		55˚C	30 seconds
		72˚C	1 minute
3	1	72˚C	10 minutes

4.3 Results

From all the genotypes tested, only PV083208 (MM + *Ol-qtl2*) showed results that could be associated with infection with *O. neolycopersici.* The rest of the genotypes tested did not show a response that could be associated with the silencing of the RLK (Supplementary material). Plants transiently transformed with the VIGS constructs targeting *SOBIR1* and *SOBIR1-like* for both tomato and *N. benthamiana* showed a higher disease index than the GUS controls (Table 4-8, Table 4-9, and Figure 4-3). Molecular markers confirmed the presence of *Ol-qtl2* and the absence of *Ol-qtl1* in PV083208. Markers analysis also confirmed the presence of *Ol-qtl1* and *Ol-qtl2* in PV073004 and PV043154 (Supplementary material).

Sample	Construct	D.I.	60kbF+R	Ol-qtl2-15kb-2F+R	P21M47	Phenotype
PV083208-3	pTRV2: GUS	1	b	b	a	
PV083208-4	pTRV2: GUS	0.5	b	b	a	shorter plant
PV083208-5	pTRV2: GUS	0	b	b	a	
PV083208-6	pTRV2: GUS	0.5	b	b	a	
PV083208-7	pTRV2: GUS	0		b		
PV083208-1	pTRV2: PDS	0.75	b	b	a	
PV083208-2	pTRV2: PDS	0	b	b	a	Dwarf
PV083208-8	pTRV2: PDS	2	b		a	no bleaching
PV083208-9	pTRV2: PDS	0.75	b		a	
PV083208-10	pTRV2: PDS	0	b		a	necrotic, no bleaching
PV083208-15	pTRV2:*SlSOBIR1/SlSOBIR1-like*	2	b	b	a	
PV083208-22	pTRV2:*SlSOBIR1/SlSOBIR1-like*	0.75	b	b	a	
PV083208-24	pTRV2:*SlSOBIR1/SlSOBIR1-like*	2	b	b	a	
PV083208-25	pTRV2:*SlSOBIR1/SlSOBIR1-like*	1	b		a	
PV083208-27	pTRV2:*SlSOBIR1/SlSOBIR1-like*	1	b	b	a	
PV083208-28	pTRV2:*SlSOBIR1/SlSOBIR1-like*	0		b	a	dwarf
PV083208-29	pTRV2:*SlSOBIR1/SlSOBIR1-like*	1	b	b	a	shorter plant
PV083208-30	pTRV2:*SlSOBIR1/SlSOBIR1-like*	1.5	b		a	shorter plant
PV083208-11	pTRV2:*NbSOBIR1/NbSOBIR1-like*	0	b	b	a	
PV083208-12	pTRV2:*NbSOBIR1/NbSOBIR1-like*	2	b	b	a	

PV083208-13	pTRV2:*NbSOBIR1/NbSOBIR1-like*	0.5	b	b	a	shorter plant
PV083208-14	pTRV2:*NbSOBIR1/NbSOBIR1-like*	0.75	b	b	a	shorter plant
PV083208-16	pTRV2:*NbSOBIR1/NbSOBIR1-like*	2		b	a	
PV083208-17	pTRV2:*NbSOBIR1/NbSOBIR1-like*	1.5			a	shorter plant
PV083208-18	pTRV2:*NbSOBIR1/NbSOBIR1-like*	1.5	b		a	
PV083208-19	pTRV2:*NbSOBIR1/NbSOBIR1-like*	0.75	b		a	
PV083208-20	pTRV2:*NbSOBIR1/NbSOBIR1-like*	1	b		a	
PV083208-21	pTRV2:*NbSOBIR1/NbSOBIR1-like*	2	b		a	

Table 4-8. Disease index (DI) scoring, genotyping and atypical phenotypes present in the PV083208 genotype. Molecular markers 60kbF+R and Ol-qtl2-15kb-2F+R were used to confirm the presence of *Ol-qtl2*. Marker P21M47 was used to confirm the absence of *Ol-qtl1*. (a: Moneymaker allele; b: *S. neorickii* allele).

Construct	Average DI	Std.error
pTRV2:*SlSOBIR1/SlSOBIR1-like*	1.16 ab	0.236
pTRV2:*NbSOBIR1/NbSOBIR1-like*	1.2 ab	0.223
pTRV2: PDS	0.7 bc	0.366
pTRV2: GUS	0.4 c	0.187

Table 4-9 Average disease index (DI) of transformed tomato plants. PV083208 plants silenced with the constructs targeting *SOBIR1* and *SOBIR1-like* showed a significantly higher DI ($P<0.05$) than the GUS controls.

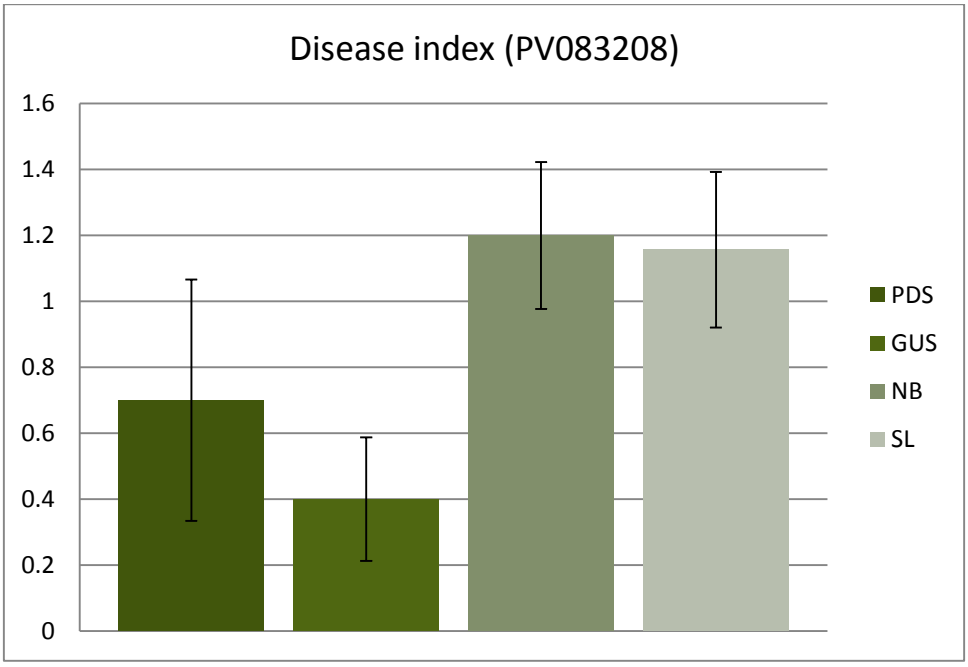

Figure 4-3. Disease index of genotype PV083208. Plants transformed with the VIGS constructs targeting PDS and GUS showed less powdery mildew symptoms than the plants transformed with the constructs targeting *SOBIR1* and *SOBIR1-like*. Error bars showing standard error (Plants transformed with constructs: PDS: TRV2:PDS; GUS: TRV2:GUS; NB: TRV2:*NbSOBIR1/NbSOBIR1-like*; and SL:TRV2 *SlSOBIR1/SlSOBIR1-like*).

4.4 Discussion

Moneymaker plants carrying *Ol-qtl2* (PV083208) transformed with the VIGS constructs targeting *SOBIR1* and *SOBIR1-like* showed a higher susceptibility to *O. neolycipersici* than the control plants. These results suggest that the molecular mechanisms of the resistance conferred by *Ol-qtl2* can be affected by the silencing of *SOBIR1* and *SOBIR1-like*, further confirming the identity of the candidate gene as an RLP that depends on the interaction with this RLK for downstream signalling. Genotyping of this family confirmed the presence of the *Ol-qtl2* and the absence of *Ol-qtl1*. This confirmed that the variation on the disease index was not due to the presence of other QRL. Genotyping using markers for *R* genes is needed to confirm that the variation in the DI is not caused by other resistance loci.

The results of this experiment gave an indication of the involvement of the RLKs SOBIR1 and SOBIR1-like in the molecular mechanisms of the resistance conferred by *Ol-qtl2*. However, more replications are needed to confirm the significance of the variation (Figure 4-3) in the disease index and its relation to the silencing of the *SOBIR* ortologues. This will further confirm the RLP identity of the candidate gene accountable for the resistance in *Ol-qtl2*. The addition of more GUS controls is also strongly suggested to obtain a more clear confirmation.

A large phenotypic variation was found across and within the different genotypes. In PV083208, plants showed a contrasting phenotype in height and branching (Figure 4-4A). This variation can be due to the segregation of the genotype. Nevertheless, symptoms of the virus were also seen in the plants and the phenotypic variation could have been caused by the viral infection. Stunt growth and necrotic lesions were found on several plants (Figure 4-4B). In some cases, the virus symptoms were very severe, especially in plantlets, even causing the death of two PV083208 plants.

Response of genotypes PV073004 and PV043154 could not be associated with the silencing of *SOBIR1* and *SOBIR1-like* (Supplementary material). Molecular markers were used to confirm the presence of *Ol-qtl1* and *Ol-qtl2.* The idea of testing both of these genotypes was to compare the response upon infection with that of the genotypes only containing *Ol-qtl1*, however, the genotype PV043159 did not show variation on the DI associated with the silencing of the RLK.

Important considerations regarding the nature of the VIGS technique should be taken into account. Silencing using this technique occurs incompletely and in a patchy distribution (Orzaez *et al.*, 2009, Schilmiller *et al.*, 2012). This was also evident in the tomato plants inoculated with the PDS construct (Figure 4-5). Stable transformation is suggested to obtain a more uniform silencing and to avoid the effect of the virus symptoms.

Fungal biomass quantification is also recommended to improve the reliability of the results. Additionally, further genotyping of the PV083208 genotype can be used to confirm if the variation in

the phenotype is caused by segregation or by viral infection. The inoculation concentration and method used in this experiment proved to be appropriate to assess the disease index.

Figure 4-4. Phenotypic variation and virus symptoms in the tomato plants transformed with VIGS constructs. A) Phenotypic variation on the PV083208 genotype. Stunted growth, shorter plants and atypical branching was seen across the different plants. B) Necrotic stems were present in most of the PV083208 plants. In some cases, the virus symptoms were very sever, even causing the death of two of the plants.

Figure 4-5. Incomplete and patchy silencing of phytoene desaturase (PDS) in tomato. The photobleaching, used as control for the inoculation showed the incomplete nature of the VIGS technique.

4.5 References

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4.6 Supplementary material

4.6.1 Disease index (DI) scoring, genotyping and atypical phenotypes of all genotypes tested.

Table 4-10. Disease index (DI) scoring, genotyping and atypical phenotypes present in across all tested genotypes. Molecular markers 60kbF+R and Ol-qtl2-15kb-2F+R were used to confirm the presence of *Ol-qtl2*. Marker P21M47, 194N16 and 286N17 were used to confirm the absence or presence of *Ol-qtl1*. (a: Moneymaker allele; b: *S. neorickii* allele; h: heterozygous).

Sample	Construct	D.I.	60kb	15-2FR	2147	194N16	286N17	Notes
MM-1		3						
MM -2		3						
MM -3		3						
MM -4		3						
MM -5		3						
MM -6		3						
MM -7		3						
MM -8		3						
MM -9		3						
MM -10		3						
MM -11		3						
MM -12		3						
MM -13		3						
MM -14		3						
MM -15		3						
MM -16		3						
MM -17		3						
MM -18		3						
MM -19		3						
MM -20		3						
MM -21		3						
MM -22		3						
MM -23		3						
MM -24		3						
MM -25		3						
MM -26		3						
MM -27		3						
MM -28		3						
MM -29		3						
MM -30		3						
PV043159-1	PDS	1.5						
PV043159-2	PDS	1.5						
PV043159-3	GUS	2						
PV043159-4	GUS	2						
PV043159-5	GUS	2						
PV043159-6	GUS	1.5						

Sample	Construct	D.I.	60kb	15-2FR	2147	194N16	286N17	Notes
PV043159-7	GUS	2						
PV043159-8	N. benthamiana	1.5						
PV043159-9	N. benthamiana	1.5						
PV043159-10	N. benthamiana	2						
PV043159-11	N. benthamiana	2						
PV043159-12	PDS	2						
PV043159-13	PDS	2.5						
PV043159-14	PDS	1.5						
PV043159-15	S. lycopersicum	1.5						
PV043159-16	N. benthamiana	1.5						
PV043159-17	N. benthamiana	1.5						
PV043159-18	N. benthamiana	1.5						
PV043159-19	N. benthamiana	1.5						
PV043159-20	N. benthamiana	1						
PV043159-21	N. benthamiana	1						
PV043159-22	S. lycopersicum	1.5						
PV043159-23	S. lycopersicum	1.5						
PV043159-24	S. lycopersicum	1.5						
PV043159-25	S. lycopersicum	2						
PV043159-26	S. lycopersicum	1						
PV043159-27	S. lycopersicum	1.5						
PV043159-28	S. lycopersicum	1						
PV043159-29	S. lycopersicum	2						
PV043159-30	S. lycopersicum	1						
PV073004-1	PDS	0.5	b	b	b	b	b	
PV073004-2	PDS	0.75	b	b	b	b	b	
PV073004-3	GUS	0.5	b		h	b	b	shorter plant
PV073004-4	GUS	0.5	b		h	b	b	shorter plant
PV073004-5	GUS	0.5	b		h	b	b	
PV073004-6	GUS	0.5	b	b	b	b	b	
PV073004-7	GUS	0	b	b	b	b	b	shorter plant
PV073004-8	N. benthamiana	1.5	b			b	b	shorter plant
PV073004-9	N. benthamiana	0.75		b	b	b		
PV073004-10	N. benthamiana	0.5		b		b	b	
PV073004-11	N. benthamiana	0.5	b	b	b	b		
PV073004-12	PDS	1	b		h		b	
PV073004-13	PDS	0.5			a	b	b	Dwarf
PV073004-14	PDS	0.5	b	b	b	b	b	shorter plant
PV073004-15	S. lycopersicum	1	b	b	b	b	b	shorter plant
PV073004-16	N. benthamiana	0.75	b	b	b	b		
PV073004-17	N. benthamiana	0.75		b	b	b	b	shorter plant
PV073004-18	N. benthamiana	0.5		b	b	b	b	
PV073004-19	N. benthamiana	0.5	b			b	b	
PV073004-20	N. benthamiana	0.5	b			b	b	

Sample	Construct	D.I.	60kb	15-2FR	2147	194N16	286N17	Notes
PV073004-21	N. benthamiana	0.5		b	b	b		
PV073004-22	S. lycopersicum	0.5	b	b	b	b	b	
PV073004-23	S. lycopersicum	0.75	b		h	b	b	Dwarf
PV073004-24	S. lycopersicum	0.75	b		h	b	b	shorter plant
PV073004-25	S. lycopersicum	0.75	b		h	b	b	shorter plant
PV073004-26	S. lycopersicum	0.5	b	b	b	b	b	
PV073004-27	S. lycopersicum	1	b	b	b	b	b	
PV073004-28	S. lycopersicum	0.5	b	b	b	b	b	
PV073004-29	S. lycopersicum	1	b		h	b	b	
PV073004-30	S. lycopersicum	1	b		h	b	b	
PV043154-1	PDS	1	b	b	b	b	b	
PV043154-2	PDS	2		b	b	b	b	
PV043154-3	GUS	0	b	b	b	b	b	
PV043154-4	GUS	0.5	b	b	b	b	b	shorter plant
PV043154-5	GUS	2		b	b	b	b	
PV043154-6	GUS	1.5	b			b	b	
PV043154-7	GUS	0.75	b			b	b	
PV043154-8	PDS	0.5		b	b	b	b	
PV043154-9	PDS	0.5	b	b		b	b	shorter plant
PV043154-10	PDS	1	b			b	b	
PV043154-11	N. benthamiana	0	b	b	b	b	b	shorter plant
PV043154-12	N. benthamiana	1.5	b	b	b	b	b	
PV043154-13	N. benthamiana	2	b	b	b	b	b	
PV043154-14	N. benthamiana	1.5	b			b	b	
PV043154-15	S. lycopersicum	0.5	b	b	b	b	b	shorter plant
PV043154-16	N. benthamiana	0.5	b			b	b	shorter plant
PV043154-17	N. benthamiana	0.5	b	b		b	b	shorter plant
PV043154-18	N. benthamiana	0	b	b	b	b	b	shorter plant
PV043154-19	N. benthamiana	1.5	b			b	b	
PV043154-20	N. benthamiana	0.75	b			b	b	shorter plant
PV043154-21	N. benthamiana	0.75	b			b	b	
PV043154-22	S. lycopersicum	0.75	b	b	b	b	b	
PV043154-23	S. lycopersicum	0.5	b			b	b	
PV043154-24	S. lycopersicum	0.75	b	b		b	b	
PV043154-25	S. lycopersicum	0.75	b			b	b	shorter plant
PV043154-26	S. lycopersicum	0.75	b	b	b	b	b	
PV043154-27	S. lycopersicum	1	b	b	b	b	b	
PV043154-28	S. lycopersicum	0.5	b			b	b	shorter plant
PV043154-29	S. lycopersicum	2	b			b	b	
PV043154-30	S. lycopersicum	1.5	b		b			
PV083208-1	PDS	0.75	b	b	a			
PV083208-2	PDS	0	b	b	a			Dwarf
PV083208-3	GUS	1	b	b	a			

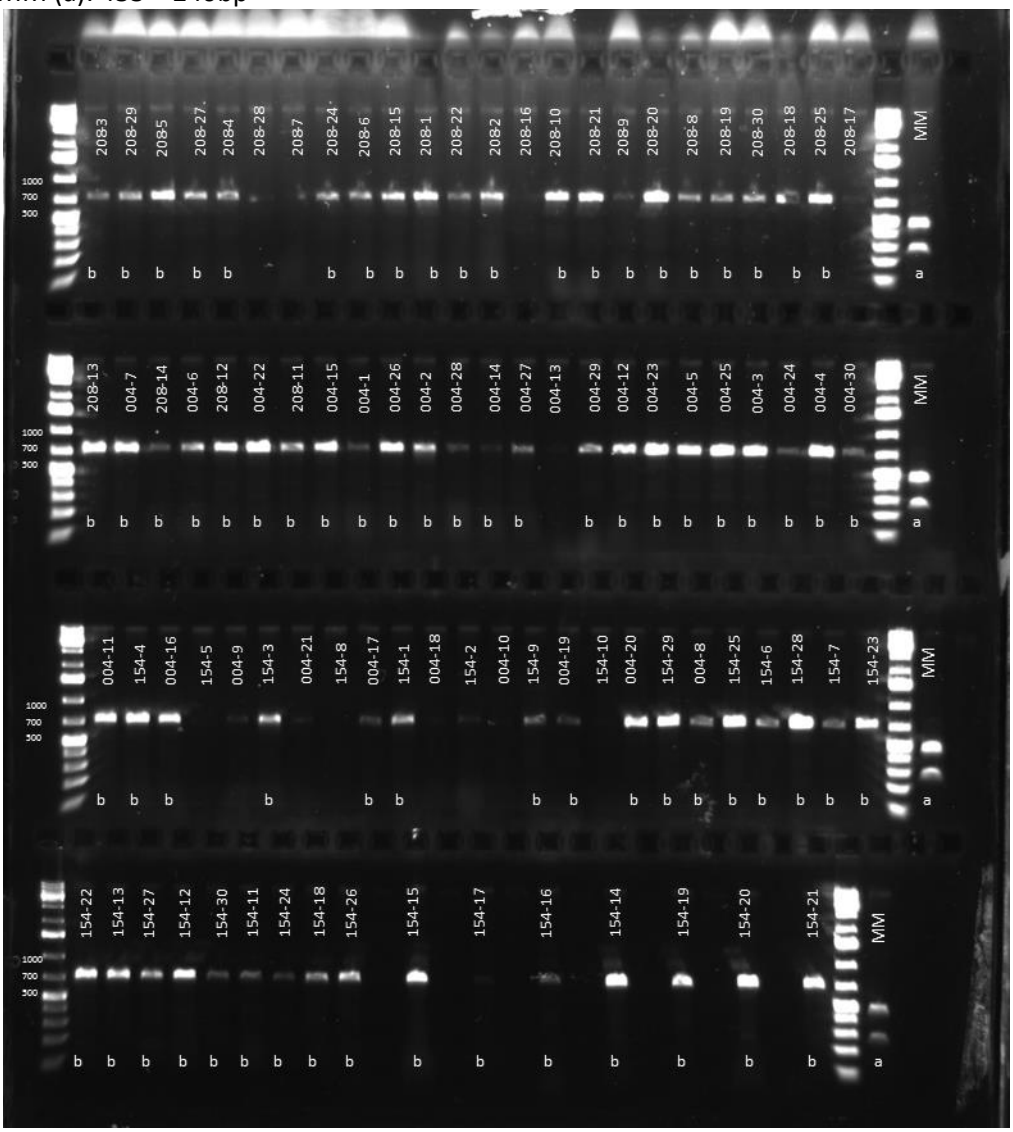
Sample	Construct	D.I.	60kb	15-2FR	2147	194N16	286N17	Notes
PV083208-4	GUS	0.5	b	b	a			shorter plant
PV083208-5	GUS	0	b	b	a			
PV083208-6	GUS	0.5	b	b	a			
PV083208-7	GUS	0		b				
PV083208-8	PDS	2	b		a			no bleaching
PV083208-9	PDS	0.75	b		a			
PV083208-10	PDS	0	b		a			necrotic, no bleaching
PV083208-11	N. benthamiana	0	b	b	a			
PV083208-12	N. benthamiana	2	b	b	a			
PV083208-13	N. benthamiana	0.5	b	b	a			shorter plant
PV083208-14	N. benthamiana	0.75	b	b	a			shorter plant
PV083208-15	S. lycopersicum	2	b	b	a			
PV083208-16	N. benthamiana	2		b	a			
PV083208-17	N. benthamiana	1.5			a			shorter plant
PV083208-18	N. benthamiana	1.5	b		a			
PV083208-19	N. benthamiana	0.75	b		a			
PV083208-20	N. benthamiana	1	b		a			
PV083208-21	N. benthamiana	2	b		a			
PV083208-22	S. lycopersicum	0.75	b	b	a			
PV083208-24	S. lycopersicum	2	b	b	a			
PV083208-25	S. lycopersicum	1	b		a			
PV083208-27	S. lycopersicum	1	b	b	a			
PV083208-28	S. lycopersicum	0		b	a			dwarf
PV083208-29	S. lycopersicum	1	b	b	a			shorter plant
PV083208-30	S. lycopersicum	1.5	b		a			shorter plant

4.6.2 Genotyping information

Codominant CAPS markers ran in 1% agarose gels. For each marker, expected sizes are shown after digestion with the respective enzyme (Table 4-6).

60kbF+R (*Ol-qtl2*) x DdeI

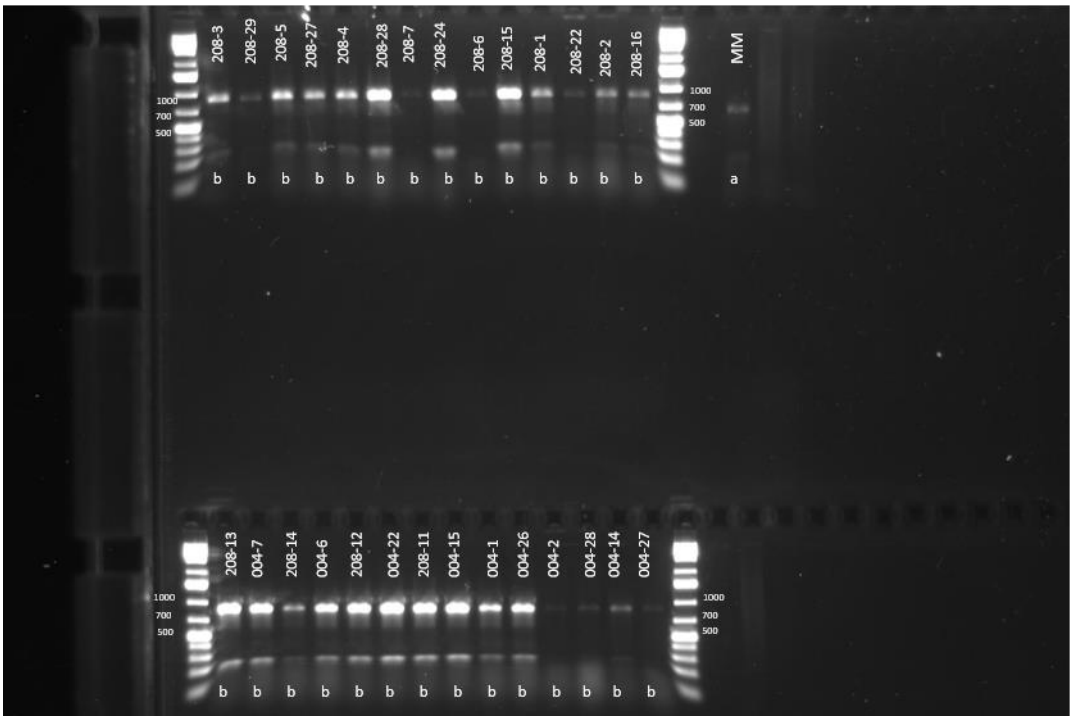
S. neorickii (b): 661bp
MM (a): 433 + 240bp

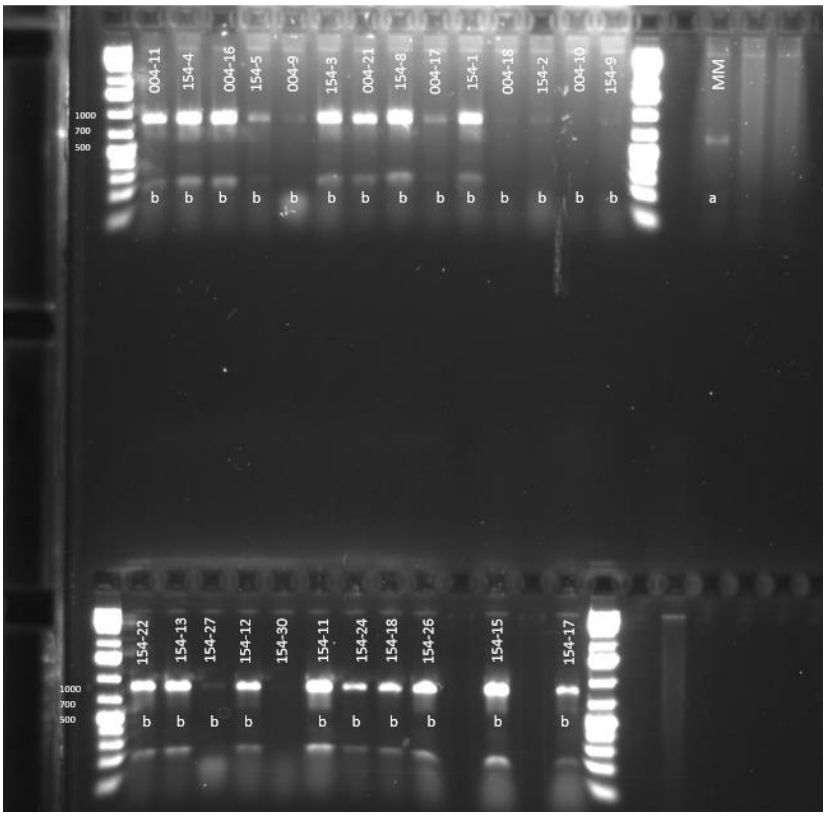


Ol-qtl2-15kb-2F+R (*Ol-qtl2*) x DdeI

S. neorickii (b): 813 + 167bp

MM (a): 613 +167 + 123bp

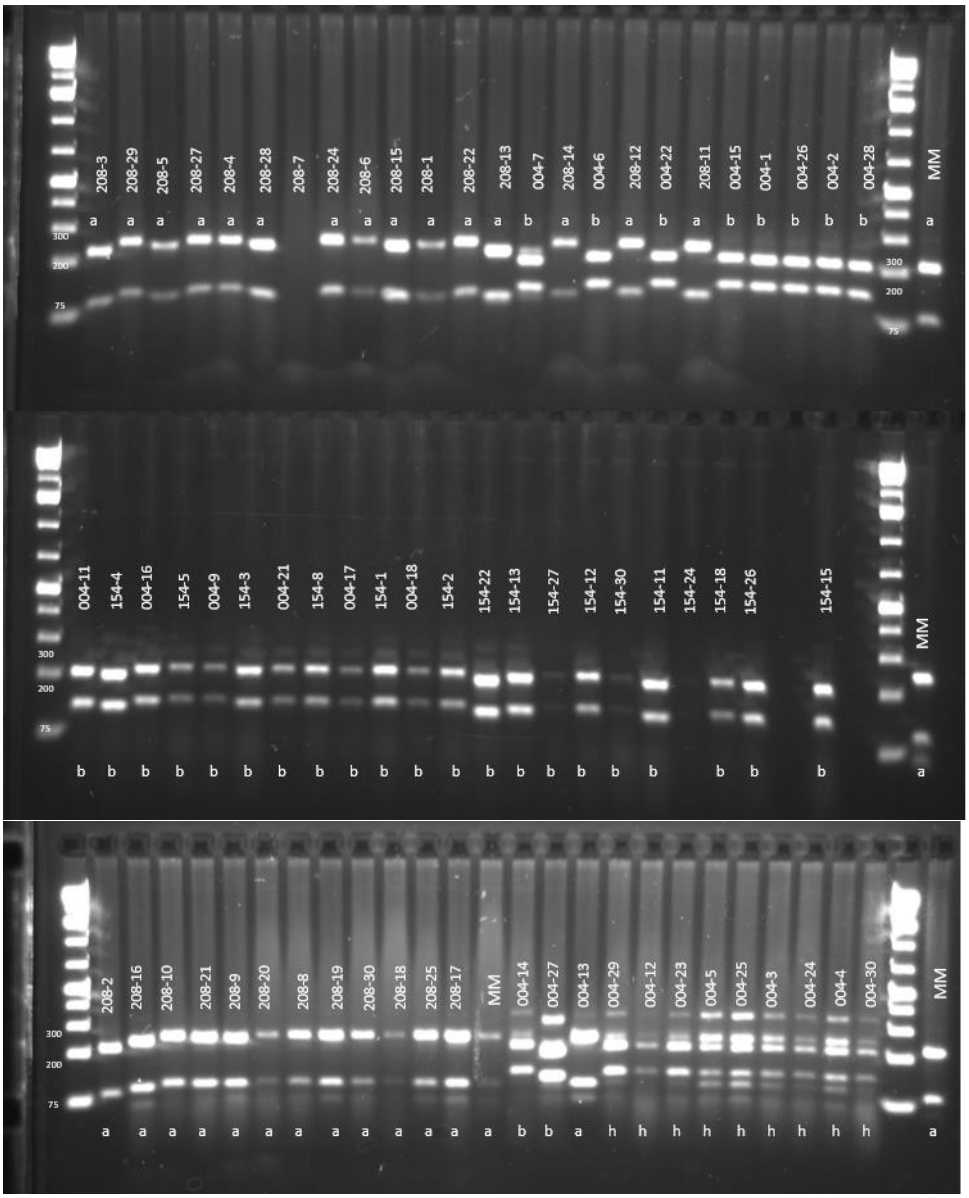




P21M47 (*Ol-qtl1*) x HaeIII

S. neorickii (b): 226 + 90bp

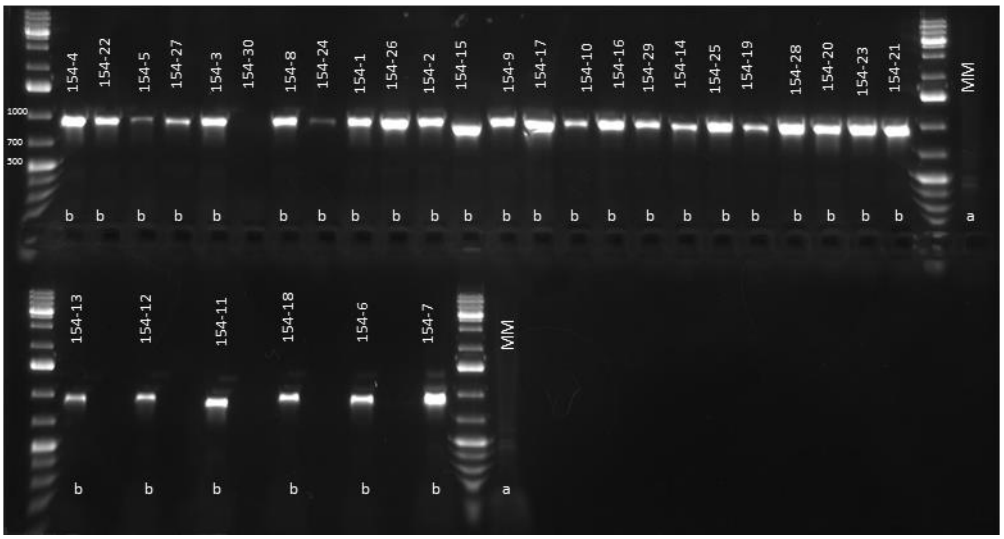
MM (a): 196 + 120bp

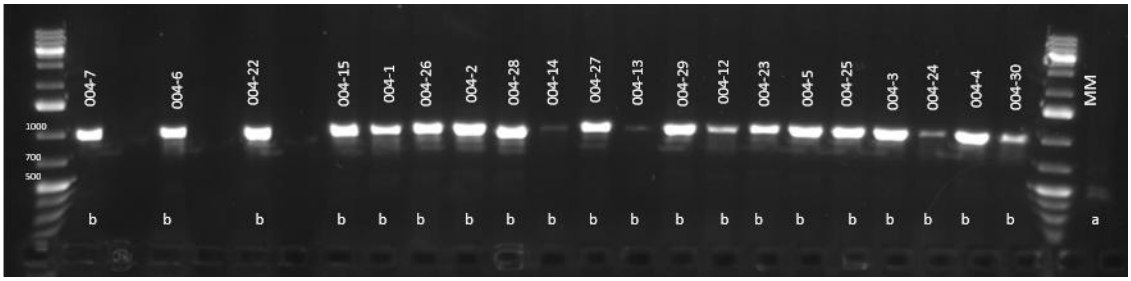


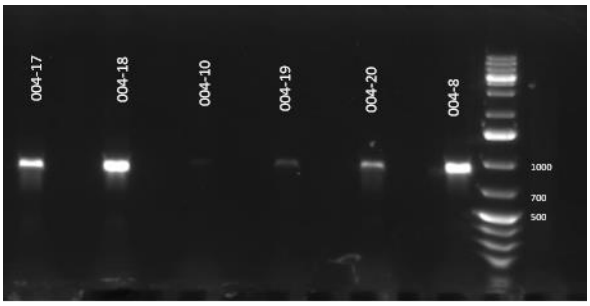
286N17 (*Ol-qtl1*) x ApoI: Only ran in PV73004 and PV43154

S. neorickii (b): 888bp

MM (a): ~500+470bp



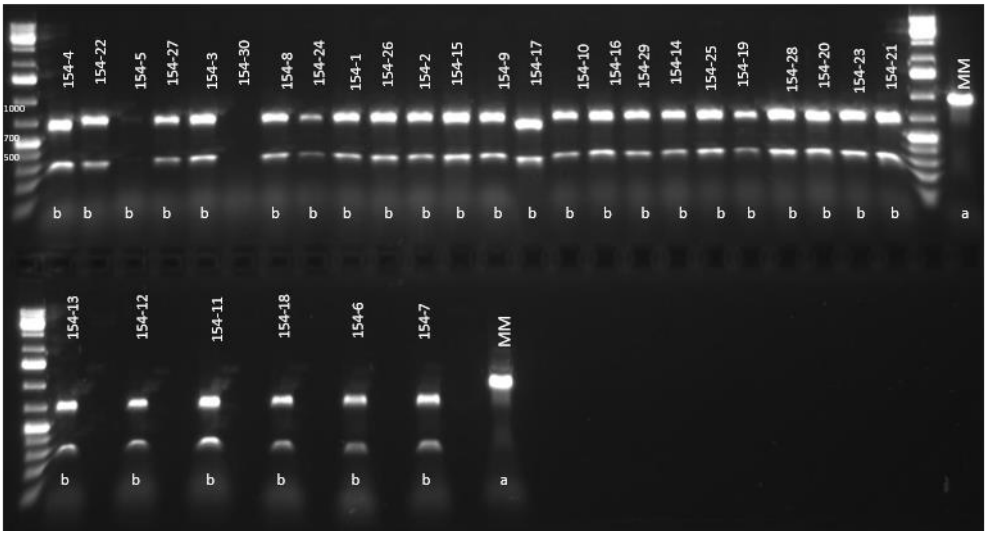


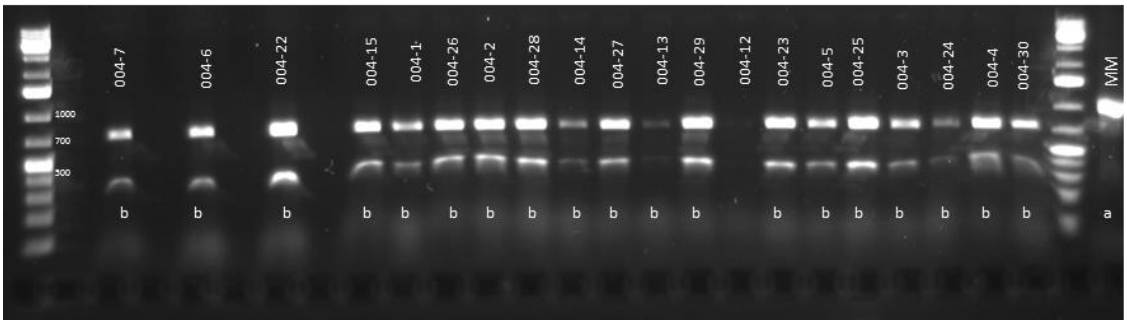


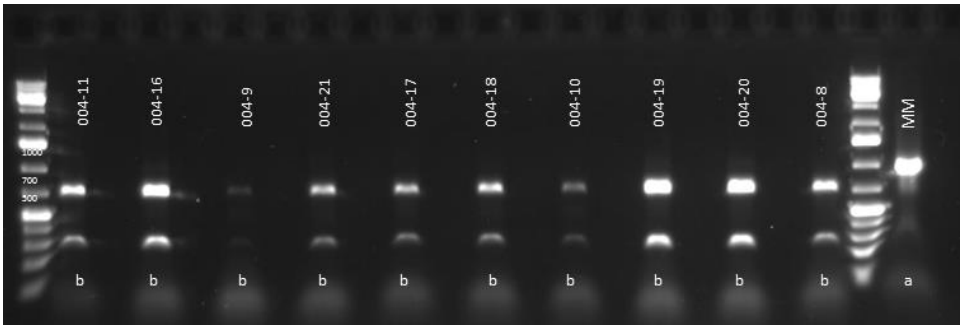
194N16 (*Ol-qtl1*) x ApoI: Only ran in PV73004 and PV43154

S. neorickii (b): 861bp

MM (a): ~1000bp







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