Role of *MLO* genes in susceptibility to powdery mildew in apple and grapevine

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CHAPTER 1

General Introduction

Economical relevance of apple and grapevine

Apple (*Malus domestica*) and grapevine (*Vitis vinifera*) are two of the most important cultivated fruit species for human consumption. Grapevine is the 8th most valuable cultivated plant species in the world and the most valuable fruit plant, with a global value of 39.5 billion dollars and a total production of 69 million tons on 7.6 million of hectares of land (OIV 2012; FAO, 2013). It has been cultivated for more than 7000 years and few other crops have had the same cultural impact. Italy is the second producer of grape worldwide after China and the first in Europe, with 8 million tons per year. Of these 8 million tons, 4.1 are intended for wine production, making Italy the second wine producer in the world after France (FAOSTAT, 2013). The region of Trentino Alto Adige produces 2.7% of the Italian wine, mostly high quality white wines (ISTAT, 2014).

Apple is the 10th most valuable cultivated plant species in the world and the second most valuable fruit plant after grapevine, with a global value of 31.7 billion dollars and a total production of 75 million tons (FAO, 2012). Italy is the fifth producer of apple worldwide and the second in Europe after Poland, with 2.41 million tons per year (FAOSTAT, 2013). The 80% of this production is concentrated in northern Italy, with the region of Trentino accounting for 46% of the total (Agraria.org).

Both apple and grapevine are susceptible to many different diseases including the fungal disease powdery mildew (PM).

Powdery Mildew pathogens

Powdery mildew is one of the most common plant diseases, caused by ascomycete fungi belonging to the order *Erysiphales*, which comprises approximately 100 species (Glawe, 2008). These obligate biotrophic pathogens can affect nearly 10,000 species of angiosperms, including all major crops, fruit and ornamental plants (Braun *et al.,* 2002). The name Powdery Mildew is due to the powdery aspect

of the fungal colonies growing on the infected tissues. Leaves, stems, flowers and fruits can all be infected (Braun *et al.,* 2002).

The conidia, asexual airborne spores spreading from nearby infected plants, are the most common source of inoculum. In the first 60 seconds after landing on the host, the conidium produces a liquid extracellular matrix with esterase and cutinases activity that fasten the spore to the host (Carver *et al,* 1999; Wright *et al*., 2002). The spore germinates and starts forming the hypha within 30-60 min (Kunoh, 2002), and in about 10 hours, lateral outgrowths called appressoria start the penetration of the cuticle (Zhang *et al*. 2005). In two more hours, the appressoria produce narrow protrusions, called penetration pegs, which penetrate the cell-wall of the host by turgor pressure and enzymatic activity (Glawe, 2008). If the penetration is successful, the penetration pegs extend into the host cells and invaginate the cytoplasm to form the haustoria, which function is to keep the parasitic relationship with the host. It is a dynamic exchange that provides the pathogen with resources shunted from the plant, such as hexoses, amino acids, and vitamins, while it delivers to the host proteins that suppress defence (Fotopoulos *et al.,* 2003; Qiu *et al.,* 2015). Powdery mildew pathogens are even able to interfere with the expression of specific host genes to improve the delivery of resources, as documented for a nitrite/nitrate transporter of grapevine (Pike *et al.,* 2014).

After the parasitic relationship has been successfully established, the hyphae elongate, spread on the surface of the host and form circular whitish or transparent colonies that eventually turn grayish, reddish or brownish (Braun *et al.,* 2002). The production of conidia begins several days after the infection (Glawe, 2008).

Powdery mildew in apple

The causing agent of PM of apple is *Podosphaera leucotricha*, a pathogen occurring in all major growing regions of the world, especially in semiarid ones and nursery

productions (Turecheck, 2004). Losses caused by PM depend on several factors, such as susceptibility of the cultivar, management practices and environmental conditions, but can reach up to 50% if the infection is not properly treated (Yoder, 2000).

P. leucotricha favors young green tissues and blossoms. Leaves are extremely susceptible, particularly in the first few days after opening (Turecheck, 2004). Infections on the underside of the leaf cause chlorotic patches, whereas on the upper surface they appear as powdery white spots that eventually turn brown (Fig. 1A). Infected leaves have the tendency to crinkle, curl and, in case of severe infection, drop prematurely (Fig. 1b) (Turecheck, 2004). Blossoms, petals, sepals, receptacles, and peduncles can also be targeted, although it is not as common as infections of the leaves. However, the infections of blossoms are particularly dangerous, as they will produce small, stunted fruits or no fruits at all (Turecheck, 2004).

P. leucotricha overwinters in buds infected during the previous season. If *P. leucotricha* overwinters in floral buds, it can cause severe reductions of yields the next year (Turecheck, 2004). With the reprise of the vegetative growth in spring, infected buds break dormancy later than healthy ones and the growing shoots appear stunted, misshapen and completely covered by conidia. Therefore, they are called flag shoots and they are the primary infections, whereas the secondary infections are caused by conidia spreading from flag shoots to nearby plants. In late summer, *P. leucotricha* produces sexual spores (ascospores) carried by the fruit bodies (cleistothecia), masses of small black structures that play a minor role in spreading the infection (Turecheck, 2004).

The environmental conditions that favor PM infection are relative humidity higher than 70% and temperatures between 10 and 25°C, with an optimum between 19 and 22°C. Leaf wetting is not required for *P. leucotricha*, as spores will not

germinate if immersed in water (Turecheck, 2004). Under optimum conditions, grey spots can appear after just 48 hours and the period between the beginning of the infection and the first production of conidia (latency period), can be of just five days (Turecheck, 2004).

Figure 1. Apple leaves infected by *P. leucotricha*.

Powdery mildew in grapevine

The causing agent of PM in *V. vinifera* is *Erysiphe necator* (syn. *Uncinula necator*), a pathogen native of North America able to infect several species within the *Vitaceae*

(Pearson and Gadoury, 1992). It was introduced in Europe in 1850 and since then it caused significant grapevine losses (Campbell, 2004). *E. necator* can infect all green tissues of grapevine (Gadoury, 2012). Leaves are always susceptible to PM (Fig. 2), particularly when half expanded, but susceptibility declines with age, a process called ontogenic resistance (Doster and Schnathorst, 1985). On the other hand, fruits are susceptible only for a short period, i.e. the first two weeks after fruit setting (Gadoury *et al*., 2003). Infections caused by sexual ascospores can be found on the lower surface of recently formed leaves and they cause chlorotic spots on the upper surface, whereas asexual spores (conidia) cause the formation of greyish powdery spots on the upper surface (Gadoury, 2012). Severe infections cause the development of necrotic blotches on the leaves and premature drop (Gadoury, 2012). *E. necator* survives winter by two means: the first is overwintering in buds; the second is as cleistothecia, which are formed primarily on leaves, but also on berries and shoots (Pearson and Gadoury, 1987). In colder climates, where the pathogen cannot survive to low temperatures in dormant buds (Moyer *et al*., 2010), cleistothecia are the only source of infection in early spring (Gadoury *et al*., 1997). The environmental conditions that favor *E. necator* growth and sporulation are relative humidity of approximately 85% (Carroll and Wilcox, 2003), but spores immersed in water do not germinate (Gadoury, 2012). The range of temperature has to be between 23 and 30°C, with the optimum at 26°C and the limits for disease development at 6 and 32°C (Delp, 1954). A temperature of 35°C inhibits germination and the exposure of the spores at 40°C can kill them (Delp, 1954). Temperature affects also the duration of the latency period: at optimal temperature (26°C) it can be as short as five days, whereas at 9 °C it increases to 25 days (Delp, 1954). *E. necator* is particularly vulnerable to UV radiation (Austin, 2010), therefore shading from direct sunlight is positive for the pathogen. This is the reason why atypically rainy seasons are accompanied by severe mildew

epidemics: the reduced germination due to the immersion of spores in water is more than compensated by the protection from direct sunlight granted by the clouds (Gadoury *et al*., 2011). Rain also favors dispersal of ascospores (Gadoury and Pearson, 1988).

Figure 2. Grapevine leaf infected by *E. necator*.

Controlling powdery mildew

For both apple and grapevine, the economic impact of PM is significant. A study on apple cultivation in Virginia (USA) showed that even the least effective PM treatment can result in extra incomes for the growers of more than 2,000 $/ha, assuming that all the production is sold for fresh consumption (Yoder, 2000). The profit can increase to 4,500 $/ha when adopting the most effective treatment (Yoder, 2000). A study on grapevine production in California (USA), showed that the

use of resistant varieties would save to the growers up to 720 $/ha (Fuller *et al*., 2014). These numbers clearly show how PM control is of primary interest for apple and grapevine growers. Pruning and training systems can help to reduce microclimate humidity and promote direct sunlight exposure (Gadoury *et al.,* 2012), but they are not sufficient and the two main approaches to effectively control PM are the application of fungicides and the use of resistant cultivars.

Fungicides

Both *P. leucotricha* and *E. necator* can be controlled with frequent applications of a variety of organic and chemical fungicides. Those based on sulphur have several advantages: they are moderately effective at a limited cost, they do not favor the development of resistance in the pathogen and they can be used in organic agriculture (Gadoury *et al.,* 2012). Other fungicides used to control *E. necator,* such as benzimidazoles, ergosterol biosynthesis inhibitors and quinone-outside inhibitors, are more effective than sulphur ones, but they have single-site modes of action and the probability that the pathogens will develop resistance through a single base pair mutation is high, especially when the fungicides are used repeatedly (Gadoury *et al.,* 2012). *E. necator* strains resistant to a variety of fungicides are not uncommon (Dufour *et al.,* 2011).

A pivotal aspect of fungicides application is the timing, which depends on several factors, such as weather, phenology and development of ontogenic resistance in plants (Gadoury *et al*., 2011). For instance, in grapevine the time span of berries susceptible to PM is brief, therefore fungicides application should be particularly diligent in this period (Gadoury *et al.,* 2012). The control of PM in apple requires applications every 7-10 days starting just before bloom and proceeding until terminals no longer produce new leaves (Turecheck, 2004).

The amount of fungicides necessary to control PM is extremely high. The case of grapevine is explanatory: a report of the EU showed that in the period between 2001 and 2003, 67% of all fungicides applied to crops were used on grapevine, when viticulture accounted for only 3.3% of the agricultural land (EUROSTAT, 2007). Powdery mildew is the main fungal disease of grapevine and the amount of fungicides necessary to control it accounted for a large part of the total fungicide delivery. Considering the high costs of fungicide application (Fuller *et al.,* 2014) and their known effects on the environment (Wightwick *et al.,* 2010), on the health of vineyard beneficial organisms (Gadino *et al.,* 2011) and on the health of vineyard workers (Le Moal *et al.,* 2014), it is clear that fungicides should not be the major mean to control PM.

Resistant varieties

The majority of most cultivated apple cultivars, like "Granny Smith", "Gala", "Golden Delicious" and "Jonagold", are susceptible to PM (Turecheck, 2004), as well as all *V. vinifera* cultivars, including the most cultivated ones, such as 'Chardonnay', 'Pinot Noir', 'Merlot' and 'Cabernet Sauvignon'.

Despite the fact that the genus *Vitis* is composed of many species, nearly all the world's commercial grapevine comes from *V. vinifera*. Grapevine originated in Eurasia, whereas the pathogen *E. necator* originated in North America, where it colonized other *Vitis* species. Their evolution in isolation from each other is the cause of the extreme susceptibility of European grapevine, whereas wild grapevines native to North America are often partially or completely resistant (Cadle-Davidson *et al.,* 2011). Crosses between *V. vinifera* and North American species, such as *Vitis rotundifolia* (syn *Muscadiana rotundifolia*)*, Vitis rupestris, Vitis riparia* and *Vitis aestivalis*, resulted in resistant hybrids. However, the wine

produced from these resistant hybrids is low-quality and the market never supported their commercialization (Fuller *et al.,* 2014).

Like in *Vitis*, also in *Malus* wild species resistance to PM is not rare. The main difference is that apple production is less conservative than grapevine and the use of hybrids or cultivars that have wild *Malus* species in their lineage, is widely accepted. This can explain why several apple cultivars, some of which are widely cultivated like "McIntosh", are resistant or moderately resistant to PM (Swensen, 2006). However, even some resistant cultivars can be moderately infected under favorable conditions for the inoculum (Turecheck, 2004).

Resistance and susceptibility

The use of resistant cultivars can significantly reduce the amount of fungicides necessary to control PM. PM Resistance and susceptibility are the result of complex host-pathogen interactions. Today, the main focus is on resistance genes (R-genes), but an alternative approach based on susceptibility genes (S-genes) is here discussed.

Resistance genes

The interaction between a pathogen and its host is a complex process. Pathogens secrete a variety of effectors and among them there are the avirulence factors (Chen *et al.,* 2000). Resistant hosts, but not susceptible ones, have in their genomes resistance R-genes coding for specific proteins able to recognize avirulence factors and trigger defense response (Dodds and Rathjen, 2010). The largest family of R-genes is the NB-LRR family, which has three core domains: a C-terminal leucine-rich repeat (LRR), responsible for avirulence factor recognition, a central nucleotide binding site (NB) with a regulatory function and a variable effector domain at the N-terminal (Jones, 2000). The recognition of the avirulence factor by the R-gene

leads to a reaction called effector-triggered immunity, usually associated with programmed cell death (Bari and Jones, 2009; Qiu *et al.,* 2015).

Perennial woody plants, including *Vitis* and *Malus* wild species, are rich in NB-LRR genes because they have to face a wide range of pathogens during their lifespan (Patzak *et al.,* 2011; Feechan *et al.,* 2013a; Perazzolli *et al*., 2014; Tobias and Guest, 2014). Two families of PM R-genes, *REN* and *RUN,* were found in *V. rotundifolia* (Qiu *et al*., 2015) and several R-genes of various origins are known in apple. *Pl-1,* from *M. robusta* and *Pl-2* from *M. zumi* have been included in breeding programs since the seventies (Bus *et al*., 2010) and others have been introduced more recently, such as *Pl-m* (*M. domestica* accession MIS; James *et al.,* 2004), *Pl-w* (crab apple 'White Angel'; Evans and James, 2003) and *Pl-d* (*M. domestica* accession D12; James *et al.,* 2004).

R-genes and avirulence factors are a perfect example of co-evolution, as they can be found mostly in *Vitis* and *Malus* species native of the areas where *E. necator* and *P. leucotricha* originated. However, the direct consequence of co-evolution is that resistance granted by R-genes is frequently overcome by mutations of the pathogen (Parlevliet *et al.,* 1993). Clonally propagated crops, such as apple and grapevine, are genetically uniform and the progeny is not free to spread in the environment, therefore there will be no or few new mutations. In these conditions, the advantage for the pathogens is huge and R-genes quickly lose their efficacy, as documented for apple *Pl-2* and *Pl-m*, which resistance has been overcome by several strains of *P. leucotricha* (Caffier and Laurens, 2005). A possible solution is combining more R-genes, an approach called gene pyramiding, but it is very time-consuming and complex in perennial woody species like apple and grapevine.

Susceptibility genes

Resistance and susceptibility are two sides of the same coin. Moving the focus from resistance to susceptibility allows approaching the topic with a different perspective, centered on S-genes. A gene is considered an S-gene if its loss-of-function results in recessively inherited resistance (Pavan *et al*., 2010). Adapted pathogens are able to suppress plant defence by secreting effectors that interact with specific host proteins, also known as effector targets (Jones and Dangl, 2006; Chisholm *et al.,* 2006). The effectors secreted by pathogens can inactivate a positive regulator of plant defense or they can stimulate a negative regulator, but in both cases the final result is the inactivation of defense (Pavan *et al*., 2010). Several of these targets have been identified and genes coding for them could be knocked-out to achieve resistance (Pavan *et al*., 2010). However, resistance conferred by loss-of-function mutations in S-genes is recessive, meaning that the non-functional allele has to be homozygous, otherwise in heterozygosity the other (functional) allele will still cause susceptibility.

Resistance conferred by S-genes often has a price. S-genes, despite being responsible for susceptibility to pathogens, haven't been lost by the plants during evolution. This suggests that they have important physiological functions and the fitness costs associated to their knock-out would be too high for the plant (Pavan *et al*., 2010). The knock-out of S-genes often causes pleiotropic phenotypes, although not all S-genes cause them in all species (Pavan *et al.,* 2010). If resistance conferred by knocking out S-genes comes with a cost, why should S-genes be preferred over R-genes? There are two reasons: the first is that recessive resistance identified so far is more durable and effective against many strains of the pathogen, whereas resistance granted by a single R-gene is usually not durable in the agricultural environment and it is effective against only one or few strains (Pavan *et al.,* 2010). The second reason is that evolutionary cost does not automatically

mean agricultural cost. A theoretical example: if the knock-out of an S-gene causes aberrant roots growth, it would not be relevant for species like apple and grapevine that are grafted on rootstocks.

A known example of durable PM resistance governed by mutations in the *MLO* gene family will be discussed in detail in the next paragraph.

***MLO* genes**

The story of the study of MLO genes started in Germany in 1942, when the first PM resistant barley was obtained during an X-ray random mutagenesis experiment. In the following years, other resistant lines were obtained by random mutagenesis and ten of them were discovered to be mutated in the same locus, called *Mildew Locus O* (*MLO*) (Jørgensen, 1992). Further studies led to the discoveries of several accessions with natural loss-of-function mutations in locus *MLO* (Jørgensen, 1992). Resistance of these barley lines was recessively inherited, durable (it is still used nowadays) and effective against all *Blumeria graminis* f.sp. *hordei* isolates (Jørgensen, 1992; Büschges *et al.,* 1997). For many years barley *mlo* resistance was considered unique, but it was later discovered that *MLO* genes constitute a large family with several members, not all of them acting as S-genes, and largely conserved across the plant kingdom. More important, loss-of-function of specific *MLO* genes resulted in PM resistance in tomato (Bai *et al.,* 2008), pea (Pavan *et al.,* 2011), *Arabidopsis* (Consonni *et al.,* 2006), wheat (Wang *et al.,* 2014), and pepper (Zheng *et al.,* 2013). An important difference among these species is the number of genes that are required to be knocked-out to obtain complete resistance: in tomato and pea the knock-out of a single gene is sufficient (Bai *et al.,* 2008; Pavan *et al.,* 2011), whereas in *Arabidopsis* the knock-out of three genes is required for complete resistance (Fig. 3) (Consonni *et al.,* 2006). One of the three genes,

AtMLO2, plays a major role and *AtMLO6* and *AtMLO12* add a synergistic effect (Consonni *et al.,* 2006).

Figure 3. *A. thaliana* wild-type (on the left) and *Atmlo2/6/12* mutant (on the right) infected with PM species *Oidium neolycopersici*.

Structure of MLO proteins

MLO proteins have seven trans-membrane domains, an extra-cellular N-terminal and a cytosolic C-terminal (Devoto *et al.,* 2003). The C-terminal is highly variable in length and amino acids sequence (Devoto *et al.,* 2003) and it contains a calmodulin-binding domain that is required for full activity of barley HvMLO (Kim *et al.,* 2002). A series of C-terminal replacement experiments suggested that the three-dimensional conformation is more important for MLO activity rather than sequence identity (Elliot *et al.,* 2005).

MLO proteins do not have significant sequence similarities with other proteins and do not have obvious motifs, so it is hard to speculate what their biochemical activity could be. They have some similarities with G-protein-coupled-receptors, but they function independently from G-proteins (Kim *et al.,* 2002). The alignment of 38 MLO

proteins allowed identifying 30 invariant and 17 highly conserved amino acids (Elliot *et al.,* 2005). Site-directed mutagenesis of six invariant amino acids showed that they are required for the role in PM susceptibility of HvMLO (Elliot *et al.,* 2005).

Phylogenetic analysis of MLO proteins

MLO is a sizeable family, with considerable differences among species. *Arabidopsis* has 15 members, grapevine 17, wheat, which is the species with the smallest number of members, has eight, and soybean, the species with the highest number, has 39 (Acevedo-Garcia *et al.,* 2014). For years the phylogenetic analysis of the MLO family grouped the proteins in five clades, which were increased to six when grapevine MLOs were included in the analysis (Feechan *et al.,* 2008; Winterhagen *et al.,* 2008). Two of the clades, IV and V, are of particular interest: all dicots MLO proteins associated to PM susceptibility, namely *Arabidopsis* AtMLO2, 6 and 12 (Consonni *et al.,* 2006), tomato SlMLO1 (Bai *et al*., 2008) and pea PsMLO1 (Pavan *et al.,* 2011), group in clade V, whereas all monocot MLO proteins associated to PM susceptibility, namely barley HvMLO (Jørgensen*,* 1992) and wheat TaMLO-A1, B1 and D1 (Wang *et al.,* 2014), group in clade IV. No MLO protein involved in PM susceptibility has so far been found outside these two clades. Clades I to IV are populated by members of both monocot and dicot species, although clade IV is constituted mostly by monocot MLO proteins, whereas clade V and VI are represented exclusively by dicot MLOs (Acevedo-Garcia *et al.,* 2014). This suggests that clades I-IV originated before the divergence between monocots and dicots and clades V and VI are recent dicot innovations (Acevedo-Garcia *et al.,* 2014). Clade VI is probably the most recent, as only few proteins cluster in it (Acevedo-Garcia *et al.,* 2014), whereas clade I seems to be the most ancient, as MLO proteins from mosses and ferns can be found only in it (Jiwan *et al*., 2013). A seventh clade was recently

proposed, represented by cucumber CsMLO11 (Zhou *et al.,* 2013) and tomato SlMLO2 (Chen *et al.,* 2014).

Role of *MLO* genes in susceptibility to powdery mildew

The specific function of MLO proteins is not clear yet, but the information available allows drawing some conclusions. What follows in this chapter is a recap of what is known to date.

The mechanism of resistance in *mlo* mutants seems linked to the formation of cell wall appositions called papillae (Consonni *et al.*, 2006). Papillae consist in a callose matrix enriched in proteins and various compounds (Vanacker *et al.* 2000) and they constitute a pre-penetration defense system that thickens the cell wall to stop the penetration pegs of PM fungi (Fig. 4). Formation of papillae at the cell wall depends on the delivery through endomembrane transport of the materials that constitute them (Hückelhoven, 2014). The formation of papillae is not restricted to *mlo* mutants, but it is part of the normal reaction of the host to PM infection. However, papillae of susceptible plants are not effective. Three factors determine the difference between effective and non-effective papillae: timing of formation, composition and size. In barley, rapid formation of papillae (Lyngkjær *et al.* 2000) and increased size (Stolzenburg *et al.*, 1984) correlate with *mlo* resistance. Moreover, it was recently suggested that the composition plays a major role, with effective papillae containing a higher concentration of callose, cellulose and arabinoxylan (Chowdhury *et al*., 2014).

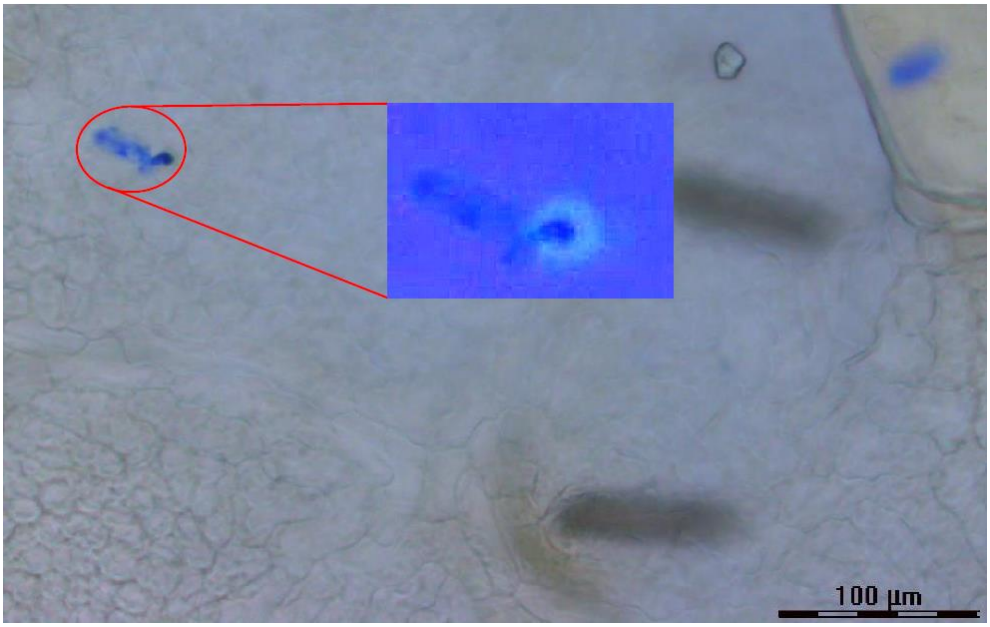

Figure 4. Papilla in a grapevine leaf after three days from the inoculation with *Erysiphe Necator.* The light blue fluorescence is the papilla.

Miklis *et al.* (2007) showed that *mlo* resistance depends on actin cytoskeleton. The depolymerization of actin cytoskeleton resulted in increased susceptibility to PM in both normal and *mlo* barley plants. However, *mlo* barley, even with depolymerized actin, was still less susceptible to PM than wild-type barley, suggesting that *mlo* resistance does not entirely depend on actin and there is at least one actin-independent component (Miklis *et al.,* 2007).

The knock-out of three genes of Arabidopsis, *AtPEN1, 2* and *3*, resulted in increased PM susceptibility (Collins *et al.,* 2003; Lipka *et al.,* 2005; Stein *et al*., 2006). *AtPEN1* is a syntaxin, a class of protein involved in exocytosis*, AtPEN2* is a glycosil hydrolase, a class of enzymes that assist the hydrolysis of glycosidic bonds, and *AtPEN3* is an ABC transporter, a class of trans-membrane proteins involved in the transportation of a wide variety of substrates (Collins *et al.,* 2003; Lipka *et al.,* 2005; Stein *et al*., 2006). Moreover, when *AtPEN1, 2* and *3* were knocked-out in combination with

Atmlo2, they restored *Arabidopsis* PM susceptibility to nearly wild-type levels (Consonni *et al.,* 2006). These findings suggest that *AtPEN1, 2* and *3* are involved in the defense mechanism against PM. *AtPEN1* and its homologous from barley (*HvROR2*) and grapevine (*VvPEN1)*, code for proteins that were found to localize at attempted PM penetration sites (Collins *et al.,* 2003; Bath *et al.,* 2005). A study carried out in the *Arabidopsis* heterologous system showed that VvPEN1 was co-trafficked with VvMLO3 and VvMLO4 at PM infection sites through the same transport system (Feechan *et al.,* 2013b).

In sum, MLO are trans-membrane proteins which function partially depends on actin cytoskeleton. Some of them are co-trafficked and co-localized at attempted PM penetration sites with a syntaxin, a class of proteins involved in exocytosis. The knock-out of specific *MLO* genes results in the formation of effective cell-wall appositions able to prevent fungal penetration. Taken together, these observations suggest that MLO proteins are involved in the regulation of actin-dependent transport to the cell wall, particularly in the negative regulation of vesicle-associated defense mechanisms necessary for the formation of effective cell-wall appositions at the sites of attempted PM penetration (Panstruga, 2005).

Other functions of *MLO* genes

The specific biochemical activity of MLO proteins is, to date, unknown. The role in susceptibility is a peculiarity of a limited number of them and the function of the majority of the members of the *MLO* family has not been unraveled yet. *MLO* genes are known to be differentially expressed in different tissues (Feechan *et al.,* 2008), suggesting the involvement in a variety of physiological processes. However, what these processes precisely are is still unknown. The only information available is with regards to three *Arabidopsis* genes: *AtMLO7* is required for pollen tube perception

from the embryo sac (Kessler *et al*., 2010) and *AtMLO4* and *AtMLO11* are involved in root thigmomorphogenesis (Chen *et al.,* 2009).

Up-regulation of specific *MLO* genes in response to powdery mildew infection

The majority of the members of the *MLO* family do not have a role in PM susceptibility and those that have are restricted to clades IV and V. Again, not all members of clades IV and V are S-genes, but candidates can be identified during early stages of PM infection because their expression increases in response to the pathogen within the first 24 hours, with a peak at around 6 hours, as documented in tomato (Bai *et al.,* 2008), barley (Piffanelli *et al.,* 2002), pepper (Zheng *et al.,* 2013) and grapevine (Feechan *et al*., 2008; Winterhagen *et al.,* 2008). In grapevine, three clade V genes are up-regulated upon PM inoculation (*VvMLO7, VvMLO11* and *VvMLO13*), while a fourth one (*VvMLO6*) is not responsive to the pathogen (Feechan *et al*., 2008; Winterhagen *et al.,* 2008).

Summarizing, selection of candidate S-genes from the group of *MLO* genes can be performed using two filters: 1) For dicots, the *MLO*-gene should cluster in Clade V; 2) The *MLO* gene should be induced by PM.

The costs of the knock-out of *MLO* genes

As previously explained, the knock-out of S-genes can result in pleiotropic phenotypes. This appears to be particularly true for S-genes coding for negative regulators of defense, which is the case of *MLO* genes. As a matter of fact, pleiotropic phenotypes due to MLO genes knock-out have been documented in three species. In barley, early senescence-like leaf chlorosis in non-optimal conditions was observed, as well as reduced grain yield, although this last phenotype was eliminated through breeding (Jørgensen, 1992). *Arabidopsis mlo* triple mutants show a senescence-like phenotype similar to barley and are more

susceptible to necrotrophic pathogens (Consonni *et al.,* 2006). In pepper, the knock-down of *CaMLO1* and *CaMLO2* resulted in reduced size compared to wild-type plants (Zheng *et al.,* 2013). However, the insurgence of pleiotropic phenotypes in *mlo* plants may not be a general rule, as for *mlo* tomato fitness costs have not been discovered yet (Bai *et al.,* 2008).

Thesis outline

The goal of this thesis is to identify the *MLO* genes responsible for susceptibility to PM in apple and grapevine. Both these species are pivotal for the economy of Italian agriculture, with a particular regard for Trentino, the region where Fondazione Edmund Mach, the institute that funded this thesis, is located.

Such a valuable piece of information could be used to develop resistant apple and grapevine varieties. This could be done either with traditional technologies, like marker assisted selection, or with modern gene technologies. Genetic manipulation is perceived as unacceptable by a majority of the European public, but a new generation of gene editing systems, like TALEN and CRISPR/CAS9, could change this perception and allow developing resistant plants using GM technologies.

In Chapter 2 of this thesis, we describe the characterization of the *MLO* gene family in three rosaceae species (*M. domestica, Prunus persica* and *Fragaria vesca*) and the identification through gene expression analysis of three candidate S- genes of apple, namely *MdMLO11, MdMLO18* and *MdMLO19*.

In Chapter 3 we describe the knock-down through RNA interference of two *MLO* genes of apple, *MdMLO11* and *MdMLO19*, and the complementation test with *MdMLO18* in *Arabidopsis.* The phenotypic and molecular characterization of the transgenic plants showed that *MdMLO19* is the sole *MLO* gene responsible for PM susceptibility in apple.

In Chapter 4 we describe the screening of the Fruitbreedomics (an EU supported international research program) re-sequencing dataset of 63 apple cultivars. A particularly interesting mutation was found in *MdMLO19*: it is an insertion that causes a frameshift and an early stop codon, which results in an MLO protein lacking the final 185 amino acids. The estimation of the frequency of this mutation in 159 apple genotypes revealed that the insertion is more common than anticipated.

In Chapter 5 we described the knock-down through RNA interference of four *MLO* genes of grapevine and the phenotypic and molecular characterization of the resulting transgenic plants. One of the genes, *VvMLO7* has a major role, whereas *VvMLO6* and *VvMLO11* have a putative additive effect, although they are not effective on their own. *VvMLO13* is not involved in susceptibility.

The results described in the separate chapters of this thesis are discussed in the general discussion, where we analyse our results in the light of previous studies on the topic, and we propose additional experiments for better understanding the nature of *mlo* resistance, with a particular focus on fruit crops.

CHAPTER 2

Characterization of the *MLO* gene family in Rosaceae and gene expression analysis in *Malus domestica*

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ABSTRACT

Background: Powdery mildew (PM) is a major fungal disease of thousands of plant species, including many cultivated Rosaceae. PM pathogenesis is associated with up-regulation of MLO genes during early stages of infection, causing down-regulation of plant defense pathways. Specific members of the MLO gene family act as PM-susceptibility genes, as their loss-of-function mutations grant durable and broad-spectrum resistance.

Results: We carried out a genome-wide characterization of the MLO gene family in apple, peach and strawberry, and we isolated apricot MLO homologs through a PCR-approach. Evolutionary relationships between MLO homologs were studied and syntenic blocks constructed. Homologs that are candidates for being PM susceptibility genes were inferred by phylogenetic relationships with functionally characterized MLO genes and, in apple, by monitoring their expression following inoculation with the PM causal pathogen *Podosphaera leucotricha.*

Conclusions: Genomic tools available for Rosaceae were exploited in order to characterize the MLO gene family. Candidate MLO susceptibility genes were identified. In follow-up studies it can be investigated whether silencing or a loss-of-function mutations in one or more of these candidate genes leads to PM resistance.

Keywords: Rosaceae, MLO, Powdery Mildew, *Malus domestica*

INTRODUCTION

Powdery mildew (PM) is a major fungal disease for thousands of plant species (Glawe *et al*., 2008), including cultivated Rosaceae such as apple (*Malus domestica)*, peach (*Prunus persica),* apricot *(Prunus armeniaca)* and strawberry (*Fragaria x ananassa)*. It occurs in all major growing regions, leading to severe losses (Turechek *et al*., 2004). Main PM causal agents are *Podosphaera leucotricha*

in apple (Turechek *et al*., 2004), *Sphaerotheca pannosa* var. *persicae* in peach (Foulongne *et al*., 2003), *Podosphaera tridactyla* in apricot (Boesewinkel 1979) and *Podosphaera aphanis* (syn. *Sphaerotheca macularis* f. sp. *fragariae)* in strawberry (Xiao *et al.*, 2001). The disease shows similar symptoms in the four species: white spots appear on young green tissues, particularly leaves in the first days after opening, whereas mature leaves show some resistance. Infected leaves crinkle, curl, and prematurely drop. Blossoms and fruits are not the primary targets of PM fungi, but infections of these tissues are possible (Turechek *et al*., 2004; Foulongne *et al*., 2003; Xiao *et al*., 2001)**.** In peach, apricot and apple, PM spores overwinter in buds and in spring, with the reprise of vegetative growth, spores start a new infection (Foulongne *et al*., 2003; Turechek *et al*., 2004).

The availability of resistant cultivars is fundamental to reduce the amount of pesticides used to control PM in agricultural settings. The usual strategy in breeding focuses on plant resistance genes (R-genes). However, R-genes often come from wild-relatives of the cultivated species, and thus interspecific crossability barriers could prevent their introgression (Fu *et al*., 2009). Moreover, in case of a successful cross, several unwanted traits are carried along with the R-gene and this makes extensive backcrossing necessary, which is time-consuming in woody species. Finally, the durability of R-genes is generally limited because of the appearance of virulent strains of the pathogen, which can overcome resistance in a few years (Parlevliet, 1993). Two examples are *Venturia inaequalis* race 6, able to overcome *Rvi6* resistance to scab in apple (Parisi *et al.*, 1998), and *P. leucotricha* strains able to breakdown *Pl-1* and *Pl-2*, two major PM R-genes of apple (Krieghoff, 1995).

A breeding approach alternative to the use of R-genes is based on plant susceptibility genes (S-genes), defined as genes whose loss-of-function results in recessively inherited resistance (Pavan *et al*., 2010). Barley *mlo* PM resistance, first

characterized in 1942, is a remarkable example of immunity due to the lack of an S-gene, as it derives from loss-of-function mutation of a gene called *MLO* (*Mildew Locus O*), encoding for a protein with seven transmembrane domains (Büschges *et al*., 1997; Devoto *et al.,* 1999). *Mlo* resistance has been considered for a long time as a unique form of resistance, characterized by durability, broad-spectrum effectiveness and recessive inheritance (Jørgensen, 1992). However, the characterization of resistance sources in other plant species, like *Arabidopsis* (Consonni *et al*., 2006), pea (Humphry *et al*, 2011; Pavan *et al*., 2011) and tomato (Bai *et al*., 2008), which are due to loss-of-function mutations of *MLO* functional orthologs, made clear that *mlo* resistance is more common than previously thought. Therefore, it has been suggested that the inactivation of *MLO* susceptibility genes could represent a valid strategy to introduce PM resistance across cultivated species (Pavan *et al*. 2010).

The histological characterization of *mlo* resistance revealed that it is based on a pre-penetration defense system, associated to the formation of cell-wall appositions (Aist and Bushnell, 1991; Consonni *et al*., 2006) and at least partially dependent on actin cytoskeleton (Miklis *et al*., 2007). It has been suggested that functional MLO proteins negatively regulate vesicle-associated and actin-dependent defense pathways at PM attempted penetration sites (Panstruga, 2005), and are targeted by PM fungi as a strategy to induce pathogenesis. Early stages of PM infection are associated with an increase of the transcript abundances of *MLO* susceptibility genes, showing a peak at 6 hours after inoculation. This has been shown to occur in tomato (Bai *et al*., 2008), barley (Piffanelli *et al*., 2002), pepper (Zheng *et al.,* 2013) and grape (Feechan *et al.*, 2008; Winterhagen *et al*., 2008).

MLO susceptibility genes are members of a gene family which shows tissue specific expression patterns and are involved in different physiological processes, besides

the response to PM fungi: one of the 15 *MLO* genes of *Arabidopsis, AtMLO7,* is involved in pollen tube reception by the embryo sac and its mutation results in reduced fertility (Kessler *et al*., 2010). Two other *Arabidopsis* genes, named *AtMLO4* and *AtMLO11,* are involved in the control of root architecture, as mutants with null alleles of these two genes show asymmetrical root growth and exaggerated curvature (Chen *et al.,* 2009).

Previous phylogenetic analysis of the MLO protein family identified six clades (Feechan *et al*., 2008). One of them, named clade V (Feechan *et al*., 2008), includes all the MLO proteins so far functionally related to PM susceptibility in dicot species (Consonni *et al*., 2006; Bai *et al*., 2008; Winterhagen et al., 2008). Similarly, Clade IV harbors monocots PM susceptibility proteins (Panstruga *et al*., 2005; Reinstädler *et al*., 2010).

MLO genes have been intensively studied in many monocots and dicots, but not in Rosaceae. We addressed this work to the characterization of the *MLO* gene family in Rosaceae, with respect to their structural, genomic and evolutionary features. Moreover, we monitored the transcript abundances of apple *MLO* homologs following *P. leucotricha* inoculation in three apple cultivars.

RESULTS

In silico and *in vitro* characterization of Rosaceae *MLO* homologs

Database search for Rosaceae *MLO* homologs produced 21 significant matches in *P. persica*, 23 *in F. vesca* and 28 in *M. domestica*. Of these, six (five from *M. domestica* and one from *F. vesca*) showed a very limited alignment region with other *MLO* genes, whereas eight (two from *M. domestica*, two from *P. persica* and four from *F. vesca*) were characterized by markedly different length with respect to *MLO* homologs reported in the genomes of *Arabidopsis* and grapevine (Devoto *et al.,* 2003; Feechan *et al*., 2008), i.e. less than 350 amino acids (aa) or more than

700 aa. Information retrieved on genomic localization amino acid number, putative transmembrane domains and predicted exon/intron structure of the remaining homologs, together with information about the *MLO* homologs nomenclature chosen in this study is provided in Tables 1, 2 and 3.

Peach and apricot are evolutionary very close to each other, and show a high degree of homology in DNA sequence. Phylogenetic analysis (see next paragraph) indicated peach homologs *PpMLO1*, *PpMLO3* and *PpMLO4* as candidates for being required for PM susceptibility. Therefore, we used the sequences of these genes to design primers to identify full-length apricot *MLO* genes. This approach resulted in the amplification and the successive characterization of three *MLO* sequences, which were by analogy named *PaMLO1, PaMLO3,* and *PaMLO4* (deposited in the NCBI database with the accession numbers KF177395, KF177396, and KF177397, respectively).

Phylogenetic relations and inference of orthology

We performed a phylogenetic study on the newly identified Rosaceae MLO proteins. The dataset was completed with four homologs recently characterized in *Rosa hybrida* (Kaufmann *et al*., 2012) (RhMLO1, RhMLO2, RhMLO3 and RhMLO4), the complete *Arabidopsis* AtMLO protein family (Consonni *et al.,* 2006) and a series of MLO homologs which have been functionally associated with PM susceptibility, namely tomato SlMLO1 (Bai *et al.,* 2008), pea PsMLO1 (Humphry *et al.*, 2011; Pavan *et al.,* 2011), pepper CaMLO2 (Zheng *et al.,* 2013), lotus LjMLO1 (Humphry *et al.,* 2011), barrel clover MtMLO1 (Humphry *et al.,* 2011), barley HvMLO (Büschges *et al*., 1997), rice OsMLO2 (Elliot *et al.,* 2002), wheat TaMLO_B1 and TaMLO_A1b (Elliot *et al.,* 2002) and grapevine VvMLO14, the only dicot MLO homolog known to belong to clade IV (Feechan *et al.* 2008).

Table 1: Members of the *MdMLO* gene family as predicted in *M. domestica* cv. 'Golden Delicious' genome sequence

Gene	Accession number [a]	Chr.	Starting position (Mb)	Clade	Introns	TM[b]	Amino acids	Conserved aa[c]
MdMLO1	MDP0000177099	2	1.02	II	11	3	487	25
MdMLO2	MDP0000240125	2	11.10	I	11	3	571	20
MdMLO3	MDP0000168575	2	11.11	I	13	7	670	22
MdMLO4	MDP0000207002	2	8.79	III	16	7	634	28
MdMLO5	MDP0000163089	9	15.26	V	14	6	579	30
MdMLO6	MDP0000119433	3	33.95	II	0	7	504	30
MdMLO7	MDP0000123907	n.d.	n.d.	V	n.d.	6	561	28
MdMLO8	MDP0000218520	2	11.11	I	9	4	390	14
MdMLO9	MDP0000320797	2	27.20	II	10	5	454	28
MdMLO10	MDP0000196373	3	26.97	I	13	6	539	28
MdMLO11	MDP0000239643	4	9.84	V	12	8	575	28
MdMLO12	MDP0000133162	6	0.81	III	13	5	516	28
MdMLO13	MDP0000142608	7	7.48	II	12	6	351	18
MdMLO14	MDP0000191469	8	29.25	II	10	5	395	23
MdMLO15	MDP0000141595	9	7.54	III	15	6	647	24
MdMLO16	MDP0000191848	9	21.12	VI	14	6	606	29
MdMLO17	MDP0000145097	11	27.97	I	13	7	523	28
MdMLO18	MDP0000928368	10	27.97	VII	12	7	502	30
MdMLO19	MDP0000168714	12	16.23	V	13	7	590	30
MdMLO20	MDP0000134649	13	11.61	VIII	13	5	589	27
MdMLO21	MDP0000133760	15	24.99	VI	15	6	560	28

[a] Available at http://genomics.research.iasma.it/gb2/gbrowse/apple/

[b] Number of transmembrane domains in the predicted protein, as determined by InterPro (http://www.ebi.ac.uk/interpro/).

[c] number of conserved amino acids out of the 30 identified by Elliott *et al.* (2005).

Table 2: Members of the *PpMLO* gene family as predicted in *Prunus persica* genome sequence

Gene	Accession number [a]	Chr.	Starting position (Mb)	Clade	Introns	TM[b]	Amino acids	Conserved aa[c]
PpMLO1	ppa003207m	6	6.82	V	14	7	593	30
PpMLO2	ppa003435m	7	18.38	III	14	8	574	30
PpMLO3	ppa003437m	6	21.99	V	13	7	574	30
PpMLO4	ppa003466m	2	21.03	V	14	7	572	30
PpMLO5	ppa003706m	4	10.92	I	14	8	555	30
PpMLO6	ppa004012m	7	22.64	II	14	6	535	29
PpMLO7	ppa004508m	8	21.17	II	0	7	506	29
PpMLO8	ppa004621m	6	22.01	VI	14	6	499	29
PpMLO9	ppa004687m	4	2.59	VII	11	7	496	29
PpMLO10	ppa004866m	2	13.73	II	11	7	488	29
PpMLO11	ppa020172m	1	43.04	I	14	4	561	30
PpMLO12	ppa020311m	5	0.82	IV	13	7	566	30
PpMLO13	ppa021048m	4	15.57	VIII	12	5	510	24
PpMLO14	ppa022847m	6	6.80	VI	14	6	550	29
PpMLO15	ppa024476m	7	17.63	I	14	8	539	26
PpMLO16	ppa024488m	5	0.76	III	14	6	504	30
PpMLO17	ppa024581m	6	8.95	II	13	6	463	27
PpMLO18	ppa026565m	6	22.00	VI	13	6	416	25
PpMLO19	ppb024523m	1	42.04	II	13	5	446	23

[a] Available at http://www.rosaceae.org/gb/gbrowse/prunus_persica/

[b] Number of transmembrane domains in the predicted protein, as determined by InterPro (http://www.ebi.ac.uk/interpro/).

[c] number of conserved amino acids out of the 30 identified by Elliott *et al.* (2005).

Table 3: Members of the *FvMLO* gene family as predicted in *Fragaria vesca* genome sequence

Gene	Accession number [a]	Chr.	Starting position (Mb)	Clade	Introns	TM[b]	Amino acids	Conserved aa[c]
FvMLO1	mrna02774.1-v1.0-hybrid	n.d.	n.d.	V	14	7	632	28
FvMLO2	mrna03210.1-v1.0-hybrid	3	14.46	II	11	5	528	20
FvMLO3	mrna09651.1-v1.0-hybrid	6	35.88	III	14	6	542	28
FvMLO4	mrna09653.1-v1.0-hybrid	6	35.90	V	14	7	573	30
FvMLO5	mrna10166.1-v1.0-hybrid	1	1.34	II	14	3	688	26
FvMLO6	mrna10346.1-v1.0-hybrid	3	12.52	II	7	2	385	15
FvMLO7	mrna10363.1-v1.0-hybrid	3	12.49	II	9	2	442	21
FvMLO8	mrna10558.1-v1.0-hybrid	2	19.08	II	n.d.	6	514	28
FvMLO9	mrna11028.1-v1.0-hybrid	n.d.	n.a.	I	10	4	434	18
FvMLO10	mrna13023.1-v1.0-hybrid	1	7.96	III	13	6	557	27
FvMLO11	mrna14592.1-v1.0-hybrid	1	8.77	I	13	7	548	28
FvMLO12	mrna23198.1-v1.0-hybrid	7	15.89	V	14	7	507	29
FvMLO13	mrna26428.1-v1.0-hybrid	7	17,79	VIII	11	5	558	20
FvMLO14	mrna28541.1-v1.0-hybrid	n.d.	n.a.	III	11	4	481	26
FvMLO15	mrna29770.1-v1.0-hybrid	3	7.36	VII	13	7	538	28
FvMLO16	mrna31264.1-v1.0-hybrid	3	30.51	I	16	8	579	28
FvMLO17	mrna31498.1-v1.0-hybrid	5	20.23	IV	11	5	531	27
FvMLO18	mrna29285.1-v1.0-hybrid	5	19.12	V	6	4	357	18

[a] Available at http://www.rosaceae.org/gb/gbrowse/fragaria_vesca_v1.0-lg/ (hybrid)

[b] Number of transmembrane domains in the predicted protein, as determined by InterPro (http://www.ebi.ac.uk/interpro/).

[c] number of conserved amino acids out of the 30 identified by Elliott *et al.* (2005).

Phylogenesis reconstruction by using a UPGMA algorithm resulted in a total of eight distinct clades and no divergent lineage (Fig. 1).

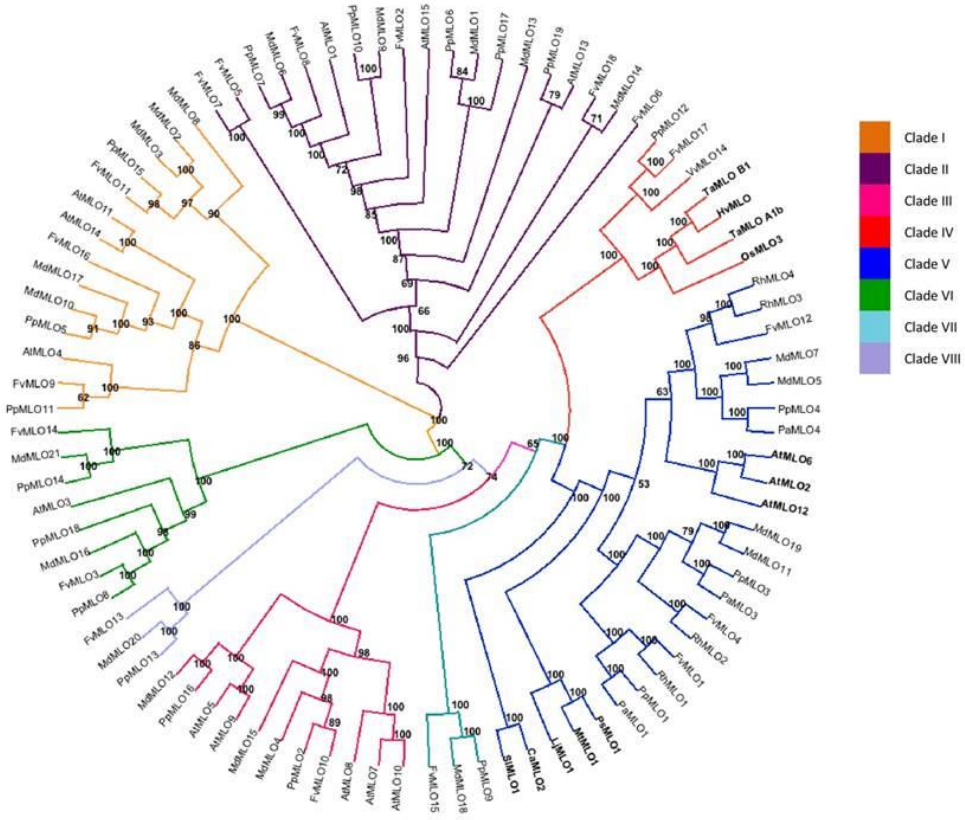


Figure 1. **Phylogenetic tree of Rosaceae MLO**. Phylogenetic relationships of predicted Rosaceae MLO amino acid sequences to MLO proteins of other plant species. The dataset includes Rosaceae MLO sequences from *Rosa hybrida* (RhMLO), *Malus domestica* (MdMLO), *Prunus persica* (PpMLO), *Prunus armeniaca* (PaMLO) and *Fragaria vesca* (FvMLO). The other proteins included are *Solanum lycopersicum* SlMLO1, *Arabidopsis thaliana* AtMLO, *Capsicum annuum* CaMLO2, *Pisum sativum* PsMLO1, *Medicago truncatula* MtMLO1, *Lotus japonicus* LjMLO1, *Vitis vinifera* VvMLO14, *Hordeum vulgare* HvMLO, *Triticum aestivum* TaMLO_B1, TaMLO_A1b and *Oryza sativa* OsMLO2. Proteins which have been functionally characterized as susceptibility genes are highlighted in bold.

Clade numbers from I to VI were assigned based on the position of *Arabidopsis* AtMLO homologs and barley HvMLO, according to the previous study of Feechan *et al.* (2008). The two additional clades (named VII and VIII) were found to include Rosaceae MLO homologs only, both having one homolog from *P. persica*, one from *F. vesca* and one from *M. domestica*. Further phylogenetic analysis with a Neighbor-Joining algorithm resulted in merging clade VII and VIII (not shown).

Four apple MLO homologs (MdMLO5, MdMLO7, MdMLO11 and MdMLO19) and three MLO homologs from peach (PpMLO1, PpMLO3 and PpMLO4), apricot (PaMLO1, PaMLO3 and PaMLO4) and woodland strawberry (FvMLO1, FvMLO4 and FvMLO12) were found to cluster together in the phylogenetic clade V, containing all the dicot MLO proteins experimentally shown to be required for PM susceptibility (e.g. Feechan *et al.,* 2008; Pavan *et al.,* 2011). One homolog from strawberry (FvMLO17) and one from peach (PpMLO12) were found to group, together with grapevine VvMLO14, in clade IV, which contains all monocot MLO proteins acting as PM susceptibility factors (Fig. 1).

We used the GBrowse-Syn tool to detect syntenic blocks encompassing *P. persica*, *F. vesca* and *M. domestica MLO* genes. As syntenic blocks derive from the evolution of the same chromosomal region after speciation, relations of orthology between *MLO* genes were inferred. In total, twelve relations of orthology were predicted between *P. persica* and *F. vesca*, nine between *P. persica* and *M. domestica* and eight between *F. vesca* and *M. domestica* (Table 4, Fig. 2 and additional material 1). The relation of orthology between *PpMLO1, PpMLO3*, *PpMLO4* and apricot *PaMLO1, PaMLO3*, *PaMLO4*, respectively, was clearly suggested by the high percentage of sequence identity between these homolog genes, which was 97.3%, 98.8% and 96.7 %, respectively.

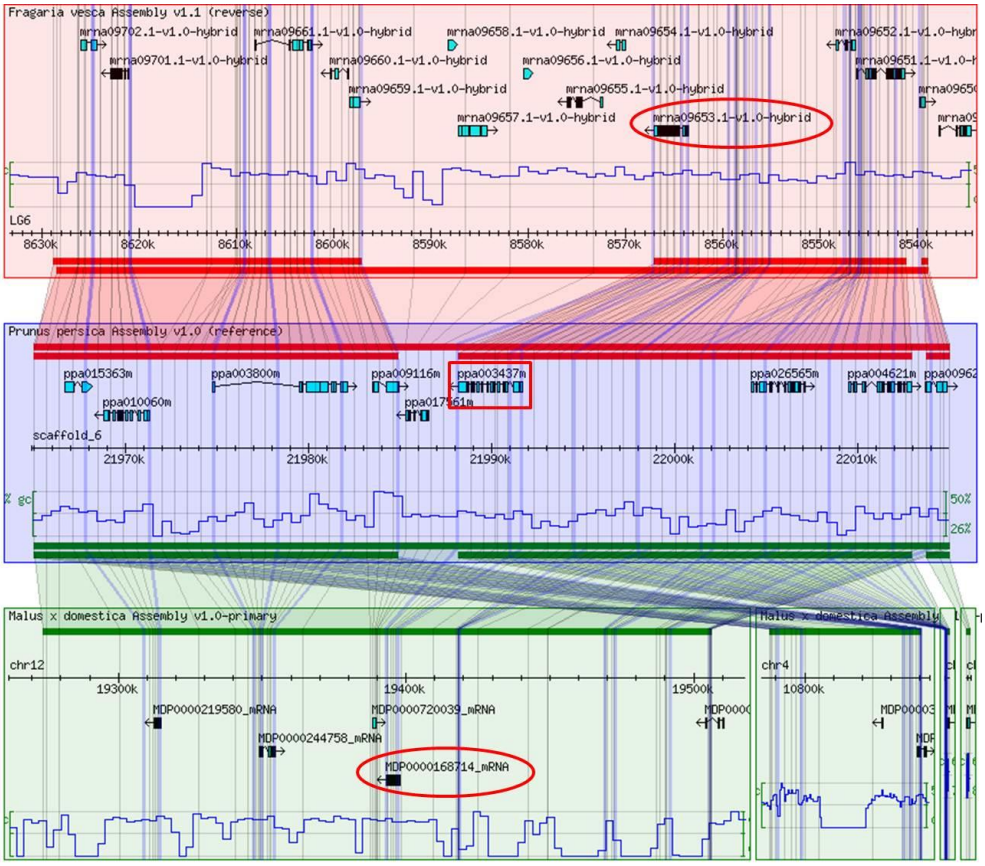


Figure 2. Synteny between apple, peach and strawberry. Results of search for *F. vesca* and *M. domestica* chromosomal regions syntenic to a *P. persica* 50 kb stretch including the *MLO* homolog *PpMLO3* (corresponding to ppa003437m in the genomic database of Rosaceae), boxed. Shaded polygons indicate aligned regions between genomes. Grid lines are meant to indicate insertions/deletions between the genomes of *F. vesca* and *M. domestica* with respect to the *P. persica* reference sequence. Strawberry *FvMLO4* and apple *MdMLO19* (named in the figure as mrna09653.1-v1.0-hybrid and MDP0000168714, according to the nomenclature provided in this paper), predicted to be *PpMLO3* orthologs, are indicated with circles.

Table 4: Relations of orthology inferred between *persica, F. vesca* and *M. domestica MLO* homologs

***P. persica* genes**	***F. vesca* orthologs**	***M. domestica* orthologs**
PpMLO2	*FvMLO10*	*MdMLO15*
PpMLO3	*FvMLO4*	*MdMLO19*
PpMLO4	*FvMLO12*	-
PpMLO5	*FvMLO16*	*MdMLO10, MdMLO17*
PpMLO6	*FvMLO5*	*MdMLO1*
PpMLO7	*FvMLO8*	-
PpMLO8	*FvMLO3*	-
PpMLO9	*FvMLO15*	*MdMLO18*
PpMLO10	*FvMLO2*	*MdMLO9*
PpMLO14	*FvMLO14*	*MdMLO21*
PpMLO15	*FvMLO11*	-
PpMLO16	-	MdMLO12
PpMLO18	*FvMLO3*	-

Transcription of apple putative *MLO* genes in response to *Podosphaera leucotricha* inoculation

To identify *MLO* genes that respond to the PM fungus *P. leucotricha*, we measured the transcript abundance of 19 out of 21 apple *MLO* genes in leaves 4, 6, 8 and 24 hours after artificial inoculation, and compared these data with the ones of non-inoculated leaves. Three cultivars, 'Golden Delicious', 'Braeburn' and 'Gala', were analysed in order to see if up-regulation was comparable among them and, therefore, results could be generalized for all apple cultivars. Three genes, namely *MdMLO11*, *MdMLO18* and *MdMLO19*, were found to be significantly up-regulated after inoculation with the pathogen (Fig. 3 and additional material 2). Up-regulation of these genes was about 2-fold compared to non-inoculated plants, with peaks of 4-fold at very early time points ('Braeburn'- *MdMLO11* - 6 hpi; 'Gala'- *MdMLO18* - 4 hpi; 'Golden Delicious'- *MdMLO19* - 6hpi). *MdMLO11* and *MdMLO18*

were up-regulated in all cultivars, *MdMLO19* only in 'Braeburn' and 'Golden Delicious'.

Two of the genes, *MdMLO11* and *MdMLO19* belong to Clade V, while *MdMLO18* belongs to the newly identified Clade VII (Fig. 1).

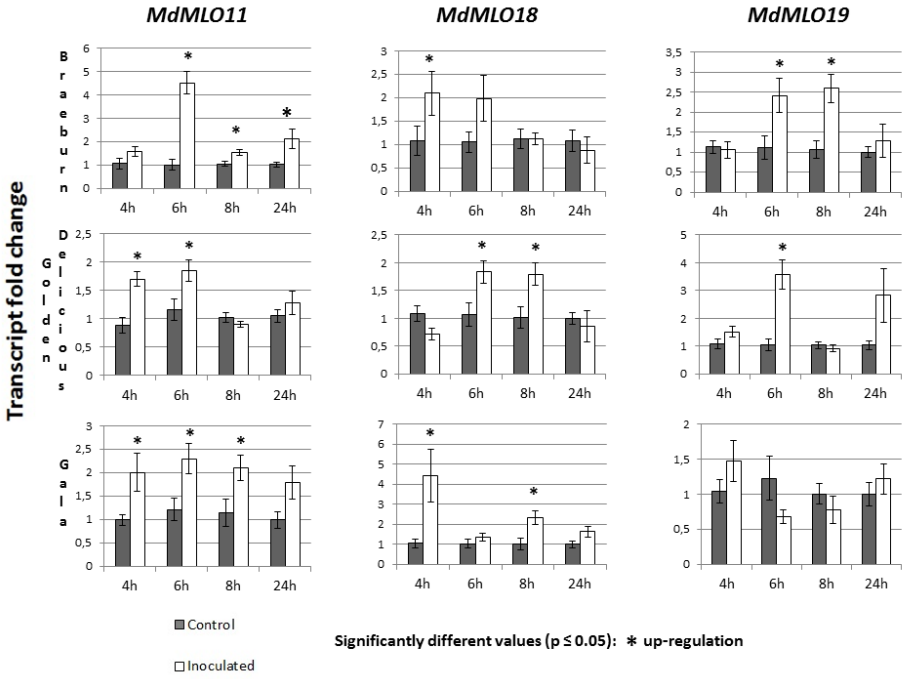


Figure 3. Transcriptional variation of three apple MLO genes following inoculation with *P. Leucotricha*. Transcript abundances of three *MLO* genes in leaves of 'Braeburn', 'Golden Delicious' and 'Gala' following PM inoculation. Here are shown only *MLO* genes that were significantly up or down regulated more than once following PM inoculation at one of the time points examined (4, 6, 8 and 24 hpi). Each bar shows the average of four to eight biological replicates. The Ct values have been normalized for three reference genes: actin, ubiquitin and elongation factor 1. Statistical significance was determined with a *t*-test for each pair of inoculated and non-inoculated samples at each time point. The error bars show standard errors of the means. Significant differences between inoculated samples and control samples are indicated with an asterisk ($P < 0.05$).

DISCUSSION

Genomic organization and phylogenetic relations between Rosaceae *MLO* homologs

We report here the identification, through an *in silico* approach, of 19 *MLO* homologs in the diploid genome of peach and 18 in the genome of woodland strawberry. This is consistent with the results of previous genome-wide studies carried out on dicotyledonous species, indicating the presence of 15 *MLO* homologs in *Arabidopsis*, 17 in grapevine and 16 in tomato (Devoto *et al.,* 2003; Feechan *et al.,* 2008; Dr. M. Appiano Wageningen UR Plant Breeding/University of Bari, unpublished results; Winterhagen *et al*.,2008). Conversely, the number of *MLO* homologs detected in apple (21) is lower than expected, considering that a relatively recent genome-wide duplication event had occurred in the Pyreae tribe (Velasco *et al.*, 2010).

Most *PpMLO, FvMLO* and *MdMLO* homologs appeared to be physically scattered within the respective genomes (tables 1, 2 and 3), indicating segmental duplication as the prevailing evolutionary mechanism for the Rosaceae *MLO* gene family. However, we also found cases of clusters of adjacent homologs (*PpMLO3, PpMLO8* and *PpMLO18, PpMLO12 and PpMLO16, PpMLO1* and *PpMLO14, FvMLO3* and *FvMLO4, FvMLO6* and *FvMLO7, MdMLO2, MdMLO3* and *MdMLO8*), which are likely the result of tandem duplication events.

Inference of phylogenetic relationships between MLO proteins revealed the presence of apple, strawberry, peach and apricot homologs in the clade (V) containing all dicots MLO homologs shown so far to be involved in PM susceptibility, thus making them candidates to act as susceptibility factors. Although the simple clustering in clade V is not enough to recognize a gene as a susceptibility factor, it is a first clue that allows narrowing down the number of candidates for further functional analysis. Clade IV, that contains functional MLO

susceptibility homologs from monocots, was found to include one homolog from *F. vesca* (FvMLO17) and one from *P. persica* (PpMLO12). According with this finding, a MLO homolog from the dicot species *V. vinifera* also clusters in clade IV (Feechan *et al*., 2008; Winterhagen *et al*., 2008; Fig. 1). Interestingly, phylogenetic analyses carried out in this study also revealed the presence of one or two clades, depending on the type of phylogenetic reconstruction (UPGMA or Neighbor-Joining), which were not reported to occur in earlier investigations. Moreover, they appear to be characteristic of Rosaceae, since they contain only homologs from this family. Clearly, the specificity for Rosaceae of these clade(s) needs to be confirmed by further studies considering larger dataset of MLO proteins. Additional studies could be also addressed to the functional characterization of Rosaceae MLO homologs grouped in clade VII. Indeed, this appears to be basal to both clade IV and clade V (Fig. 1), and thus might have contained ancestral proteins which later on evolved into PM susceptibility factors.

Synteny between apple, peach and woodland strawberry *MLO* genes

We took advantage of recent developments in Rosaceae genomics in order to detect synteny between *P. persica*, *F. vesca* and *M. domestica* chromosomal regions containing *MLO* homologs. This allowed inferring orthology relationships between *MLO* genes in these species. Notably, all predicted *MLO* orthologs from different Rosaceae species, fall in the same phylogenetic clade (Tables 1, 2 and 3; Fig 1 and additional materials 1). This was expected, since orthologs generally share the same function and thus are characterized by a high level of sequence conservation. It is worth to point out that the localization of predicted *MLO* orthologs between *P. persica*, *M. domestica* and *F. vesca* is in accordance with the results of the synteny study performed after the release of the three genomes (Shulaev *et al.*, 2011; Jung *et al*., 2012). In particular, genes situated on peach

scaffold 2, 7 and 8 were predicted to have orthologs on strawberry chromosome 7, 1 and 2, respectively, whereas genes on peach scaffold 4 were predicted to have orthologs on strawberry chromosomes 2 or 3 (Table 4). *FvMLO3* was predicted to be orthologs to two peach *MLO* genes*, PpMLO8* and *PpMLO18*, which locate in proximity of each other on peach scaffold 6 and group together in clade VI. In this case, we hypothesize a relation of co-orthology due to the occurrence of a recent tandem duplication event in the peach genome. Similarly, *PpMLO5* and *FvMLO16* were predicted to be orthologs of two apple *MLO* genes, *MdMLO10* and *MdMLO17*, located on chromosomes 3 and 11. This is consistent with indications of duplications of large segments of these two chromosomes during the evolution of the apple genome (Velasco *et al.,* 2010).

Transcription of apple putative *MLO* genes in response to *P. leucotricha* inoculation

In barley, pea and tomato, only one of the clade V *MLO* homologs seems to be involved in powdery mildew susceptibility, whereas in *A. thaliana* three *MLO* genes in Clade V have to be inactivated in order to achieve a fully resistant phenotype (Reinstädler *et al.* 2010; Pavan *et al.* 2011). This implies that, within Clade V *MLO* genes, a further selection might be required to identify PM susceptibility genes. Accumulating evidence indicates that *MLO* susceptibility genes are up-regulated upon challenge with powdery mildew fungi (Bai *et al*., 2008). Therefore, we analysed the expression level of apple *MLO* genes identified in this study in response to the interaction with *P. leucotricha*. Three pathogen-dependent gene up-regulations were detected. Two up-regulated *MLO* homologs, *MdMLO11* and *MdMLO19*, encode for proteins falling in clade V, thus making them obvious candidates to act as PM susceptibility genes in apple. *MdMLO11* and *MdMLO19* are located on chromosomes 4 and 12 respectively, that both generated from a

duplication event in the 9-chromosome ancestor of apple (Velasco *et al*., 2010). Moreover, considering the high degree of identity of aa sequences, we conclude that these two genes are paralogs. A third pathogen-dependent up-regulated gene, *MdMLO18*, was found, which encodes a protein grouping in the newly identified Clade VII (Fig. 1). The presence of a powdery mildew upregulated gene outside clade V is consistent with transcriptome analyses recently performed in tomato (Appiano *et al*., unpublished). Apple clade V also contains two genes, *MdMLO5* and *MdMLO7*, which show no significant changes in expression following inoculation. Accordingly, the lack of up-regulation of some clade V *MLO* genes has been observed in grapevine and tomato (Feechan *et al*., 2008; Winterhagen *et al*., 2008; Appiano *et al*., unpublished), but the possible role of these genes as susceptibility factors has not been highlighted yet. The different response of cultivar 'Gala', where *MdMLO19* is not up-regulated, does not seem to be particularly relevant: the paralog gene of *MdMLO19*, *MdMLO11* is up-regulated and the cultivar does not show resistance to *P. leucotricha*.

PpMLO3, *PaMLO3* and *FvMLO4* are likely to represent true orthologs of *MdMLO19* (Tab. 4). Since orthologs usually maintain the same function during evolution, we predict that the expression of these genes might also be responsive to powdery mildew fungi attacking corresponding species. Moreover, *FvMLO15* and *PpMLO9* are likely orthologs of *MdMLO18*, so they should also be considered as putative responsive genes to PM fungi attack. Further studies aimed to the functional characterization of these genes (e. g. through the application of reverse genetic approaches of targeted mutagenesis or gene silencing), in apple but also in peach and strawberry, might lead to the identification of resistant phenotypes, which could be used for the development of PM resistant cultivars. Particularly, studies on *MdMLO18* could lead to the characterization of a possible role for clade VII in the interaction with PM fungi.

CONCLUSIONS

Our work led to the identification of 19 *MLO* homologs in peach, 17 in strawberry and 21 in apple. Three, three and four homologs, respectively, belong to clade V and therefore are candidates for being S-genes. Thanks to the similarity between peach and apricot, we were able to amplify and characterize three Clade V apricot *MLO* genes.

The phylogenetic analysis revealed two new Rosaceae specific clades (possibly one) for the MLO family, although this needs to be confirmed by the use of a larger MLO proteins dataset.

Through inoculation of apple with *P. leucotrica*, we identified three up-regulated genes, i.e. *MdMLO11*, *MdMLO18* and *MdMLO19*. *MdMLO11* and *MdMLO19,* which belong to Clade V, are positioned in duplicated regions and have high sequence identity, therefore they are paralogs. *MdMLO18* belongs to the newly identified Clade VII.

MATERIALS AND METHODS

***In silico* identification and comparison of MLO predicted proteins in peach, woodland strawberry and apple**

Predicted peptides from the peach genome (v. 1.0) and the strawberry genome (v.1.0) gene model databases, available at the website of the Genomic Database for Rosaceae (GDR) (www.rosaceae.org), were searched for the presence of MLO homologs protein sequences. First, a BLAST search, using the tomato SlMLO1 amino acid sequence as query was carried out. A further search was performed with the HMMER program, which uses a method for homolog searches based on the **profile hidden Markov** probabilistic model (Finn *et al.* 2011). The sequences obtained with the previously mentioned BLAST search, were used together with other known *MLO* sequences from dicot and monocot species, namely: four

RhMLOs from *Rosa hybrida*, 15 AtMLOs from *Arabidopsis thaliana* , SlMLO1 from *Solanum lycopersicum*, CaMLO2 from *Capsicum annuum*, PsMLO1 from *Pisum sativum*, MtMLO1 from *Medicago truncatula*, LjMLO1 from *Lotus japonicus*, VvMLO14 from *V. Vinifera*, HvMLO *from Hordeum vulgare*, TaMLO1_A1b and TaMLO_B1 from *Triticum aestivum* and OsMLO2 from *Oryza sativa*. MLO protein sequences from apple (*Malus* x *domestica* Borkh cv. 'Golden Delicious') were identified by searching the MLO domain profile (IPR004326) in the apple genome available at FEM-IASMA computational biology web resources (http://genomics.research.iasma.it). The resulting list was integrated with a BLAST search, carried out with the amino acid sequences previously listed for the HMMER search in peach and strawberry.

Chromosomal localization and predicted introns/exons structure of each *MLO* gene of apple, peach and strawberry was deducted based on the available genomic informations at the GDR database. The presence and number of membrane spanning helices was predicted using the online software InterPro (http://www.ebi.ac.uk/interpro). Alignments for conserved amino-acids analysis were performed with the CLC Sequence Viewer v. 6.9 software (http://clcbio.com). Ninety (90) MLO protein sequences, including three apricot *MLO* sequences isolated *in vitro* (see next paragraph), were used for Clustal alignment (http://www.ebi.ac.uk/Tools/msa/clustalw2/). UPGMA-based and Neighbor-Joining-based phylogenetic trees were obtained with the CLC sequence viewer software. The UPGMA phylogenetic tree was further used as input for the Dendroscope software, suitable for the visualization of large phylogenetic trees (Huson *et al*., 2007).

Relationships of orthology between *MLO* candidate genes from peach, woodland strawberry and apple were inferred by running the GBrowse-Syn tool available at GDR (http://www.rosaceae.org/gb/gbrowse_syn/peach_apple_strawberry)

(McKay *et al.*, 2010; Jung *et al.,* 2014). This displays syntenic regions among the three available genomes of Rosaceae, as detected by the Mercator program (Dewey 2007; Jung *et al.*, 2014). For 50 Kb chromosomal stretches flanking each *P. persica PpMLO* homolog, syntenic regions from *F. vesca* and *M. domestica* were searched. Orthology was called upon the identification of *F. vesca* or *M. domestica MLO* homologs within syntenic blocks.

***In vitro* isolation of apricot *MLO* homologs**

RNA from apricot leaves (cultivar 'Orange Red') was extracted by using the SV Total RNA Isolation System Kit (Promega), and corresponding cDNA was synthesized by using the QuantiTect Reverse Transcription Kit (Qiagen) with oligo(dT) primers. Sequences of the peach *MLO* homologs *PpMLO1*, *PpMLO3* and *PpMLO4*, are phylogenetically close to *MLO* homologs functionally associated to PM susceptibility, and were therefore used to design the primer pairs 5'-ATGGCAGCCGCAACCTCAGGAAGA-3' / 5'-TTATATACTTTGCCTATTGTCAAAC-3', 5'-ATGGCAGGGGGAAAAGAAGGACG-3' / 5'-TCAACTCCTTTCTGATTTCTCAA-3' and 5'-ATGGCCGAACTAAGTAAAGA-3' / 5'TCAACTTCTTGATTTTCCTTTGC-3', respectively. These were employed to amplify full-length cDNA sequences of apricot putative orthologs, by using the AccuPrime Taq polymerase (Invitrogen). Amplicons were purified by using the NucleoSpin Extract II kit (Macherey-Nagel) and ligated (molar ratio 1:1) into the pGEM-T Easy vector (Promega). Recombinant plasmids were cloned in *E. coli* DH10β chemically competent cells and recovered by using the Qiaprep spin miniprep kit (Qiagen). Sequencing reactions were performed twice, by using universal T7 and SP6 primers (Eurofins MWG Operon).

Glasshouse test with apple cultivars

One hundred and ninety-two (192) apple plants from three cultivars ('Braeburn', 'Golden Delicious' and 'Gala') were used to measure transcript abundances of *MLO* genes. Budwoods from these cultivars were grafted on M9 rootstocks in January 2012. The grafts were kept at -1°C for 2 months, and potted at the beginning of March in greenhouse. The plants grew for 6 weeks in the greenhouse at 20°C during the day, 17°C during the night, relative humidity of 70% and natural day/night cycle.

P. leucothrica was collected from apple trees in an unsprayed test orchard and used to infect greenhouse grown apple seedlings from 'Gala Galaxy' seeds. Four weeks after inoculation, conidia were used for the inoculation experiment, or transferred to new seedlings, to keep them viable. We inoculated by touching the plants with heavily infected apple seedlings. Control plants were not inoculated and kept separated in the same greenhouse. Inoculated and control plants were kept in the greenhouse with the same growing conditions previously mentioned. The samples were collected 4, 6, 8 and 24 hours post-inoculation (hpi).

Eight experimental repeats were performed and each sample contained three or four young leaves collected from each single plant. Every plant was used for sampling only once, to avoid any possible effect of wounding on the expression *of MLO* genes. The smallest statistical unit was a plant. The leaves were flash-frozen and ground in liquid nitrogen, and stored at -80°C until RNA extraction.

qPCR analysis of transcript levels

RNA extraction was carried out with the MagMAX-96 Total RNA isolation kit (Applied Biosystem) that includes DNAse treatment. The kit yielded between 50 and 200 ng/µl, with a good quality of resulting RNA.

Primers for gene expression analysis were designed with NCBI Primer Designing Tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Four serial dilutions of cDNA (1/5 - 1/25 – 1/125 – 1/625) were used to calculate the efficiency of each primer pair with iCycler software (Biorad). In case of efficiency lower than 1.80 or greater than 2.20, the primer pair was discarded and a new one tested, with the exception of *MdMLO9*, for which was not possible to design a primer pair with better efficiency. It was possible to analyse only 19 *MLO* genes because for *MdMLO12* and *MdMLO16* was not possible to design specific and efficient primer pairs, despite numerous attempts. Presence of a specific final dissociation curve was determined after each qPCR amplification reaction with progressive increment of temperature from 65°C to 95°C (0.5°C each step, 5 sec) and the size of the product was confirmed by agarose gel electrophoresis.

Quantitative Real Time-PCR (qPCR) was performed with SYBR greenER mix (Invitrogen) in a 15-µL reaction volume, using a Bio-Rad iCycler iQ detection system, run by the Bio-Rad iCycler iQ multicolor 3.1 software. The software applies comparative quantification with an adaptive baseline. Samples were run in two technical replicates with the following thermal cycling parameters: 95°C 3 min – 95°C 15 sec, 60°C 1 min (repeated 40 times) – 95°C 10 sec.

Reference genes β-actin (NCBI accession number DT002474; Plaza accession number MD00G171330 - http://bioinformatics.psb.ugent.be/plaza/), ubiquitin (Plaza accession number MD05G001920) and elongation factor 1 (Plaza accession number MD09G014760) were used as reference genes (Table 5). All these three genes were used in previous works (Kürkcüoglu *et al*., 2007; Giorno *et al*., 2012; Dal Cin *et al.,* 2005). For additional control, we assessed the stability of our genes with the software geNorm (medgen.ugent.be/~jvdesomp/genorm/). An M-value lower than 1.5 is generally considered as stable enough (Ling and Salvaterra, 2011; Van Hiel *et al*., 2009; Strube *et al.,* 2008) and all three reference genes in all three

cultivars considered are within this threshold. We saw differences in stability between cultivars: 'Golden Delicious' was the most stable cultivar (actin: 0.824 – ubiquitin: 0,852 – elongation factor 1: 0,926), whereas 'Braeburn' was the less stable (actin: 1.246 – ubiquitin: 1,293 – elongation factor 1: 1,369) and 'Gala' showed intermediate stability (actin: 1.039 – ubiquitin: 1,152 – elongation factor 1: 1,078).

Each of the biological replicates was analysed in duplicate and the average of these two replicates was used for further analysis. In case of excessive difference between the two replicates (one Ct or more), the run was repeated. Considering the high number of samples and genes of interest, we opted for this approach in order to reduce the number of total runs. Data analysis was performed according to Hellemans *et al.* (2007), using the statistical package SPSS (IBM). This analysis method takes into account the efficiency value of each primer pair. For some genes it was necessary to apply a natural log transformation to the data, in order to obtain normal distribution of residues. To investigate the differences between control and inoculated samples, we used T-test ($p \leq 0.05$).

AKNOWLEDGMENT

The authors would like to thank Remmelt Groenwold (WUR) for helping with apple inoculation with *P. leucotricha* and Lorenza Dalla Costa (FEM) for all the valuable advices about qRT-PCR data analysis.

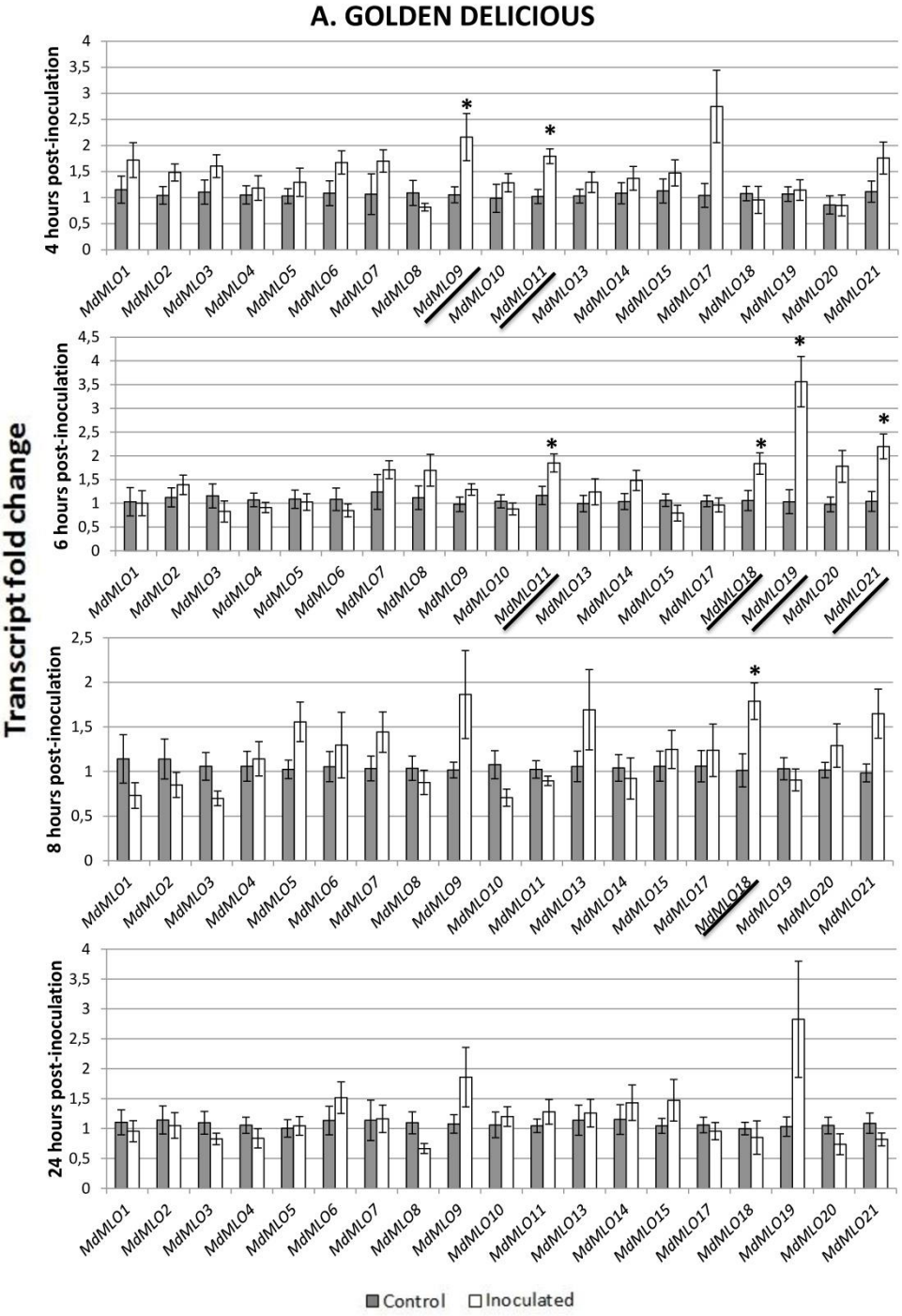
Table 5: Gene-specific primers and amplicon sizes in qRT-PCR detection of 19 *MdMLO*-like genes based on *Malus domestica* cv. 'Golden Delicious' genome sequence*

Gene	Forward primer (5' – 3')	Reverse primer (5' – 3')	Size (bp)	Efficiency
MdMLO1	GTGGGCTCGGTCGGCCAAAA	CCAGCACCAGCACCAGAACCA	81	2.06
MdMLO2	CGTTGGATCAACCACTGCGCCT	TGAGCTGCAGCCAGTGGGATCT	87	1.83
MdMLO3	CCACTGCGCCTCTCTGAAGCA	CCACCAAAACGGCTCTCCAGGT	93	2.12
MdMLO4	TGTTGCAGACACTATGCTGCCATGT	GGCAGCAGCTAAAGATCTGCGT	109	1.87
MdMLO5	TCGTCAGGCTCTCATTCGGGGT	GTGCTGCTGCCACTCCCTC	132	1.80
MdMLO6	TTCGCGGAGGAGGGGTCGTT	TTCGAGCGACAGCAACGGCA	72	2.15
MdMLO7	TGGAGCAAGTCACCAGTCTCCAT	CGCTTCCTGGTGCCAAATGTGC	127	2.12
MdMLO8	GTCAAGCTAATCTTACCACGCGCT	GGCTGGAAGGAAGGACAGCCA	85	1.95
MdMLO9	GCTGCAACACGTAATCACCC	AGAACGCCATTTCGAAAGCA	173	2.30
MdMLO10	GCGATCGTTGGCCTTGACTCC	TTCCGCGCTCGACAAGCAGA	86	1.92
MdMLO11	AGCAAGGTACAAACCGGCAGGG	GCATTGCTCTCCGAGTTACGCCC	70	1.82
MdMLO13	ACATTGTCCCCAGGCTTGTT	GCCCAACCAATAAGTCCCGA	151	2.00
MdMLO14	TGCACTTGTCAGCCAGATGGG	GCATCTCCCACCCACGAACCG	81	2.15
MdMLO15	GCGCCTTTCTCTCTGCTGGGT	CGCGTGCGAGGTGGTCTCTT	90	2.01
MdMLO17	TTGCCACTGTATGCCTTGGT	TGCTTGCTTCTGTGCGAATG	163	2.15
MdMLO18	AAGGAAGGCTCTCATTCAGGCTCT	TGCAATTGGCTTTTGACCAACGGT	100	2.22
MdMLO19	CAGAGTGGCGACTGCACTTA	GGGACATGGAGTGCAAAGGA	110	1.97
MdMLO20	AAAAAGCTCCACCAACCCCA	TTTCTCTCCCATGACGCTCG	165	2.11
MdMLO21	CCTTGTTCGAGGCCGTAGAG	ACCAAGTGCTTTGGTGGTTT	176	1.95
β-actin	CTATGTTCCCTGGTATTGCAGACC	GCCACAACCTTGATTTTCATGC	82	1.90
Ubiquitin	CATCCCCCCAGACCAGCAGA	ACCACGGAGACGAAGCACCAA	349	1.91
Elongation Factor 1	TACTGGAACATCACAGGCTGAC	TGGACCTCTCAATCATGTTGTC	308	2.07

SUPPORTING MATERIAL

Figure S1. Synteny between apple, peach and strawberry. Results of search for *F. vesca* and *M. domestica* regions syntenic to 50 kb *P. persica* chromosomal stretches containing the *PpMLO* homologs identified in this study. Shaded polygons indicate aligned regions between genomes. Grid lines are drawn to indicate insertions/deletions between the genomes of *F. vesca* and *M. domestica* with respect to the *P. persica* reference sequence. *P. persica*, *F. vesca* and *M. domestica MLO* homologs, named according to the nomenclature of the Genomic Database of Rosaceae, are boxed.

http://www.biomedcentral.com/content/supplementary/1471-2164-15-618-s1.pdf


A. GOLDEN DELICIOUS
Transcript fold change
4 hours post-inoculation
6 hours post-inoculation
8 hours post-inoculation
24 hours post-inoculation
MdMLO1
MdMLO2
MdMLO3
MdMLO4
MdMLO5
MdMLO6
MdMLO7
MdMLO8
MdMLO9
MdMLO10
MdMLO11
MdMLO13
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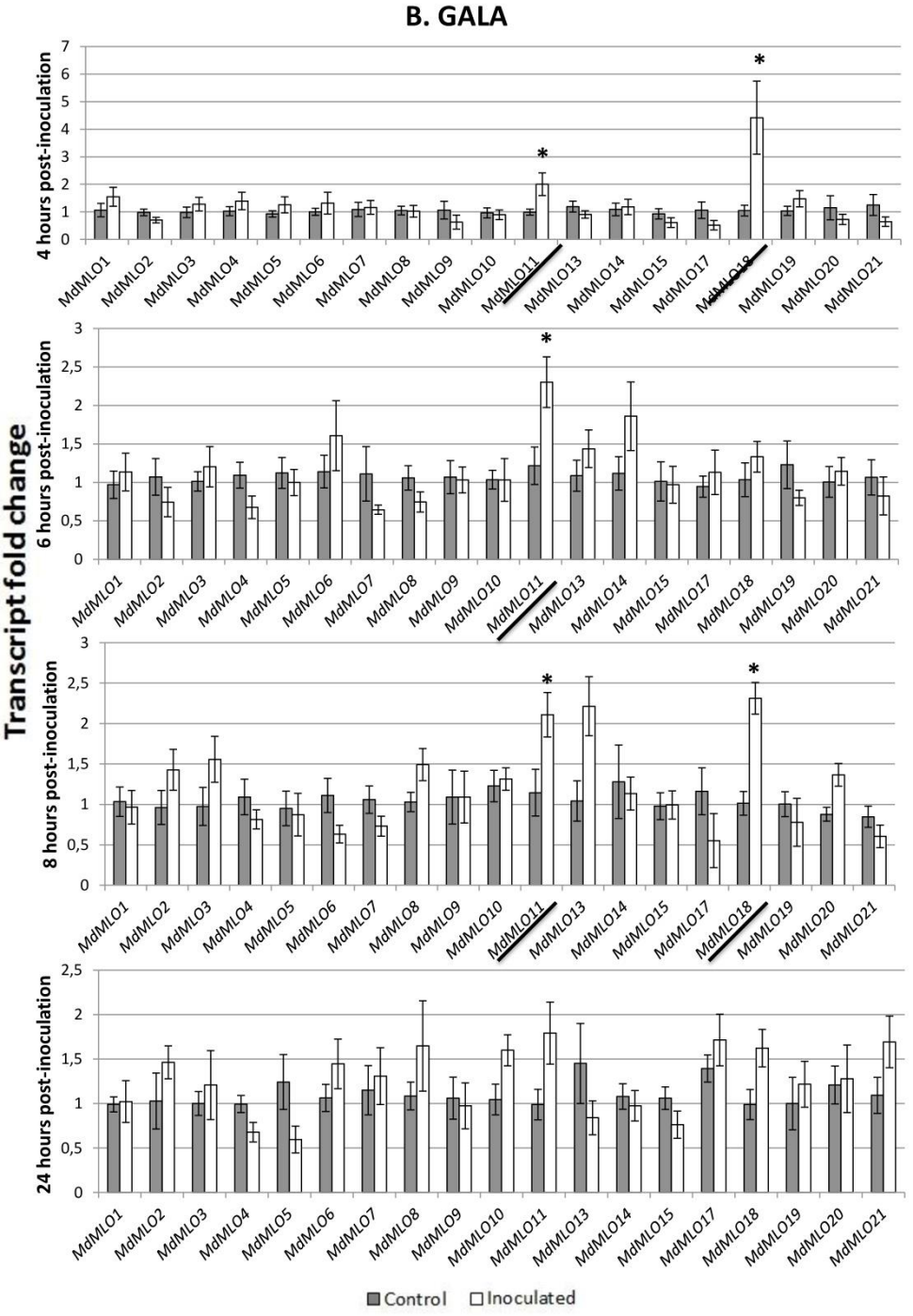
MdMLO18
MdMLO19
MdMLO20
MdMLO21
Control
Inoculated


B. GALA
Transcript fold change
4 hours post-inoculation
6 hours post-inoculation
8 hours post-inoculation
24 hours post-inoculation
MdMLO1
MdMLO2
MdMLO3
MdMLO4
MdMLO5
MdMLO6
MdMLO7
MdMLO8
MdMLO9
MdMLO10
MdMLO11
MdMLO13
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MdMLO18
MdMLO19
MdMLO20
MdMLO21
Control
Inoculated

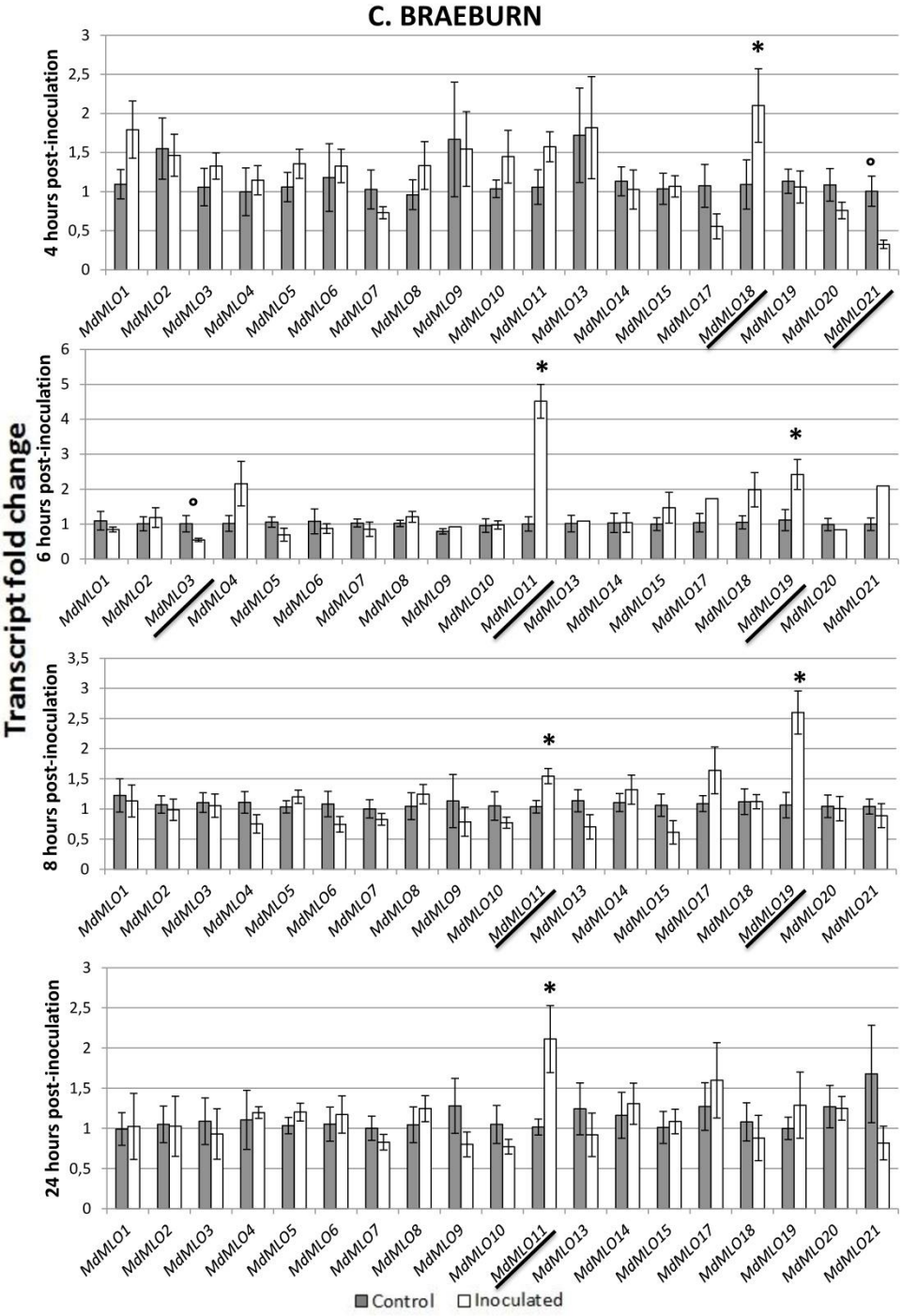
C. BRAEBURN
Transcript fold change
4 hours post-inoculation
6 hours post-inoculation
8 hours post-inoculation
24 hours post-inoculation
MdMLO1
MdMLO2
MdMLO3
MdMLO4
MdMLO5
MdMLO6
MdMLO7
MdMLO8
MdMLO9
MdMLO10
MdMLO11
MdMLO13
MdMLO14
MdMLO15
MdMLO17
MdMLO18
MdMLO19
MdMLO20
MdMLO21
Control
Inoculated

Figure S2. Transcriptional variation of 19 apple MLO genes in three cultivars following inoculation with *P. Leucotricha*. Transcription abundances of 19 *MLO*-like genes following powdery mildew (PM) inoculation in 'Golden Delicious' (1a), 'Gala' (1b) and 'Braeburn (1c) leaf samples. The graphs show expression values of inoculated samples relative to control samples, averaged from four to eight biological replicate, normalized, that are in turn the average of two experimental replicates. The Ct values have been normalized with three reference genes: actin, ubiquitin and elongation factor 1. Statistical significance was determined with a *t*-test for each individual pair of inoculated and control samples at each time point (4, 6, 8 and 24 hpi). The error bars show standard errors of the means. Significant differences between inoculated samples and control samples are indicated with a * ($P < 0.05$).

CHAPTER 3

Knock-down of the *MdMLO19* gene expression reduces susceptibility to powdery mildew (*Podosphaera leucotricha*) in *Malus domestica*.

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ABSTRACT

Powdery mildew (PM), caused by *Podosphaera leucotricha,* is a major disease of apple. The development of PM resistant varieties is a necessity for sustainable apple production. Resistance can be achieved by knocking-out susceptibility S-genes, such as specific members of the *MLO* gene family (*Mildew Locus O*) that was first identified in barley. Phylogenetic clade V *MLO* S-genes of dicots are usually up-regulated upon PM inoculation, as evident for apple genes *MdMLO11* and *19*. The two other clade V genes of apple, *MdMLO5* and *7,* are not up-regulated. In apple, the clade VII gene *MdMLO18* is also up-regulated upon *P. leucotricha* infection. Before adopting a gene editing approach to knock-out candidate S-genes, the evidence that loss-of-function of *MLO* genes can reduce PM susceptibility is necessary. This paper reports the knock-down through RNA interference of *MdMLO11* and *19*, as well as complementation of *MdMLO18* in the *Arabidopsis thaliana* triple *mlo* mutant, *Atmlo2/6/12*, which excluded a role of the gene in PM susceptibility. The knock-down of *MdMLO19* resulted in reduction of PM disease severity up to 75%, whereas the knock-down of *MdMLO11,* alone or combined with *MdMLO19,* did not cause a reduction or an additional reduction of susceptibility compared to *MdMLO19* alone. Cell wall appositions (papillae), a response to PM infection, were found in both PM resistant and susceptible plants, but were larger in *mlo* lines. The expression analysis of 17 genes related to plant defense, and quantification of phenolic metabolites in *mlo* silenced lines revealed line-specific changes compared to the control.

INTRODUCTION

Powdery mildew (PM), caused by the obligate biotroph fungus *Podosphaera leucotricha,* is a major disease of *Malus domestica* present in all major apple growing areas of the world. Leaves are the most susceptible organs, particularly in

the first days after opening. Powdery white lesions present on the upper leaf side eventually turn brown, whereas infections on the underside result in chlorotic patches. Infected leaves tend to crinkle, curl and drop prematurely. Blossom infections are less common but important because infected fruits are small and stunted if not dropping. *P. leucotricha* survives the winter as mycelium in vegetative tissues or in infected flower buds. The primary infection starts when infected buds break dormancy: the fungus resumes growth and colonizes developing shoots. Primary infections of flower buds cause severe yield losses. Spores growing on infected shoots spread nearby and initiate secondary infections (Turechek *et al.,* 2004).

Yield losses caused by PM can be limited with frequent applications of fungicides. However, fungicides, besides their significant cost for the growers, affect the environment negatively (Wightwick *et al.,* 2010). Moreover, agrochemical treatments select fungicide-resistant strains of the pathogen, as known for *Erysiphe necator*, the PM causing agent of grapevine (Dufour *et al.,* 2011), and *Venturia inequalis,* the agent of apple scab (Pfeiffer *et al.,* 2015). Therefore, the development of PM-resistant varieties is a valuable option to improve economic and environmental sustainability of apple cultivation.

Apple germplasm, including domesticated and wild *Malus* species, is rich of dominant resistance genes (R-genes). About 868 R-genes have been identified in the apple genome, which are effective against a large number of pathogenic organisms (Perazzolli *et al.,* 2014,). They encode proteins that recognize pathogen effectors and activate the defense response (Pavan *et al*., 2010; Dodds and Rathjen, 2010), manifested as localized hypersensitive response at the site of infection (Bari and Jones 2009). Two PM R-genes, *Pl-1,* from *Malus robusta* and *Pl-2* from *Malus zumi*, have been used since the seventies of the last century, in a variety of breeding programs (Bus *et al*., 2010), later together with *Pl-m*, *Pl-w* and *Pl-d* (Lespinasse,

1983; James *et al.,* 2004). Unfortunately, the durability of R-genes is limited due to new pathogen strains able to overcome the resistance (Parleviet*,* 1993), as noted for apple *Pl-2* and *Pl-m* (Caffier and Laurens, 2005). Considering how time-consuming breeding of woody species is, a more durable source of PM resistance is a necessity. This source can be based on mutations in plant susceptibility genes (S-genes), which are defined as plant genes that are required by pathogens to promote diseases. Some S-genes encode negative regulators of the plant immunity system, which impairment prevents the suppression of plant defense and leads to resistance (Pavan *et al.,* 2010). However, knocking-out S-genes may induce pleiotropic phenotypes in the plant, which may result in negative effects (Pavan *et al.* 2011; Van Schie and Takken, 2014).

The barley *MLO* gene is an example of an S-gene for promoting PM infection. The *mlo* recessive resistance caused by the knock-out of a dominant *MLO* allele was discovered in barley in 1942 (Jørgensen, 1992), and was for a long time considered a unique form of resistance. Further studies revealed that *MLO* genes are largely conserved across the plant kingdom, as proven in *Arabidopsis thaliana* (Consonni *et al.,* 2006), pea (Pavan *et al.,* 2011), tomato (Bai *et al.,* 2008), wheat (Wang *et al.,* 2014), pepper (Zheng *et al.,* 2013) and grapevine (Chapter 5). Genes of the *MLO* family define seven phylogenetic clades (Acevedo-Garcia *et al.,* 2014; Pessina *et al.,* 2014) of which only two include S-genes: clade IV, with *MLO* S-genes of monocots (Panstruga, 2005; Reinstädler *et al.,* 2010), and clade V, with *MLO* S-genes of dicots (Consonni *et al.,* 2006; Bai *et al.,* 2008; Feechan *et al*., 2008; Winterhagen *et al.,* 2008). However, not all members of clades IV and V are S-genes, but nevertheless candidates can be identified during early stages of PM infection because of an increased expression, as documented in tomato (Bai *et al.,* 2008), barley (Piffanelli *et al.,* 2002), pepper (Zheng *et al.,* 2013), grapevine (Feechan *et al*., 2008; Winterhagen *et al.,* 2008) and apple (Pessina *et al.,* 2014). In the latter species, four

MLO genes belong to clade V and two of them, *MdMLO11* and *MdMLO19,* are up-regulated during PM infection, whereas *MdMLO5* and *MdMLO7* are not transcriptionally responsive to the pathogen (Pessina *et al.,* 2014). In addition, *MdMLO18*, a gene belonging to clade VII, is also up-regulated (Pessina *et al.,* 2014). To date, there are no reports of *MLO* genes of dicots acting as S-genes outside clade V, therefore *MdMLO18* should not be a strong candidate for being an S-gene.

MLO proteins have seven trans-membrane domains and are involved in a variety of physiological processes in different tissues. The proposed function for MLO S-proteins is the negative regulation of vesicle-associated and actin-dependent defense pathways at the site of attempted PM penetration (Panstruga*,* 2005). Plant *mlo*-based resistance is associated with cell wall appositions called papillae that constitute a mechanical barrier for the pathogen. Therefore, *mlo* resistance consists of a pre-penetration structural defense system (Consonni *et al.,* 2006; Aist and Bushnell*,* 1991). The formation of the papillae depends on the delivery of material through the actin-dependent vescicles traffic (Miklis *et al.,* 2007; Feechan *et al.,* 2011). In *A. thaliana MLO* genes have other functions: *AtMLO7* is involved in pollen tube reception by the embryo sac (Kessler *et al*., 2010), whereas *AtMLO4* and *AtMLO11* participate in the control of root architecture (Chen *et al.,* 2009).

The development of DNA editing tools is rapidly changing plant genetics and biotechnology, thanks to the possibility of inducing mutations in specific genes (Lozano-Juste and Cutler, 2014; Gaj *et al.,* 2013; Puchta and Fauser, 2014). Targeted knock-out of *MLO* S-genes, using DNA editing tools, may provide durable resistance to PM in apple, but, before applying the gene editing approach, evidence of which *MLO* gene(s) cause PM susceptibility in apple is required. This paper reports the functional analysis on apple *MLO* genes, *MdMLO*11, *18* and *19* for their roles in susceptibility to PM, by knocking down *MdMLO11* and *19* through RNA interference (RNAi) and overexpressing *MdMLO18* in the *Arabidopsis Atmlo2/6/12* mutant.

MATERIALS AND METHODS

MdMLO18 complementation test of *A. thaliana mlo* mutant

A full length *MdMLO18* gene was amplified from an apple (cultivar Gala, susceptible to PM) cDNA library using the primer pair: Fw 5' – ATGGCTGGAGACAACGGAGCTGCAA – 3' and Rv 5' - GAACCATTATTTTGCTGTACCTCAGCTGCC – 3'. The gene was cloned into gateway pENTR/SD-TOPO (Thermo Fisher Scientific, Waltham, USA) and pK2WG7 vector (Life Technologies, Waltham, USA). Final constructs were verified by sequencing and inserted into *Agrobacterium tumefaciens* strain AGL0 through electroporation. *A. tumefaciens* transformed cells were tested by PCR for the presence of the constructs, using primers annealing on the vector and on the *MdMLO18* sequence. The *A. thaliana Atmlo2/6/12* mutant in Col-0 genetic background (Consonni *et al.* 2006) was grown at 25°C in chambers with 16-h-light/8-h-dark cycle.

Gene transfer to *A. thaliana* was carried out with the floral dip method (Clough and Bent, 1998) and transformed seeds were selected on kanamycin. Expression of *MdMLO18* was assessed by qPCR on leaves collected from regenerated plants.

The disease severity assessment of transformed *A. thaliana* plants followed their inoculation by dry-brushing leaves with *O. neolycopersici* spores carried by diseased tomato leaves. Disease severity was visually evaluated on all leaves 7 days post-inoculation (dpi), and expressed for each plant as the mean percentage (intervals of 5%) of adaxial leaf area covered by PM mycelium.

Constructs for *MdMLO11* and *MdMLO19* knock-down in apple

Gene fragments for RNAi were amplified from *MdMLO11* and *MdMLO19* (accession numbers in Table S1) with primers listed in Table S2 and cloned in gateway pENTR/SD-TOPO (Thermo Fisher Scientific, Waltham, USA). In addition, a chimeric construct was developed joining RNAi fragments supposed to silence *MdMLO11*

and *MdMLO19* simultaneously (Abbott *et al.,* 2002). For this purpose, a restriction site for EcoRI was added at the 3' end of the *MdMLO11* RNAi fragment and at the 5' end of the *MdMLO19* one. Both fragments were restricted with EcoR1 and joined with a T4 DNA ligase (New England Biolabs, Ipswich, USA). The resulting construct was cloned into the pENTR vector. After sequencing, all fragments were cloned into the destination vector pHELLSGATE 12 (Thermo Fisher Scientific, Waltham, USA). The final constructs were verified by sequencing, and inserted into *A. tumefaciens* strain AGL0 through electroporation. *A. tumefaciens* transformed cells were tested by PCR for the presence of the constructs, using specific primers designed to anneal on vector and *MLO* sequences.

Development of RNAi apple plantlets

The RNAi-constructs were transferred into apple as described by Joshi *et al.* (2011). Explants from the top four leaves of 4-week-old *in vitro* propagated shoots of the cultivar Gala were kept on a medium with kanamycin (Joshi *et al.,* 2011), and grown in a growth chamber with 16-h-light/8-h-dark cycle at 24°C. To certify the presence of the constructs with PCR, genomic DNA from regenerated plantlets was extracted with the Illustra Nucleon Phytopure kit (GE Healthcare). The forward primer annealed on the CaMV 35S promoter (5'- CGCACAATCCCACTATCCTT – 3') and the reverse primers were specific for the RNAi fragments (Table S2). PCR was performed with GoTaq® Green Master Mix (Promega, Fitchburg, USA). Plants positive for the construct were moved to Shoot Propagation Medium (SPM): 4,4 g/L of Murashige and Skoog medium with vitamins, 30 g/L of sucrose, 0.7 mg/L of BAP, 96 mg/L of FeEDDHA, pH 5.8. To promote rooting, plants were transferred on a medium containing IBA to promote rooting. Once roots were formed, plants were progressively acclimated to greenhouse conditions (25°C, 16-h-light/8-h-dark cycle, relative humidity 70±5%) in 125 ml pots covered with plastic bags and containing

wet autoclaved turf ("Terriccio Vegetal Radic" - Tercomposti Spa, Brescia, Italy). Every 5-7 days for three weeks, air humidity was reduced to promote the formation of the foliar cuticle. Plastic bags were then removed and plants were transferred to 1 L pots. The control (untransformed *in vitro* grown 'Gala') was acclimated as described above.

***P. leucotricha* inoculation and disease severity assessment in apple**

To produce a PM inoculum, local strains of *Podosphaera leucotricha* were isolated from infected leaves of an orchard located in Trento province (Italy). The fungus was maintained by serial inoculations on *M. domestica* seedlings under greenhouse conditions. Plants were dry-inoculated by brushing the adaxial epidermis with leaves of infected seedlings. To promote the fungal penetration, plants were incubated in greenhouse at 25°C with a relative humidity of 90±5% for 6h. The plants were then maintained at 25°C and 80±10% relative humidity until the end of the evaluation.

Four inoculation experiments were carried out in different periods of the year. In each test, three to eight biological replicates of each transgenic line were considered. Lines were tested in at least three out of four experiments and the total number of replicates varied between 15 and 24 (Table 1). Disease severity was visually assessed on all inoculated leaves 7, 14 and 21 dpi. Disease severity was expressed as the percentage (intervals of 5%) of adaxial leaf area covered by the PM mycelium, and a single plant mean value was calculated. Reduction of disease severity in transformed plants was expressed as [(severity in controls - severity in transgenics)/ severity in controls] ×100%. To consider all time points together, the area under the disease progress curve (AUDPC), summarizing disease intensity over time (Campbell and Madden, 1990; Madden *et al.,* 2007), was calculated.

Table 1. PM disease severity reduction (%) in lines transformed with RNAi constructs.

	Silenced genes	Replicates	Disease severity reduction[#]		
			14 dpi	21 dpi	Average
TG0	/	17	24.1	24.8	24.5
TG11+19	*MdMLO11* and *19*	23	60.0*	52.6*	56.3
TG19	*MdMLO19*	15	72.7*	78.1*	75.4
TG11	*MdMLO11*	16	38.0	-3.2°	17.4

* Statistically significant difference compared to the control, according to the Tukey post-hoc test (*P*=0.05).

[#] Gala was used as control (19 plants) and assumed to have 0% of disease reduction.

° Line TG11 showed a higher level of infection compared to Gala at 21 dpi.

The number of *P. leucotricha* conidia present on infected leaves was assessed as in Angeli *et al.* (2012) with slight modifications: three leaves were collected from each replicate at 21 dpi and four disks of 0.8 cm diameter for each leaf were cut for a total of 12 per replicate. Leaf disks were transferred to 50 ml tubes containing 5 ml distilled water with 0.01% Tween 20 (Sigma-Aldrich, Sant Louis, USA). Tubes were vortexed for one minute and the concentration of conidia per ml was determined by counting with a hemocytometer under a light microscope (Wetzlar H 600LL, Germany). The amount of conidia was expressed as number per square centimeter (cm^2) of leaf.

Histological analysis of inoculated apple leaves

Two inoculated leaves for each replicate were collected at 3, 10 and 21 days post inoculation for bright field microscopy observations. To visualize fungal hyphae, leaves were cleared in ethanol:acetic acid (3:1 v/v) until chlorophyll removal (approximately 48 hours). Samples were stained for 15 minutes with 250 µg/ml trypan blue in lactic acid, glycerol, and water (1:1:1). After rinsing and mounting as

in Vogel and Somerville (2000), hyphae were visualized under bright field illumination of a Leica LMD7000 microscope (Wetzlar, Germany).

Leaves considered for scansion electron microscopy (Hitachi S-2300, Tokyo, Japan) were fixed in Sorensen phospate buffer 0.1 M, pH 7, 3% gluteraldehyde. After 24 hours, leaves were washed in Sorensen buffer without gluteraldehyde for two hours under mild agitation (80-100 rpm). Afterwards, samples were progressively dehydrated with four ethanol washings at concentrations from 40 to 100%, dried and kept in falcon tubes until observation. Fragment of leaves were metallized with gold before observation. Images were processed with ImageJ software (http://imagej.nih.gov/ij/).

For the detection of papillae, leaves were cleared in ethanol:acetic acid (3:1, v/v) until chlorophyll removal, and equilibrated overnight in a solution of lactic acid, glycerol and water (1:1:1). Papillae were visualized using the LMD filter (BP filter 380-420 nm excitation, 415 dichroic mirror, and BP 445-485 nm emission) of a Leica LMD6500 microscope (Leica Microsystem, Wetzlar, Germany).

Gene expression analysis

To identify lines showing silencing effects, a first gene expression study used triplicates of *in vitro* grown transgenic plants. In the second study, concerning acclimated transgenic plants, leaf samples were collected immediately before PM inoculation, at 24 hpi and at 10 dpi. For each line at each time point, leaf samples were collected from five different plants. Samples were frozen in liquid nitrogen and stored at 80°C. Total RNA was extracted with the Spectrum^{tm} Plant Total RNA kit (Sigma-Aldrich), treated with the DNAse I (Sigma-Aldrich) and reverse transcribed using the SuperScript III reverse transcriptase (Invitrogen, Life Technologies, Waltham, USA). The qPCR analyses were run according to SsoAdvanced Universal SYBR Green Supermix, (Bio-Rad, Hercules, USA) in a 15-µl

reaction volume, using a CFX96 Touch^{tm} Real-Time PCR detection system (Bio-Rad, Hercules, USA), and the CFX Manager software. Samples were run in two technical replicates according the following thermal cycling parameters: 95°C 3 min, 95°C 10 sec, 55°C 30 sec (repeated 40 times), 95°C 10 sec. For the analysis of *MdMLO19,* the primer pairs considered in previous work were used (Table S1; Pessina *et al.,* 2014). For *MdMLO11* and for the expression of 17 genes involved in the interaction between apple and *P. leucotricha*, new primer pairs were designed with the NCBI Primer Designing Tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table S1). Serial dilutions of cDNA (1/10, 1/100, 1/1000 and 1/10000) allowed to calculate the efficiency of the primer pairs; the expected sizes of the products were confirmed using agarose gel electrophoresis. Presence of a specific final dissociation curve was determined after every qPCR run, with progressive increments of temperature from 65°C to 95°C (0.5°C each step, 5 sec). The reference genes considered were elongation factor 1, ubiquitin and *8283* (Table S1). All of them are known to be stable reference genes for apple (Botton *et al.,* 2011; Pessina *et al.,* 2014). The analysis with the software geNorm (medgen.ugent.be/~jvdesomp/genorm) resulted in M-values lower than 1 for all three reference genes, in conditions where M-values lower than 1.5 are considered adequate (Ling and Salvaterra, 2011). The threshold cycles (Ct) were converted to relative expression levels as in Hellemans *et al.* (2007), using as input the average Ct of the two technical replicates. As reference Ct, the average Ct of wild-type 'Gala' at 0 hpi was adopted.

Phenolic metabolites

Quantification of phenolic metabolites in transgenic and wild-type apple plants was carried out on non-inoculated leaves from eight biological replicates. Samples were ground in liquid nitrogen and 100 mg of powder were used for the extraction

in 4 ml of 100% methanol of the target metabolites. Extraction lasted 72 hours at 4°C. The liquid phase was diluted with water to 80% methanol and filtered with 13 mm Millex-GV syringe filters (Millipore, Billerica, USA) to remove fine debris. The quantification of 18 phenolic metabolites was carried out by multiple reactions monitoring (MRM) as described by Vrhovsek *et al*. (2012).

Statistics

Disease severity

Severity data were analysed by the statistical package SPSS (IBM, Armonk, USA). For both apple and *A. thaliana,* severity data of leaves from the same plant were averaged before further analyses. Apple severity data of the eight younger leaves of a plant were considered, while *A. thaliana* data were from all leaves. Before any analysis, data were shown to be normally distributed (Kolmogorov-Smirnov and Shapiro-Wilk tests $P > 0.05$) and to have homogeneous variances (Levene's test, $P > 0.05$). One-way ANOVA with Tukey's post-hoc test was adopted to detect significant differences ($P < 0.05$) at each time point. Data were transformed according to $y=\arcsin(x)$, in order to meet the pre-requisites of ANOVA. In case of non-homogeneous variances, the Games-Howell's post-hoc test was applied. Prior to pooling data from independent experiments, the effect of single experiments was tested: no significant effect of the experiments emerged. Pooled data were analysed independently for time points 14 and 21 dpi. AUDPC data were treated as described above for severity data. Number of conidia data was analysed with one-way ANOVA, applying the Tukey post-hoc test ($P < 0.05$).

qPCR data analyses

For the evaluation of gene expression, relative expression values were transformed in logarithmic scale according to $Y=\ln(x)$ (Pessina *et al*., 2014) to meet normal distributions and homogeneities of variances, as assessed respectively with the

test of Shapiro-Wilk ($P \leq 0.05$) and Levene ($P \leq 0.05$). Pairwise comparison of homoscedastic data was carried out with Tukey's test ($P < 0.05$), whereas non-homoscedastic data were analysed with Games-Howell test ($P < 0.05$), using the statistical package SPSS (IBM). To detect significant differences in expression, one-way ANOVA with Tukey post-hoc test ($P < 0.05$) was applied to data from samples collected at 0 hpi. Defense gene expression analysis, was tested with the Fisher post-hoc test.

Correlations

The two-tailed Pearson's correlation test was adopted to investigate the correlations between AUDPC and relative expression of *MLO* genes at 10 dpi, and between degree of severity and number of conidia, both at 21 dpi. All data have been transformed following y=arcsin(x) to achieve a normal distribution.

Metabolites

The data from the phenolic metabolites were subjected to one-way ANOVA with Fisher post-hoc test. In case of non-homoscedastic data, the Games-Howell post-hoc test was applied, and the Kruskall-Wallis non-parametric test for data not normally distributed.

RESULTS

Over-expression of *MdMLO18* in *A. thaliana* triple *mlo*-mutant did not increase susceptibility

A PM-resistant *A. thaliana Atmlo2/6/12* mutant over-expressing *MdMLO18* was generated via *A. tumefaciens* transformation by floral dipping. Seedlings of *Atmlo2/6/12*, *Atmlo2/6/12-MdMLO18* and *A. thaliana* Col-0 were inoculated with *O. Neolycopersici*. Seven days after inoculation, no infection was observed on the leaves of neither *Atmlo2/6/12* nor *Atmlo2/6/12-MdMLO18*, whereas *A. thaliana*

Col-0 was heavily infected (Fig. 1). The result was interpreted as excluding a role for *MdMLO18* in PM susceptibility of apple.

Development of RNAi apple plantlets

Three RNAi constructs were generated, two aimed at knocking-down *MdMLO11* and *MdMLO19* individually (a = KD-*MdMLO11,* b = KD-*MdMLO19*), the third aimed at the simultaneous knock-down of *MdMLO11* and *MdMLO19* (c = KD-*MdMLO11+19*). Eighty regenerated lines were obtained of which 48 did carry the RNAi insert as described in materials and methods (Table S3). The 48 transgenic lines were tested by qPCR to evaluate the level of *MLO* genes expression, but a significant knock-down was observed only in three of them (Table S3). In these three lines, off-target knock-downs were not detected for the other two clade V genes of apple (*MdMLO5* and *7*). The three knock-down lines, named TG11 (Transgenic Gala *MdMLO11*), TG19 and TG11+19, were acclimated to greenhouse conditions, as well as the control wild-type 'Gala' and TG0, a line carrying the RNAi construct for *MdMLO19* but not showing significant *MLO* genes knock-down. TG0, TG11, TG19 and TG11+19 will be indicated as transgenic lines, but only TG11, TG19 and TG11+19 as *mlo* lines.

The survival rate of plants to the acclimation procedure was above 90%. Under greenhouse conditions the *mlo* lines showed a normal growth compared to 'Gala' under greenhouse conditions.

Reduced susceptibility to *P. leucotricha* of RNAi apple plants

The four transgenic lines and the control were tested for their susceptibility to PM in four independent experiments. TG0, the line not manifesting any MLO genes knock-down, showed a level of susceptibility to *P. leucotricha* comparable to that of the control. The same was noted for TG11, whereas TG11+19 and TG19 had an

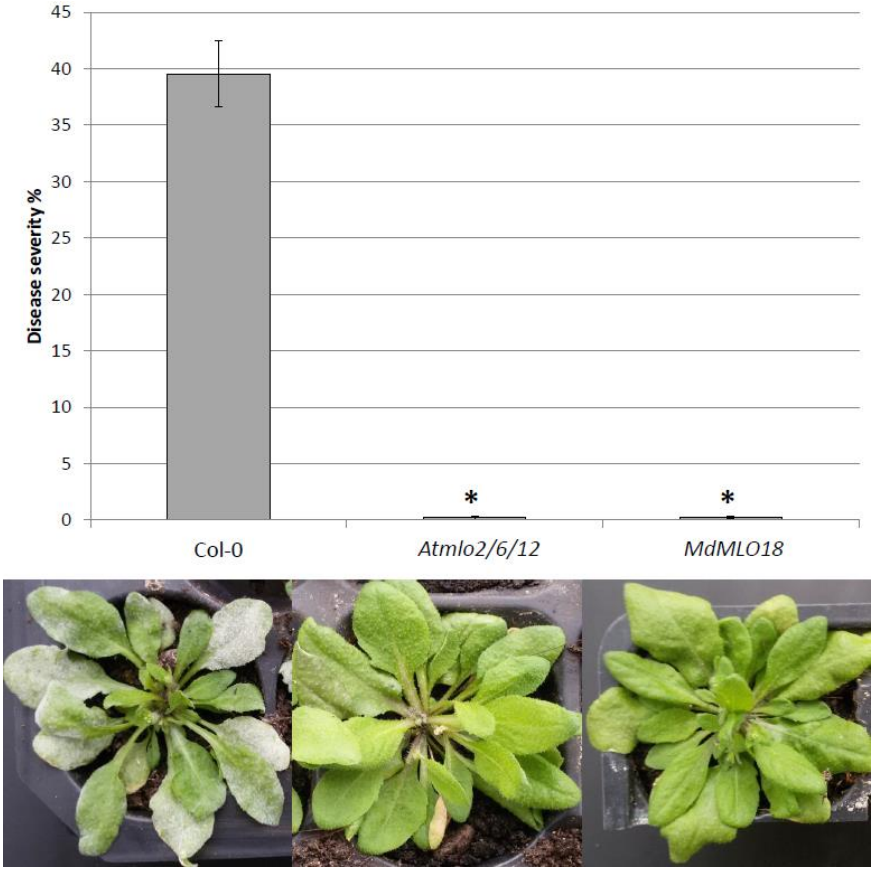

Figure 1. Disease severity recorded after 7 days from the inoculation with *O. Neolycopersici* of *A. thaliana* Col-0, *A. thaliana Atmlo2/6/12* mutant and *A. thaliana Atmlo2/6/12* mutant expressing *MdMLO18.* Histograms, representing average PM severity, were from data of 7 to 24 biological replicates. Error bars show the standard errors of the mean. The asterisks indicate statistically significant differences compared to Gala, according to the Kruskall-Wallis test (P=0.01).

evident reduction of disease severity (Fig. 2 and S1). Although leaves of TG11+19 and TG19 plants were partially infected (Fig. 2 and S1), the extension of the adaxial leaf area covered in spores was significantly reduced compared to the control (Fig. 2 and S1). Table 1 summarizes the disease severity reduction.

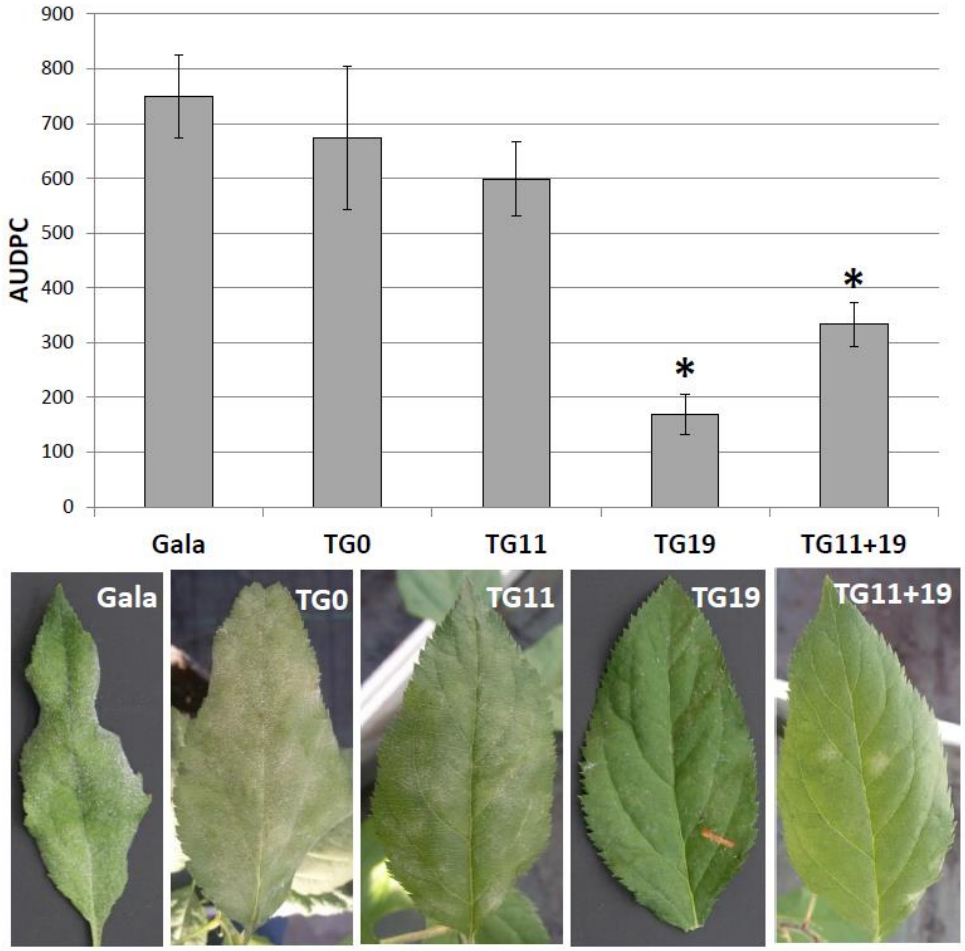

Figure 2. Area under disease progress curve (AUDPC) of four *mlo* lines and of the control 'Gala', inoculated with *Podosphaera leucotricha*. Average AUDPC was calculated from 15 to 24 biological replicates considered in four experiments. Error bars show the standard errors of the mean. Statistically significant differences in the comparisons with 'Gala', according to Tukey and Games-Howell post-hoc tests (P=0.05) are indicated with asterisks.

All the transgenic lines had a reduction in the number of conidia present on leaves (Fig. S2), but the decrease was statistically significant ($P < 0.05$) only for TG11+19 and TG19. This compares well with the disease severity assessment presented in

Figs. 2 and S1: compared to 'Gala', TG11+19 showed a 63.3% reduction in the number of conidia, and TG19 of 64.8%. A significant (P=0.01) but moderate positive correlation (Pearson coefficient of 0.525) was found between disease severity at 21 dpi and conidia count at 21 dpi.

Lines TG11+19 and TG19, together with 'Gala', were further analysed by bright field microscopy and scanning electron microscopy (SEM), to follow the development of *P. leucotricha* infection. In 'Gala', a well-developed leaf infection was observed already at 3 dpi (Fig. 3A), when fungal development was still limited in TG11+19 and TG19 (Fig. 3B and 3C). At 10 dpi, conidiophores were observed on leaves of all lines considered, but their number was higher in 'Gala' (Fig. 3). At 21 dpi, 'Gala' leaves were completely covered by spores and a large number of conidiophores were visible (Fig. 3A). The leaf surface of TG11+19 and TG19 was partially colonized by sporulating mycelium, but isolated spores unable to develop were also observed, as well as a smaller number of conidiophores compared to the situation noted for 'Gala' (Fig. 3B, C). The SEM images showed reduced growth of the mycelium on TG11+19 compared to TG0 and 'Gala' (Fig. S3).

The formation of papilla was observed at 3 dpi in all the lines, both resistant and susceptible (Fig. 4). Compared to TG11+19 and TG19, the papillae of 'Gala' (Fig. 4A, B) were smaller, the shape more defined and the fluorescence emitted was more intense (Fig. 4C, D, E).

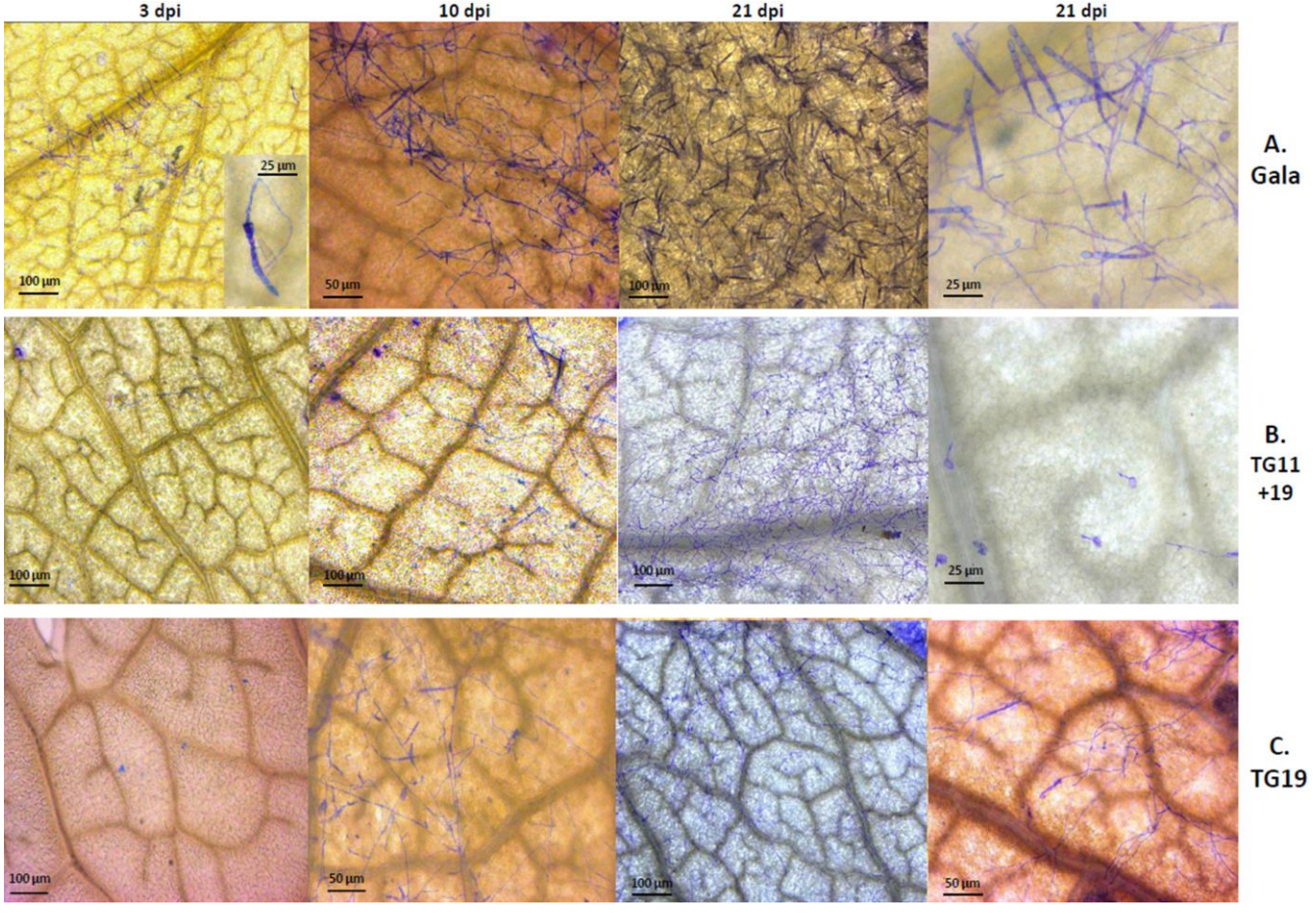

Figure 3. Bright field microscopy images of infected leaves of 'Gala' and lines TG11+19 and TG19 taken at 3, 10, and 21 dpi. For Gala at 3 dpi, at higher magnification the germination of a *P. leucotricha* spore is shown.

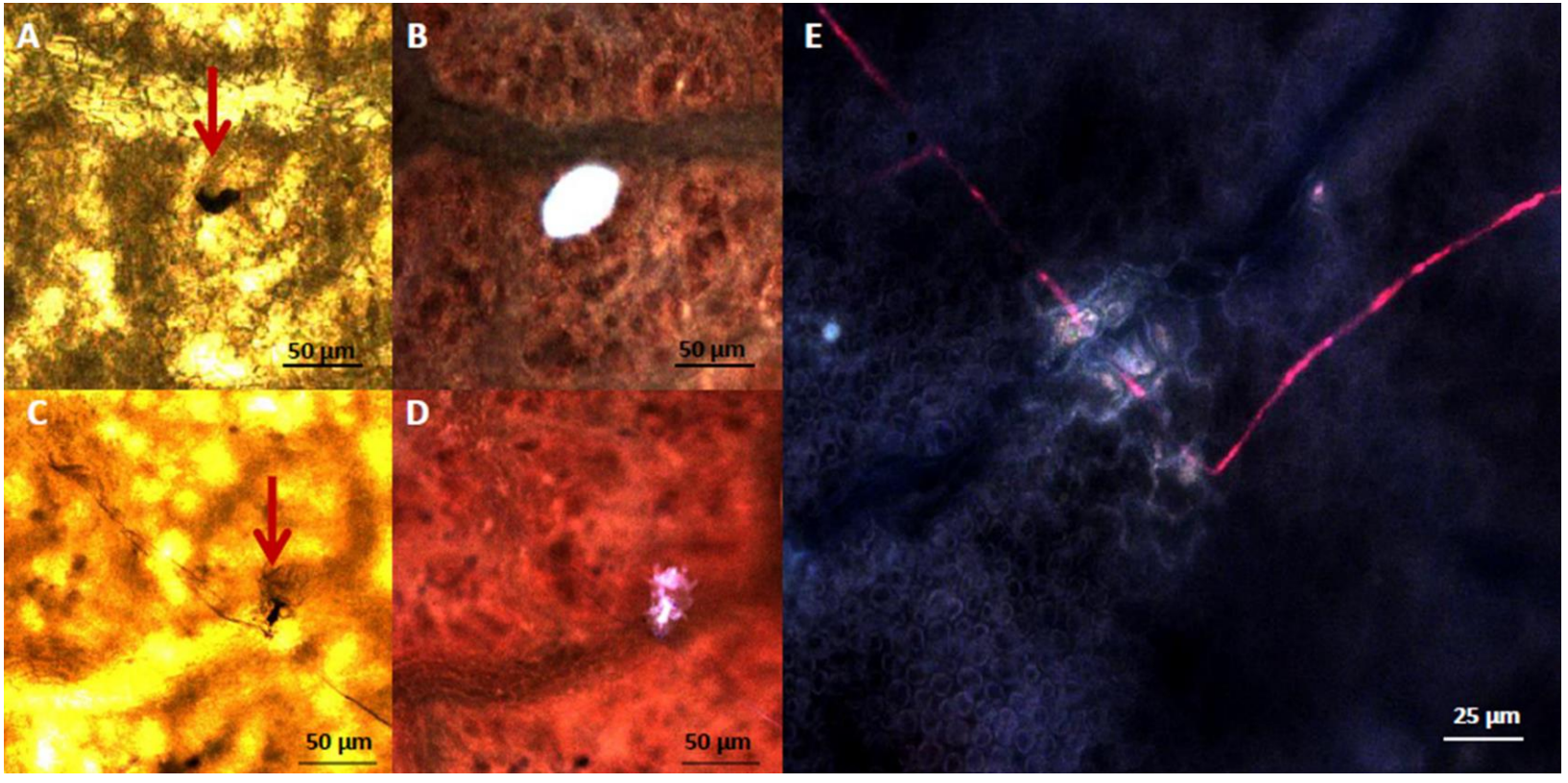

Figure 4. Formation at 3 dpi of papillae in infected leaves of 'Gala' (A, B) and in resistant lines TG11+19 (C, D) and TG19 (E). Images on the left were taken with a bright field microscope, those on the right with fluorescence microscope. For line TG19 only the image taken with the fluorescent microscope is shown.

Expression of *MLO* genes in *mlo* apple lines

Gene expression analysis of *mlo* lines previously selected was repeated in greenhouse acclimated plants. *MdMLO11* was significantly less expressed in TG11+19 (P=0.01) and TG11 (P=0.05) (Fig. 5A), whereas the expression of *MdMLO19* was reduced in TG11+19 (P=0.01) and TG19 (P=0.01) (Fig. 5B). *MdMLO5* and *MdMLO7,* the two other apple members of Clade V, were also tested but no significant reduction was observed in any transgenic line, a finding supporting the absence of off-target silencing (data not shown).

Correlation between the expression of *MdMLO19* and AUDPC – a measure of disease severity - was statistically significant (P=0.05), although moderate (Pearson coefficient=0.515). On the contrary, no significant correlation was found between AUDPC and the expression of *MdMLO11*.

Gene expression analysis of *mlo* apple lines TG11+19 and TG19

The expression profile of 17 genes related to plant disease resistance was tested at three time points in resistant *mlo* lines TG11+19 and TG19 compared to 'Gala' (Fig. 6 and S4). These genes were selected because of their role in the interaction with the PM pathogen and in defense in general. In absence of infection, five genes were down-regulated in TG11+19 compared to 'Gala' and only one in TG19 (Fig. 6A). At 24 hours post inoculation, the three lines showed only moderate differences: four genes were less expressed in TG19 than in 'Gala', whereas in TG11+19 one gene was up-regulated and two down-regulated (Fig. 6B). The scenario was slightly different at 10 dpi: three genes were less expressed than in 'Gala' and two moderately up-regulated in TG19 (Fig. 6C), whereas three genes were down-regulated in TG11+19 (Fig. 6C).

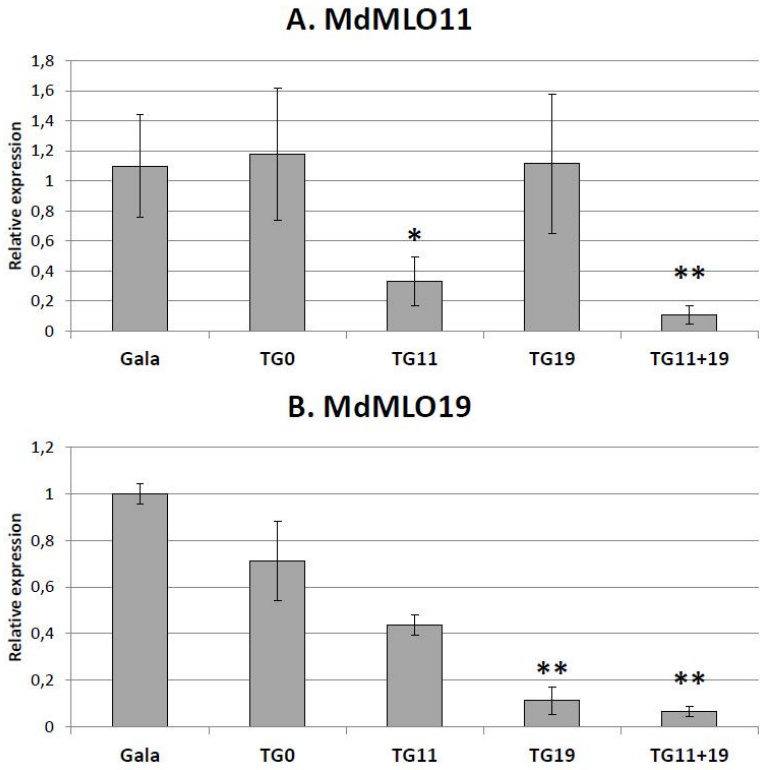

Figure 5. Expression of two apple *MLO* genes in five *mlo* lines in absence of *P. leucotricha* infection. Each bar represents the line average relative expression, evaluated from three to five plants. Error bars show the standard errors of the mean. Asterisks indicate significant differences in the comparison of *mlo* lines with 'Gala', based on Tukey or Games-Howell post-hoc tests (P=0.05).

The effect of *P. leucotricha* inoculation on single lines was different: at 24 hpi, five genes were up-regulated in 'Gala' (Fig. S4A), 13 in TG11+19 (Fig. S4B) and four in TG19 (Fig. S4C). The only gene up-regulated at 24 hpi in all lines was *MdVSP1* (*vegetative storage protein*) (Fig. S4). Of genes up-regulated at 24 hpi, only few

remained induced at 10 dpi: two in 'Gala' (Fig. S4A) and TG19 (Fig. S4C) and three in TG11+19 (Fig. S4B).

Phenolic metabolites composition of *mlo* apple leaves

Of the 135 phenolic secondary metabolites of apple identified by Vrhovsek *et al.* (2012), only 18 were found and quantified in the leaves of 'Gala', TG11+19 and TG19 (Table S4). Statistically significant differences between the *mlo* lines TG11+19 and TG19 and 'Gala' were noted for chlorogenic acid, rutin (quercetin-3-*O*-rutinoside), Kaempferol-3-*O*-rutinoside and isorhamnetin-3-*O*-glucoside (Fig. S5). Chlorogenic acid and rutin were lower in both *mlo* lines, but the difference was significant only for chlorogenic acid in TG11+19 (P=0.01) and for rutin in TG19 (P=0.01) (Fig. S5A, B). Kaempferol-3-*O*-rutinoside was higher in TG19 (P=0.05) (Fig. S5C), as for isorhamnetin-3-*O*-glucoside in both *mlo* lines (P=0.01) (Fig. S5D). Compounds derived from the same precursor were also considered together: quercetins (quercetin-3-*O*-rhamnosid + quercetin-3-*O*-glucoside + quercetin-3-*O*-galactoside + rutin) and kaempferols (kaempferol + kaempferol-3-*O*-glucoside + kaempferol-3-*O*-rutinoside) did not show any significant change (Fig. S5E, F), whereas isorhamnetins (isorhamnetin + isorhamnetin-3-*O*-glucoside + isorhamnetin-3-*O*-rutinoside) were higher in TG19 (Fig. S5G). Quercetins, kaempferols and isorhamnetins, flavonoids of the flavonols subgroup, considered together did not reveal significant differences between 'Gala' and *mlo* resistant lines (Fig. S5H).

DISCUSSION

Natural and artificial loss-of-function mutations of *MLO* S-genes reduce susceptibility to PM pathogens, as described in barley (Büschges *et al*., 1997), *A. thaliana* (Consonni *et al.,* 2006), pea (Pavan *et al.,* 2011), tomato (Bai *et al.,* 2008)

and pepper (Zheng *et al.,* 2013). In dicots, all PM-susceptibility genes belong to Clade V (Consonni *et al.,* 2006; Bai *et al.,* 2008; Feechan *et al*., 2008; Winterhagen *et al.,* 2008). In a previous contribution we identified three *MLO* genes of *M. domestica* up-regulated during early stages of PM infection (Pessina *et al.,* 2014).

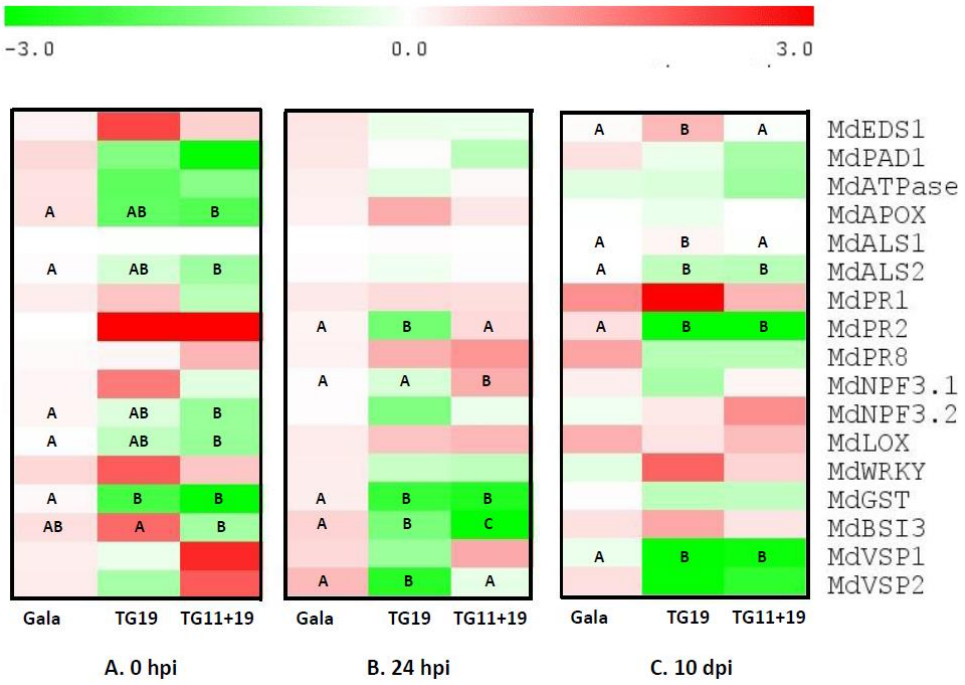

Figure 6: Relative expression in 'Gala' and in resistant *mlo* lines TG11+19 and TG19 of 17 plant genes, monitored at three time points after PM inoculation. The average values of relative expression of 'Gala' were used as reference for statistical analyses. Colour scale indicates the expression relative to 'Gala' at 0 dpi, used as reference for data normalization. The letter code indicates statistically significant differences among time points, according to Fisher post-hoc test (P=0.05). Image was prepared with the Multiexperiment Viewer software, with Log2 of relative expression data.

Two of them, *MdMLO11* and *MdMLO19*, belong to dicot clade V and *MdMLO18* to clade VII. Because *MLO* genes outside clade V acting as S-genes are not known, only *MdMLO11* and *MdMLO19* were considered reasonable candidates to be knocked-down in apple*,* whereas *MdMLO18* was tested with the quicker complementation test of the *A. thaliana* mutan*t Atmlo2/6/12,* which is completely resistant to different non-adapted PM species (Consonni *et al.,* 2006), including *O. neolycopersici*, the causal agent of tomato PM (Zheng *et al.,* 2013). Complementation is based on the ability of PM pathogens to start a successful infection harnessing *MLO* genes similar, but not identical to the resident ones (Acevedo-Garcia *et al.,* 2014). In case the resistant *Atmlo2/6/12* mutant expressing the foreign *MLO* gene becomes susceptible to PM, it is a first indication that the introduced *MLO* can functionally substitute the native *MLO* S-genes of *A. thaliana*. *MdMLO18* failed to complement, in accordance with the robust evidence that only clade V genes act as S-genes in dicots (Consonni *et al.,* 2006; Bai *et al.,* 2008; Humphry *et al.,* 2011; Pavan *et al.,* 2011; Zheng *et al.,* 2013; Acevedo-Garcia *et al.,* 2014). Therefore, we did not perform RNAi in apple for *MdMLO18*.

MdMLO11 and *MdMLO19* were knocked-down to assess their role in supporting apple susceptibility to PM. RNAi was adopted to reduce the expression of the two *MLO* genes, and in spite of the high number of transgenic 'Gala' lines generated (48), only for three of them a significant reduction of expression of the target genes was detected. In part, this was expected because short RNAi fragments of less than 150 bp, like those used in our experiments, are known for their limited knock-down efficiency. On the other hand, they have the advantage of being more specific, thus avoiding the generation of off-target silencing of other clade V *MLO* genes, as detected in our experiments.

In some species, the knock-out and knock-down of *MLO* genes causes pleiotropic phenotypes, such as necrotic spots on leaves and reduced gran yields in barley (Jørgensen, 1992), slow growth in *A. thaliana* (Consonni *et al.,* 2006) and reduced plant size in pepper (Zheng *et al.,* 2013). Such or other unexpected pleiotropic phenotypes were not observed under the greenhouse conditions specified in Materials and Methods. Greenhouse inoculation of apple transgenic lines resulted in a statistically significant reduction of disease severity in lines TG11+19 and TG19. Because of the knock-down of *MdMLO19* in both resistant lines, it was assumed that this was the most effective gene responsible for the reduction of PM susceptibility. The knock-down of *MdMLO11* did not result in a significant reduction of susceptibility and even its knock-down in combination with *MdMLO19* resulted in any additional reduction of susceptibility. The conclusion is that out of the two Clade V genes induced by PM in apple, only *MdMLO19* is a functional S-gene. Also *MdMLO18*, the Clade VII gene inducible by *P. Leucotricha* inoculation, should not be considered a PM S-gene. Line TG0 was considered with the purpose of assessing the effect on susceptibility to PM of the insertion of a "target ineffective" RNAi construct. TG0 was obtained from a transfer that aimed to knock-down *MdMLO19*. In the line, a decrease of expression of *MdMLO19* was recorded, although not significant, as well as a moderate non-significant reduction of PM susceptibility. It is concluded that the insertion of an "ineffective" RNAi construct may have functional relevance, but this cannot be statistically proven.

The precise mechanism through which the loss-of-function of *MLO* S-genes reduces susceptibility to PM pathogens is not completely clear yet. However, *mlo* resistance is known to be linked to secretory vesicle trafficking (Miklis *et al.,* 2007; Feechan *et al*., 2011) and to the formation of cell wall appositions called papillae (Consonni *et al.*, 2006). Papillae consist in a callose matrix enriched in proteins and autofluorogenic phenolics (Vanacker *et al.* 2000) whose formation depends on

actin-dependent endomembrane transport (Hückelhoven, 2014). Lines 'Gala', TG11+19 and TG19 were characterized by the presence of papillae at 3 dpi, but shape and dimensions were different in resistant and susceptible lines. Rapid papilla formation (Lyngkjær *et al.,* 2000), increased papilla size at attempted penetration sites (Stolzenburg *et al.*, 1984) and different biochemical composition (Chowdhury *et al*., 2014), may explain the noted differences between effective and non-effective papillae. In *mlo* lines, particularly in TG19, the size of papillae was larger than in the control, supporting the hypothesis that larger dimensions increase the efficacy of the papilla. Chowdhury *et al*. (2014) have shown that the difference between effective and non-effective papillae lies in the higher concentration of callose, cellulose and arabinoxylan of the effective ones. This possibly reflects the observed differences in fluorescence between papillae of resistant and susceptible lines. As a matter of fact, MLO proteins are considered negative regulators of vesicle-associated and actin-dependent defense pathways (Panstruga, 2005), which, once under the control of the fungus, induce actin filaments to supply nutrients for the growing hyphae (Miklis *et al.,* 2007). The data presented here support the view that in apple wild-types, after penetration the pathogen controls the transport of material to the cell-wall, changing the composition of the papillae and turning them into non-effective. A similar work carried out in grapevine (Chapter 5) support this interpretation: compared to the control, *mlo* grapevine lines showed larger and less defined papillae, similar to those observed in *mlo* apple.

To further understand the effect of the knock-down of *MLO* genes in apple, the expression of 17 genes involved in defense and interaction with other apple pathogens, such as *Erwinia amilovora* (fire blight), was analysed. Five genes involved in a variety of processes were down-regulated in TG11+19. Of them, *MdAPOX* (*ascorbate peroxidase*), *MdGST* (*glutathione S-transferase*) and *MdLOX*

(*lipoxygenase*), have a role in plant immunity, suggesting a moderate and unexpected inhibition of defense against PM. Other down-regulated genes were *MdALS2* and *MdNPF3.2*. Interesting was the case of *MdNPF3.2,* the homologous of a grapevine nitrite/nitrate transporter up-regulated upon PM inoculation. It has been suggested that the up-regulation of this gene is due to the PM pathogen that, lacking of nitrate transporters and nitrite and nitrate reductases, uses those of the host to obtain ammonium, amino acids and peptides (Pike *et al*., 2014). This seems not the case of apple, as neither *MdNPF3.1* nor *MdNPF3.2* was up-regulated in 'Gala' upon the inoculation with *P. Leucotricha.* These results suggest that the knock-down of *MLO* genes affected the expression of other disease-related genes in the absence of PM infection. It was no surprise that more genes were down-regulated in TG11+19 rather than in TG19, because of the double knock-down in the former. However, down-regulation in TG11+19 of three genes involved in plant defense against pathogen was unexpected: *MLO* genes are, in fact, negative regulators of defense and the expectation was that their knock-down would cause an activation of defense.

Considering again the expression of the 17 genes related to plant defense, a rationale is difficult to highlight based on the analysis of the three lines together. Nevertheless, the analysis of the PM inoculation effects on each of the three lines considered independently, clarifies some details: line TG11-19 is extremely responsive to PM with an up-regulation of 13 genes out of 17 at 24 hpi. Two of these genes are pathogenesis-related (*MdPR1* and *MdPR2)* and seven are involved in defense (*MdATPase, MdAPOX, MdLOX, MdWRKY30, MdGST, MdVSP1* and *MdVSP2*). Conversely, TG19 showed a limited transcriptional response, possibly due to its better capacity to control PM infection. The absence of gene up-regulation at 10 dpi of most of the genes tested indicated that the

transcriptional response, when evident, is more intense in early stages of pathogenesis.

In this paper results are presented concerning eighteen phenolic secondary metabolites, mostly flavonoids, identified and quantified as in Vrhovsek *et al.* (2012) in the leaves of 'Gala', TG11+19 and TG19. For chlorogenic acid, rutin, kaempferol-3-*O*-rutinoside and isorhamnetin-3-*O*-glucoside significant differences between 'Gala' and *mlo* resistant lines were found. Chlorogenic acid is known to increase potato resistance to *Streptomyces scabies*, *Verticillium alboatrum* and *Phytophthora infestans* (Lattanzio *et al.,* 2006); it was present in lower amounts in TG11+19 compared to 'Gala'. Kaempferol inhibits spore germination of the rice pathogen *Pyricularia oryzae* (Padmavati *et al.,* 1997): an accumulation of kaempferol-3-*O*-rutinoside was detected in TG19. A putative defense-related role of rutin (present in lower amount in TG19) and isorhamnetin (accumulating in both *mlo* lines) is not at the moment known. The higher amount of isorhamnetin derivatives in TG19 may indicate an increased activity of the 3'-methyl transferase that catalyses the methylation of quercetin to isorhamnetin.

CONCLUSIONS

Our results showed that *MdMLO19* is the S-gene for PM in apple and its knock-down substantially reduced PM susceptibility of *M. domestica*. The knock-down of *MdMLO11,* alone or combined with *MdMLO19,* did not cause a reduction or an additional reduction of susceptibility compared to *MdMLO19* alone, therefore the gene did not contribute to PM resistance. Immunity to PM was not observed, as expected because of the incomplete silencing of *MLO* genes in RNAi transformed plants. At the level of MLO knock-down reported, no altered pleiotrophic phenotypes were detected in *mlo* plants under the adopted greenhouse conditions.

This work provides crucial information that can be used to introduce durable resistance to *P. leucotricha* in apple. This can be done via genome editing of *MdMLO19*, resulting in knock-out mutants resistant to PM, or via the search in *M. domestica* and in wild *Malus* species of non-functional *MdMLO19* alleles.

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The authors would like to thank Jos Brinkhuis (WUR) for the help with apple gene transfer, Stefano Piazza (FEM) for the help with *in vitro* tissue culture, Manuela Campa (FEM) for building the construct for *A. thaliana* gene transfer, Franca Valentini (FEM) for the help with the management of plants and experiments in the greenhouse, Valentino Poletti (FEM) for the help with electron microscopy and Michele Butturini (FEM) for the help with qPCR and *A. thaliana* complementation. Authors would also like to thank Dr. Mauro Bernabei (CNR-IVALSA, San Michele a/Adige - Italy) for letting us use the scanning electron microscope. This work was funded by the autonomous province of Trento (Italy) and the GMPF PhD programme.

ABBREVIATIONS

AUDPC: area under disease progress curve

Dpi: days post inoculation

Hpi: hours post inoculation

PM: powdery mildew

RE: relative expression

RNAi: RNA interference

SEM: scansion electron microscopy

TG: Transgenic Gala

Table S1: Primers for qPCR

Name	Accession number	Forward (‘5 – ‘3)	Reverse (‘5 – ‘3)	Reference
EF1	MD09G014760[a]	TACTGGAACATCACAGGCTGAC	TGGACCTCTCAATCATGTTGTC	Pessina et *al.* (2014)
Ubiquitin	MD05G001920[a]	CATCCCCCCAGACCAGCAGA	ACCACGGAGACGAAGCACCAA	Pessina et *al.* (2014)
Md8283	MDP0000375455[b]	CTCGTCGTCTTGTTCCCTGA	GCCTAAGGACAGGTGGTCTATG	Botton *et al.* (2011)
MdMLO11	MDP0000239643[b]	ATCGAAGGCTGTTGGAGCAA	AAGCACGTGAAAGACGGCTA	/
MdMLO19	MDP0000168714[b]	CAGAGTGGCGACTGCACTTA	GGGACATGGAGTGCAAAGGA	Pessina et *al.* (2014)
MdALS1	MDP0000621545[b]	TACAAGTACAAGCGGCCTCG	TCTCCACACTGATCGTTGCC	/
MdALS2	MDP0000715451[b]	ATGTCTCGGTTTGTGCGGAT	GGAGGAATGGCTTCCCCAAA	/
MdNPF3.1	MDP0000606453[b]	GCTGGCCCTAATGGATCGAA	TTTTCTCCAGACTCGGCACC	/
MdNPF3.2	MDP0000549956[b]	CAACGCAGCTTCATATGCCC	ATAGTCCAGAAGCGGCCAAC	/
MdVSP1	MDP0000155698[b]	AGCTTTGCCCGAGAGTCTTC	ACAAGCTTCTCCCAGGTGTG	/
MdVSP2	MDP0000274344[b]	GGCACCAGTACAGGAAGGAC	TGAATGCCTTCGTCCCGAAT	/
MdLOX	MDP0000300321[b]	GTTGCGTATGGGAAGGAATGG	GGTAGTAGTGGTTTACATAGTCAGTG	/
MdPAD1	MDP0000158955[b]	TCAATGACTGGGCAATCGCA	TCCAGGGCAAACTCTTCGAC	/
MdWRKY30	MDP0000468391[b]	AATATACATTGGGAGCAAAAGAGTC	AGAGTTCAGCATGGAAAGCG	/
MdATPase	MDP0000494888[b]	TCTTTCTTCCACCTGCTCCT	GTTCTCACACACAATCTTCCCATCC	/
MdAPOX	MDP0000241173[b]	GCTCCAACTGACAAGGCTCTTC	CGCACAGGCATCGGCTTC	/
MdBSI3	MDP0000287919[b]	CGCCTTTGGTTAAGAATGAGCCTC	CCTCAATGTTACAAATGTCTTGGCGC	/
MDGST	MDP0000266097[b]	GAGCCCTTCATATCCCTCAATCC	GCCTCCACCTCCGACCAC	/
MdEDS1	MDP0000479863[b]	TGGAGAAAGTGATTTTGGAGAAGC	AGAACCAGATTGTGACAAACGC	/
MdPR1	AF507974[c]	AGCACACGAGTTCGACTCATAA	CACAAAACTACGCCAACCAA	/
MdPR2	AF494404[c]	GGTCGGTGGAGGATCTTTGG	TTGGAGTCCCTCCCTTCACA	/
MdPR8	DQ318214[c]	CCAAGCCCCTGTCCTAAACCTC	CAACTTGCCTTGCCTCATCAGC	/

[a] Available at http://bioinformatics.psb.ugent.be/plaza

[b] Available at http://www.rosaceae.org/gb/gbrowse/malus_x_domestica/

[c] Available at http://www.ncbi.nlm.nih.gov/genbank/

Table S2: Primers for RNAi

Gene	Accession number[a]	Primer Forward	Primer Reverse	Amplicon Lenght
MdMLO11	MDP0000239643	GCACATCGCAGCGAAGAAGCAC	AGCTTTCAGTGTCCTGTTCGGATTG	134 bp
MdMLO19	MDP0000168714	TGCACTTGCTTTCTTTGCATGGAC	AACGACATCTTCCAACTTCTCATGG	115 bp

[a] Available at http://www.rosaceae.org/gb/gbrowse/malus_x_domestica/

Table S3: summary of gene transfer results

Gene transfer	Knocked-down gene	Regenerated lines	Confirmed transgenic	Selected lines
A	*MdMLO11*	39	23	TG11
B	*MdMLO19*	33	19	TG0, TG19
C	*MdMLO11* + *MdMLO19*	8	5	TG11+19

Table S4: identified and quantified phenolic metabolites	
Compound	**Group (subgroup)**
Chlorogenic acid	Benzoic acids
Coniferyl alcohol	Phenylpropanoids
Phloretin	Polyketides (Dihydrochalcones)
Phlorizin	Polyketides (Dihydrochalcones)
Naringenin	Flavonoids (Flavanones)
Catechin	Flavonoids (Flavan-3-ols)
Epicatechin	Flavonoids (Flavan-3-ols)
Procyanidin B2 + B4	Flavonoids (Proanthocyanidins)
Kaempferol	Flavonoids (Flavonols)
Kaempferol-3-*O*-glucoside	Flavonoids (Flavonols)
kaempferol-3-*O*-rutinoside	Flavonoids (Flavonols)
Isorhamnetin	Flavonoids (Flavonols)
Isorhamnetin-3-*O*-glucoside	Flavonoids (Flavonols)
Isorhamnetin-3-*O*-rutinoside	Flavonoids (Flavonols)
Quercetin-3-*O*-rhamnosid	Flavonoids (Flavonols)
Quercetin-3-*O*-glucoside	Flavonoids (Flavonols)
Quercetin-3-*O*-galactoside	Flavonoids (Flavonols)
Rutin	Flavonoids (Flavonols)
Arbutin	Hydroquinones

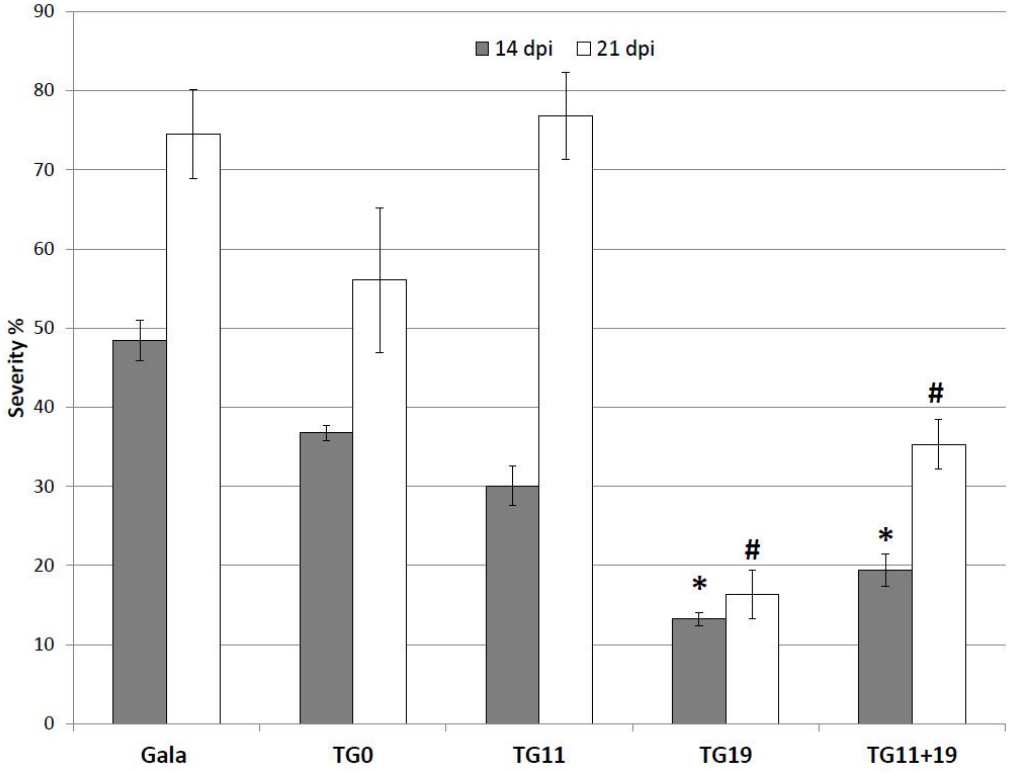

Figure S1. Infection severity at 14 and 21 dpi of four apple *mlo* lines inoculated with *P. leucotricha*. Each bar represents the average severity of infection at the given time point, calculated on 15-23 biological replicates and four experiments. Error bars show standard errors of the mean. Dark grey bars represent severity at 14 dpi, white bars at 21 dpi. Each time point has been analysed independently. For each time point, symbols highlight significant differences compared to the control Gala, according to Tukey or Games-Howell post-hoc test (P = 0.05): * for 14 dpi, # for 21 dpi.

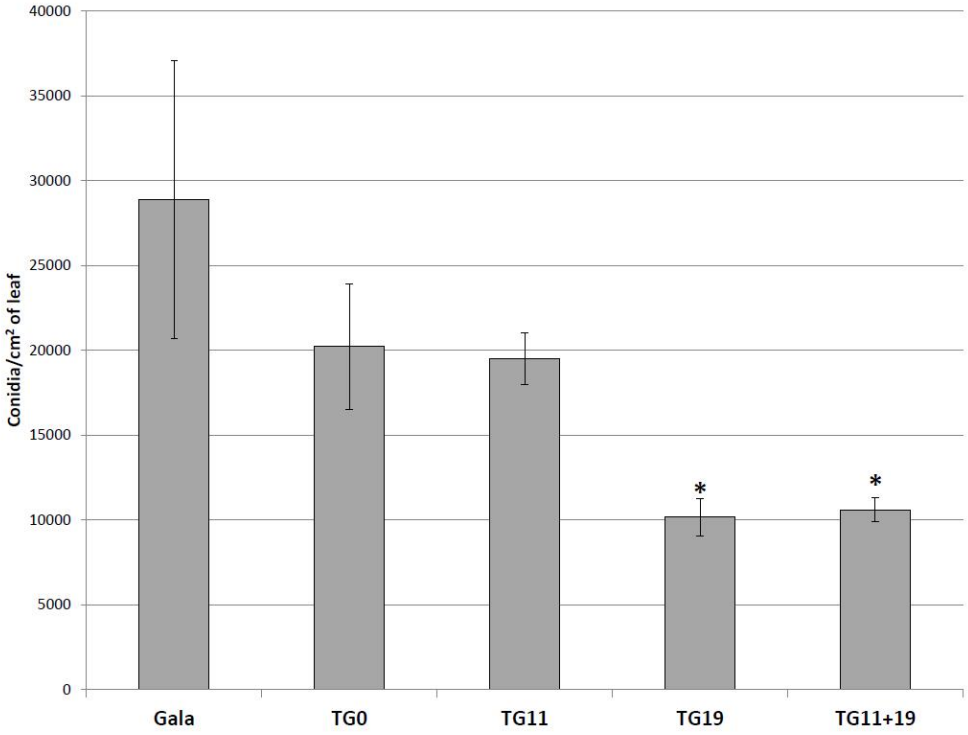

Figure S2. Number of conidia per cm^2 leaf surface of 'Gala' and *mlo* lines TG0, TG11, TG19 and TG11+19 inoculated with *P. leucotricha* at 21 dpi. Bars indicate the average number of conidia, measured in two experiments. Error bars show standard errors of the mean. Asterisks indicate statistically significant differences compared to 'Gala' according to Tukey post-hoc test (P = 0.01).

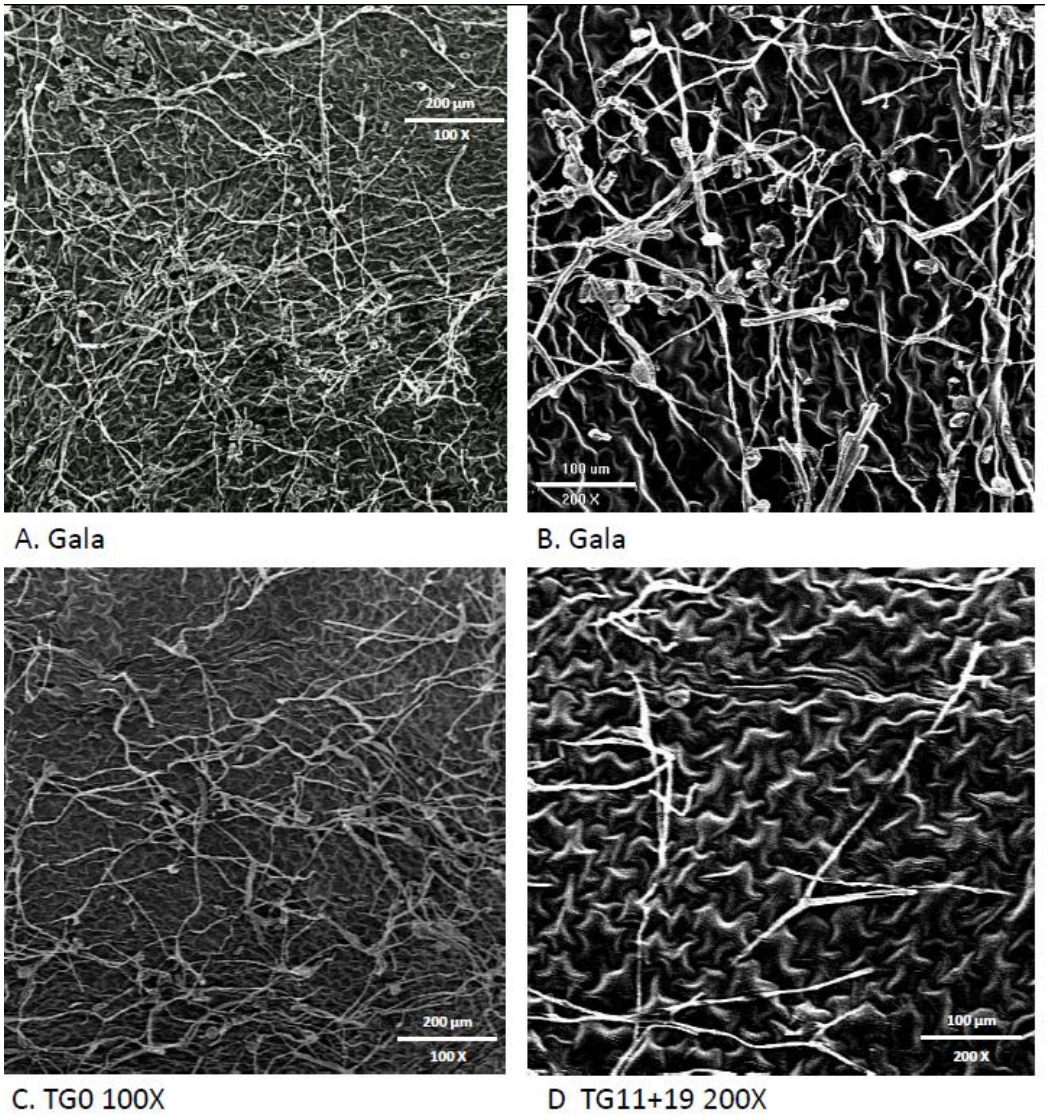

Figure S3: SEM microscopy images of infected leaves of 'Gala', the susceptible line TG0 and the resistant line TG11+19. Pictures were taken at 21 dpi.

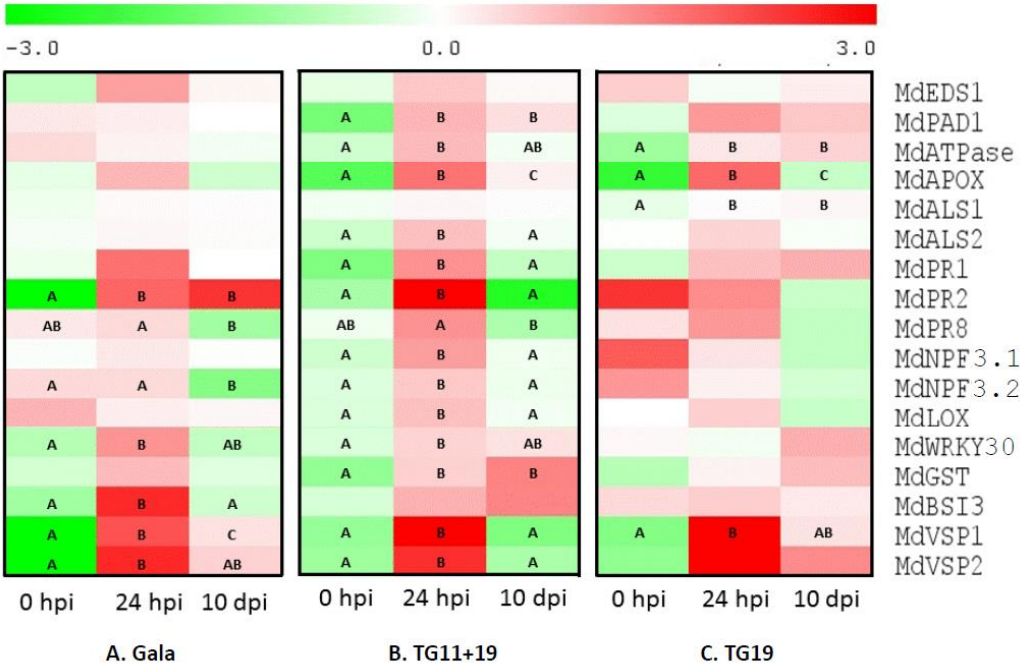

Figure S4: Relative expression at three time points in Gala and resistant *mlo* lines TG11+19 and TG19 of 17 genes related to plant disease resistance . Each line point was analysed independently and the average Ct of all samples was used as reference for the statistical analysis. The letter code indicates statistically significant differences among time points according to Fisher post-hoc test (P=0.05). The image was prepared with the Multiexperiment Viewer software with the Log2 of relative expression data.

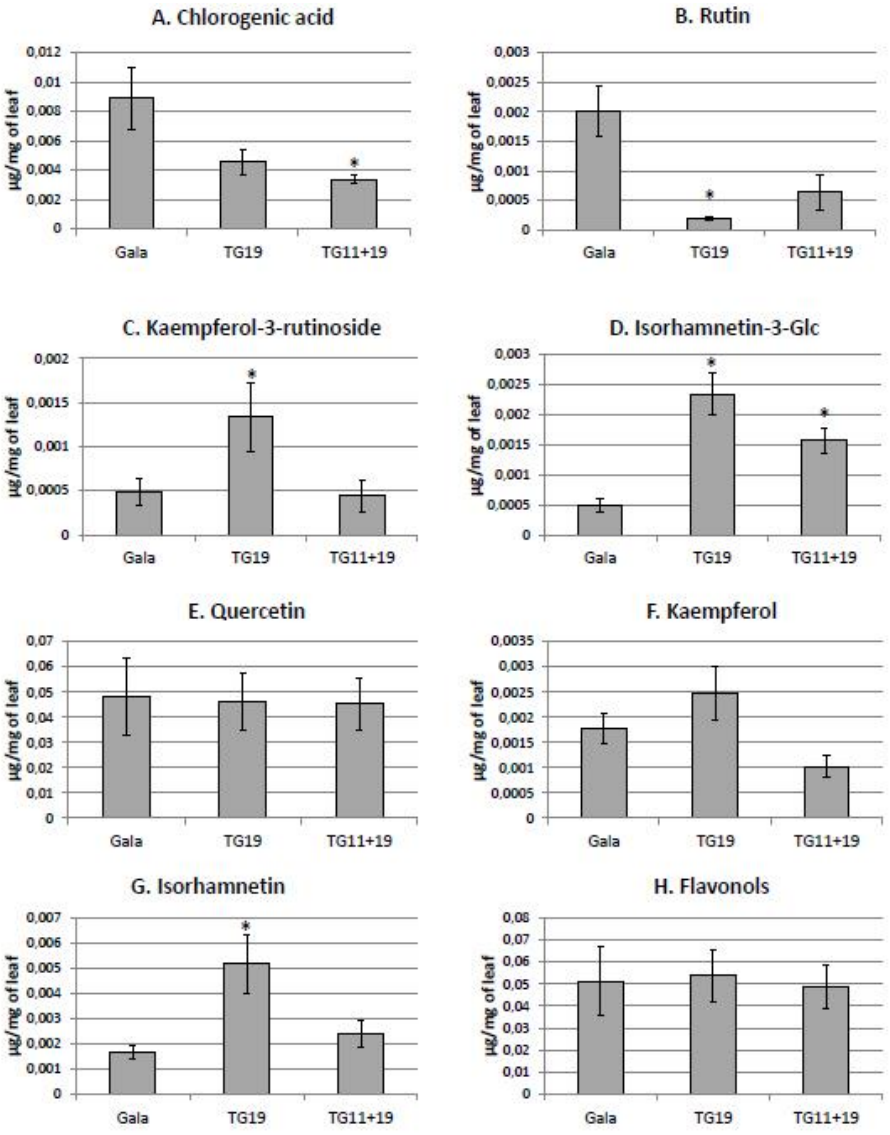

Figure S5: Phenolic metabolites content in leaves of 'Gala' and resistant lines TG11+19 and TG19. The average level of chlorogenic acid (A), rutin (B), Kaempferol-3-*O*-rutinoside (C), isorhamnetin-3-*O* glucoside (D), quercetins (E), kaempferols (F), isorhamnetins (G) and Flavonols (H) from eight samples is shown. Error bars show standard errors of the mean. Asterisks indicate significant differences (P = 0.05) according to Fisher or Games-Howell post-hoc tests or Kruskall-Wallis test.

CHAPTER 4

A truncated allele of *MdMLO19* in *Malus domestica* genotypes causes resistance to powdery mildew

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ABSTRACT

Podosphaera leucotricha is the causal agent of powdery mildew (PM) in apple. To reduce the amount of fungicides required to control this pathogen, the development of resistant apple varieties should become a priority. Resistance to PM can be achieved by knock-out of specific members of the *MLO* gene family, which are responsible for PM susceptibility (S-genes). In apple the knock-down of *MdMLO19* resulted in PM resistance. However, since gene transfer technologies are perceived unfavorably in Europe, a different approach to exploit this resistance is needed. This chapter evaluates the presence of non-functional alleles of *MdMLO19* in apple germplasm. The screening of the resequencing data of 63 apple genotypes led to the identification of 627 SNP in five *MLO* genes (*MdMLO5, MdMLO7, MdMLO11, MdMLO18* and *MdMLO19)*. Insertion T-1201 in *MdMLO19*, caused the formation of an early stop codon, resulting in a truncated protein lacking 185 amino-acids and the calmodulin-binding domain. The presence of the insertion was evaluated in a collection of 159 apple genotypes: it was homozygous in 53 genotypes, 45 of which were resistant or very resistant to PM, four partially susceptible and four not assessed. These results strongly suggest that this insertion is causative for the observed PM resistance. The absence of a clear fitness cost associated to the loss-of-function of *MdMLO19*, might have contributed to the high frequency of the mutation. Among the genotypes containing the homozygous insertion, 'McIntosh' and 'Fuji' are commonly used in apple breeding. After barley and tomato, apple would be the third species with a natural non-functional *mlo* allele in its germplasm, with the important difference that the allele is present in a plurality of genotypes.

INTRODUCTION

Powdery mildew (PM) is a relevant disease of apple that, in absence of chemical control, can reduce yield up to 50% (Yoder, 2000). The disease is caused by the obligate biotroph fungus *Podosphaera leucotricha* and it occurs in all major apple-growing regions of the world (Turechek *et al.,* 2004). Leaves are the most susceptible organ, particularly in the first days after opening, but blossom infections, although less common, are extremely severe because they result in small and stunted fruits, or in no fruit at all (Turechek *et al.,* 2004).

PM is a serious problem for thousands of plant species (Glawe *et al.,* 2008), but a source of durable resistance exists: the knock-out or knock-down of *MLO* genes lead to PM resistance in barley (Jørgensen, 1992), *Arabidopsis* (Consonni *et al.,* 2006), pea (Pavan *et al.,* 2011), tomato (Bai *et al.,* 2008), wheat (Wang *et al.,* 2014), pepper (Zheng *et al.,* 2013), apple (Chapter 3) and grapevine (Chapter 5). *MLO*s are susceptibility S-genes whose loss-of-function results in durable and broad-spectrum resistance (Pavan *et al*., 2010). The *MLO* gene family comprises a variable number of members, grouped in seven clades (Acevedo-Garcia *et al.,* 2014; Pessina *et al.,* 2014). Genes for PM susceptibility belong to clade IV, which contains monocot S-genes (Panstruga, 2005; Reinstädler *et al.,* 2010; Wang *et al.,* 2014), and clade V, which contains dicot S-genes (Consonni *et al.,* 2006; Bai *et al.,* 2008; Feechan *et al*., 2008; Winterhagen *et al.,* 2008). It is possible to identify S-genes through gene expression analysis: at early stages of PM infection, specific *MLO* S-genes have their expression increased. This was documented in tomato (Bai *et al.,* 2008), barley (Piffanelli *et al.,* 2002), pepper (Zheng *et al.,* 2013), grape (Feechan *et al*., 2008; Winterhagen *et al.,* 2008) and apple (Pessina *et al.,* 2014). Of the four *MLO* apple genes of clade V*, MdMLO11* and *MdMLO19* are up-regulated during PM infection, whereas *MdMLO5* and *MdMLO7* are not (Pessina *et al.,* 2014). *MdMLO18*, a gene of clade VII is also responsive to PM infection. Among these PM-

inducible apple genes only *MdMLO19* can be considered an S-gene because its knock-down reduced PM infection up to 75% (Chapter 3).

Gene transfer technologies, as those used to knock-down *MdMLO19* (Chapter 3), are currently not accepted by a large majority of the European public (Einsele, 2007); accordingly, the EU has the strictest regulation in the world on GMOs (Davidson, 2010). Therefore, a realistic alternative to gene transfer technologies becomes a necessity when the purpose is to take advantage of the resistance granted by *MLO* genes knock-out or knock-down. Marker-assisted breeding is a valuable option, but non-functional alleles of the S-gene are required. Moreover, non-functional recessive *mlo* alleles need to be homozygous in order to achieve PM resistance. Nature is a huge source of genetic diversity and the consideration of this variability is a key step to isolate useful alleles necessary to develop PM resistant varieties. In the case of apple, the FruitBreedomics project (http://www.fruitbreedomics.com) opened interesting possibilities making available 63 re-sequenced *Malus domestica* genotypes representing the genetic diversity present in the apple germplasm (Dr. R. Velasco, FEM; personal communication). We here report the screening of the 63 re-sequenced genomes, searching for non-functional alleles of five *MLO* genes, particularly in the four members of clade V and *MdMLO18*. A non-functional allele of *MdMLO19* was found and the link to PM resistance investigated. The possibility of using this allele to introgress durable resistance in apple varieties is discussed as well.

MATERIALS AND METHODS

FruitBreedomics data analysis

The genomic regions hosting genes *MdMLO5, MdMLO7, MdMLO11, MdMLO18* and *MdMLO19* (Pessina *et al.,* 2014) were screened in the FruitBreedomics re-sequencing dataset. For the 63 genotypes, single nucleotide polymorphisms (SNPs)

were retrieved from the variant calling format (.vcf) file of the Axiom Apple480K genotyping array of Bianco *et al.* (unpublished). A custom bioinformatic script was then written to retrieve all polymorphic sites of the five genes in the 63 apple genotypes. Data were stored in a tab separated value file (.tsv) for further processing. Only SNPs falling in exons were considered. SNP-based mutated nucleotide sequences were produced, as well as gene-encoded amino acids (aa) sequences, using EMBOSS transeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq/). Mutations were grouped in seven categories, according to the nucleotide change present in the sequence: silent substitutions (no aa changes), conservative substitutions (aa substituted with one of similar chemical and sterical properties), semi-conservative substitutions (substitution with an aa with similar sterical properties), non-conservative substitutions (substitution with an aa with different properties), insertions (insertion of one or more aa), deletions (removal of one or more aa) and nonsense mutations (formation of an early stop codon).

DNA extraction and PCR analysis

All leaf samples for DNA extraction were grinded in liquid nitrogen and extracted with Illustra Nucleon Phytopure kit (GE Healthcare, Buckinghamshire, UK). Resulting DNA was quantified with nanodrop and the quality assessed through PCR on the target *MLO* genes with GoTaq Green (Promega, Fitchburg, USA).

SNP validation by sequencing

To validate the presence of the insertion in *MdMLO19*, a total of 20 apple genotypes were sequenced. A 186 bp region of *MdMLO19* was amplified (Fw: 5' – GCATCTTGTCCTCGTATGTAGAATG – 3'; Rv: 5' – CGACATCTTCCAACTTCTCATGG – 3')

and sequenced twice from both ends. Sequences were visualized with Chromas lite (Technelysium) and aligned with the Staden package software (Staden, 1996).
To validate the heterozygous state of insertion T-1201, the 186 bp fragment of MdMLO19 (see 'SNP validation by sequencing') amplified from cultivar Durello di Forlì was cloned into the gateway vector pENTR/SD-TOPO (Thermo Fisher Scientific, Waltham, USA). and inserted into *Escherichia coli*, that was plated on a selective media. Eight colonies were picked, the plasmids extracted with Qiaprep Spin Miniprep kit (Qiagen, Venlo, the Netherland) and sequenced.

Selection of genotypes

Wädenswil

An orchard including 1195 apple genotypes, located at the Agroscope of Wädenswil (Switzerland), was yearly evaluated for four years, after being left completely untreated for pest and diseases. PM symptoms were scored every spring using a scale from 1 to 9 (1: complete absence of symptoms; 9: tree completely affected). Based on this scoring, genotypes were grouped in four categories: very resistant (average score: 1 - 3), resistant (average score: 3.01 - 5), susceptible (average score: 5.01 - 7) and very susceptible (average score: > 7.01). For each of these categories, the genotypes with the lowest standard deviation between replicates and years were selected. DNA was extracted and used for HRM analysis.

Fondazione Edmund Mach

Two groups of genotypes were collected from the orchard of FEM (Trento province, Italy): genotypes of the first group were collected based on the classification of Swensen (2006), which divided the genotypes in four categories: very resistant, resistant, susceptible and very susceptible. The second group was collected to increase the total number of genotypes considered. They were categorized as resistant or susceptible by the breeders of FEM. For some of the genotypes of this

second group collected in FEM, no information about the phenotype were available. Therefore, when possible, they were retrieved from the website http://www.orangepippin.com/apples.

Laimburg

Three orchards located at Laimburg research center (Bolzano province, Italy) were evaluated every year for two years after being left completely untreated for pest and diseases. For each scoring, a value of one was assigned to individuals with no or almost no PM symptoms, whereas a value of two was assigned to individuals with clear symptoms visible. The average score of twelve individuals in three locations in two years was used for further analysis. Based on scoring, genotypes were grouped in four categories: very resistant (average score: 1 - 1.25), resistant (average score: 1.26 – 1.5), susceptible (average score: 1.51 - 1.75) and very susceptible (average score: 1.76 – 2).

Primer design for high resolution melting

The high resolution melting (HRM) variant proposed by Kristensen *et al.* (2011), named competitive amplification of differentially melting amplicons (CADMA), was adopted. CADMA-HRM is a three primers amplification system, based on the competition between two forward primers. The competition increases the specificity of the amplification (Kristensen *et al.,* 2011). Two competitive forward primers were designed for one of the SNPs identified in *MdMLO19*, insertion T-1201 (Table 1). The first forward primer, Ins.MS-Fw (Insertion-Mutation Specific) annealed on the sequences containing the insertion, whereas Ins.Ov-Fw (Insertion–Overlapping) annealed on both the normal and the mutated sequences (Fig. 1). The sequences containing insertion T-1201 were amplified by primer Ins.MS-Fw (87 bp amplicon), whereas the sequences without the insertion were amplified by primer Ins.Ov-Fw (89 bp amplicon). According to both FruitBreedomics and Sanger re-

sequencing, insertion T-1201 was always linked to three other SNPs, located nearby the insertion. Two of these SNPs were inside the target sequence for primer Ins.MS-Fw (Fig. 1). The presence in the final amplicon of three mutations, in addition to the target insertion, would have made the HRM results extremely difficult to understand. Thus, primer Ins.MS-Fw was designed with two mismatches aimed at removing two of the SNPs from the amplicon of the mutated sequence (Fig. 1).

Table 1. CADMA-HRM primers	
Ins.MS-Fw	GTCAGGAAAATGTGGCTTACATTTTTACC
Ins.Ov-Fw	CGGTCAGGAAAAGGTGGCTTACATT
Ins-Rv	CGACATCTTCCAACTTCTCATGG

Figure 1. Annealing sites of forward primers Ins.MS-Fw and Ins.Ov-Fw, used for CADMA-HRM. WT corresponds to the sequence of 'Golden Delicious' *MdMLO19,* retrieved from https://www.rosaceae.org/gb/gbrowse/malus_x_domestica_v1.0-primary), whereas the Mut sequence (Mutated) was obtained from the FruitBreedomics re-sequencing dataset. The arrows indicated the two mismatches inserted in primer Ins.MS-Fw, aimed at removing the two SNPs T-1188-C and G-1181-A.

High resolution melting

PCR cycling and HRM analysis were performed with Precision Melt Supermix (Bio-Rad, Hercules, USA), using a CFX96 Touch Real-Time PCR detection system (Bio-Rad, Hercules, USA), run by CFX Manager software. The final reaction mixtures consisted

of 50 ng of DNA, 0.4 µL of Ins.MS-Fw primer, 0.2 µL of Ins.Ov-Fw primer, 0.4 µL of Ins-Rv, 5 µL of Precision Melt Supermix and water to a final volume of 10 µL. Final primers concentration was 200 nM for Ins.Ov-Fw and 400 nM for Ins.MS-Fw and Ins-Rv.

Samples were run with the following thermal cycling parameters: 98°C 2 min, 45 cycles of 98°C 5 sec and 58°C 10 sec, final step at 95°C 1 min. The Melting curve was determined immediately after PCR amplification with a step at 70°C 1 min, followed by progressive increment of temperature from 70°C to 95°C (0.2°C each step, 10 sec). All runs were repeated twice and when results were in disagreement, a third run was carried out. The amplification data were analyzed using the Bio-Rad HRM software.

Statistical analyses

Canonical correspondence analysis

Canonical correspondence analysis (CCA) used PAST software v. 2.17c (Hammer *et al.*, 2001), to determine the relative importance of phenotypical factors in the spatial organization of genetic diversity among genotypes. This analysis, designed to relate species composition to different predictive variables (Ter Braak, 1986), has been successfully used to describe relationships between environmental or phenotypical variables and genetic composition (Angers *et al.,* 1999; Girard and Angers, 2006; Dell'Acqua *et al.*, 2014). The analysis was based on a phenotypical variables/genetic data matrix, where susceptibility and resistance were used as phenotypical factors. The arrows emerging from the origin of the two axes represent the phenotypes and their position indicates the correlation with the genetic composition: the closer the arrow is to the dot indicating the genetic composition, the stronger is the correlation.

Disease severity

The level of PM resistance/susceptibility was known for 144 of the 159 genotypes included in this study. Based on the results of the HRM analysis, they were divided in four categories regarding the insertion in *MdMLO19*: homozygous insertion, heterozygous insertion, no insertion and other. The To investigate the differences in the level of disease severity scores between the groups that differed regarding the insertion, the non-parametric test of Kruskall-Wallis was used as the residues were not normally distributed.

RESULTS

Presence of SNPs in the target genes

The screening of the re-sequencing data returned 678 SNPs in five *MLO* genes, 127 located in exons (Table 2). The cumulative length of the five genes was 23063 bp, corresponding to one SNP every 34 bp (Table 2). Exons accounted for 36.6% (8436 bp) of the five genes and for 18.7% of all SNPs, with one SNP every 66 bp. Introns contained one SNP every 27 bp (Table 2). The *MLO* gene with the highest number of SNPs located in exons was *MdMLO19* with 48 SNPs. The gene with the lowest number of SNPs was *MdMLO5* with six SNPs. *MdMLO5* was also the gene with the lowest number of total SNPs (Table 2).

Sixty-one out of 127 SNPs caused silent mutations, and another 30 and 9 caused conservative and semi-conservative mutations, respectively. Twenty-two mutations were non-conservative (Table 3) and two insertions and two deletions were found. One insertion was located at the very end of *MdMLO7,* in position 1676-1680, causing a frameshift that changed the last three amino acids of the protein. The other insertion, T-1201, was located in *MdMLO19* and caused a frameshift with the formation of an early stop codon (Table 3). The resulting protein would be 405 amino acids long, instead of 590, and would lack a trans-membrane domain and the calmodulin binding domain at the C-terminal (Fig. 2).

Table 2. SNPs in the FruitBreedomics dataset

	Lenght (bp)	Exons (bp) (%)	No. Of SNPs	SNPs in exons No. (%)	Bp/SNP*	Bp exons/SNP°	Bp introns/SNP#
MdMLO5	4196	1740 (41.5)	36	6 (16.7)	117	290	82
MdMLO7	4296	1686 (39.2)	80	24 (30.0)	54	70	47
MdMLO11	5643	1728 (30.6)	194	23 (11.9)	29	75	23
MdMLO18	4673	1509 (32.3)	192	26 (13.5)	24	58	19
MdMLO19	4255	1773 (41.7)	176	48 (27.3)	24	37	19
Total	23063	8436 (36.6)	678	127 (18.7)	34	66	27

* Average distance in bp between two SNPs.

° Average distance in bp between two SNPs in exons.

Average distance in bp between two SNPs in introns.

Table 3: Mutations due to SNPs present in the FruitBreedomics dataset

	No. SNPs (exons)	Silent	Conservative	Semi-conservative	Non-conservative	Nonsense	Insertions	Deletions
MdMLO5	6	0	4	0	2	0	0	0
MdMLO7	24	10	9	2	2	0	1	0
MdMLO11	23	9	5	3	6	0	0	0
MdMLO18	26	12	6	1	7	0	0	0
MdMLO19	48	30	6	3	5	1	1	2
Total	127	61	30	9	22	1	2	2

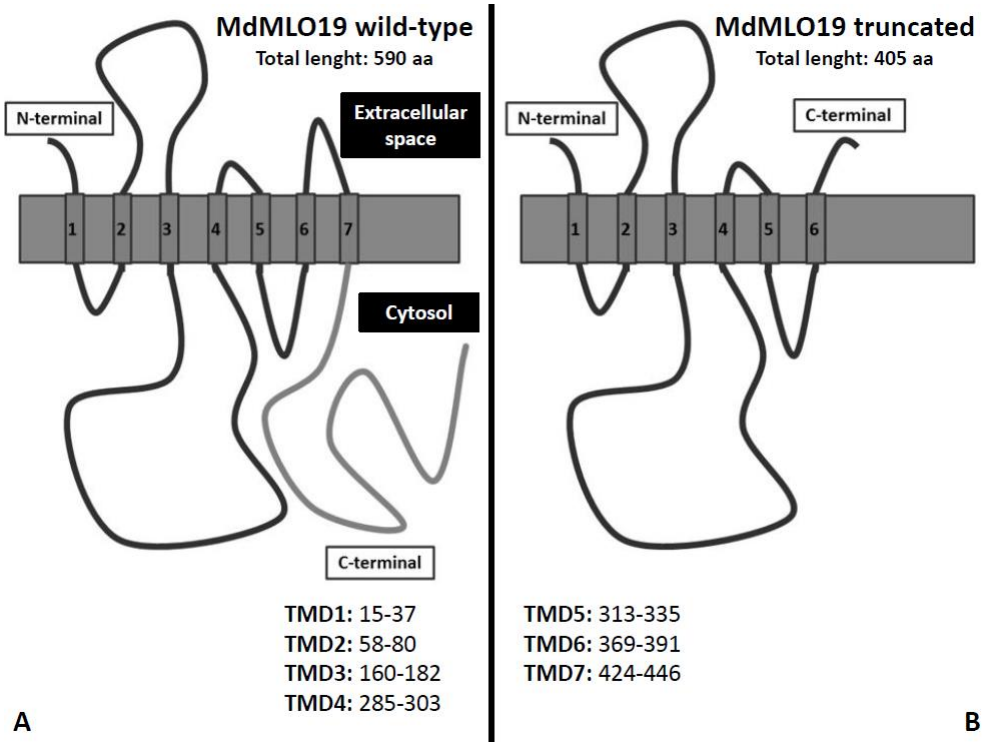

Figure 2. Structures of wild-type (A) and truncated (B) MdMLO19 proteins. The trans-membrane domains (TMD) are indicated in yellow. The wild-type MdMLO19 contains at the C-terminal a calmodulin-binding domain.

According to FruitBreedomics data, this insertion was present in 12 of the 63 FruitBreedomics genotypes. In six of them it was homozygous ('Busiard', 'Patte de Loup', 'McIntosh', 'Pepino Jaune', 'Young America and 'Kronprins'), in the other six heterozygous ('Mela Rozza', 'Priscilla', 'Abbondanza', 'Jonathan', Alfred Jolibois' and 'Filippa'). Insertion T-1201 was always linked to three SNPs, G-1181-A, T-1188-C and C-1205-T. The two deletions were both found in *MdMLO19*. One was a three-bp deletion in position 1545-1547, resulting in the removal of a proline, whereas the deletion in position 1181 caused the formation of an early stop codon. However, deletion G-1181 was present only in 'Pepino Jaune', where insertion T-

1201 was also present. The combination of deletion G-1181 and insertion T-1201 caused the substitution of five amino acids (two semi-conservative substitutions and three non-conservative), but no early stop codon. The nonsense mutation G-1176-A was found in *MdMLO19* and caused the substitution of a tryptophan with an early stop codon. This SNP was found in the genotype 'Ajmi'.

According to the data summarized above, insertion T-1201 and nonsense mutation G-1176-A, both located in *MdMLO19,* were selected for further analysis

Validation of SNPs

Sanger re-sequencing of a *MdMLO19* fragment in cultivars Busiard, Pepino Jaune, McIntosh, Patte de Loup, Mela Rozza, Alfred Jolibois and Golden Delicious, revealed that the first five of them contained the insertion T-1201, whereas 'Busiard' did not (Fig. 3). One of the two sequencing of 'Mela Rozza' did not have the insertion, and the same was noted for 'Alfred Jolibois'. The electropherograms of these two genotypes showed an overlapping in position 1201 of the peaks for A and T, suggesting that the insertion was heterozygous, as expected from FruitBreedomics data (Fig. S1).

Sequencing confirmed that SNPs G-1181-A, T-1188-C and C-1205-T were always present with insertion T-1201 but not in its absence (Fig. 3). The SNP in position 1181 was different in 'Pepino Jaune', as it was a deletion instead of a substitution, confirming the data from FruitBreedomics (Fig. 3).

The sequencing of 'Ajmi', did not confirm the presence of the mutation in the genotype, therefore no further analysis were carried out.

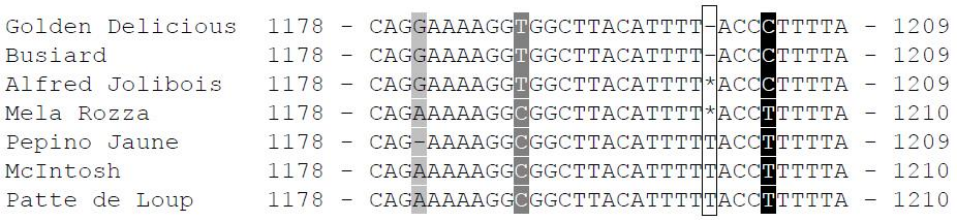

Figure 3. Sequences of a fragment of *MdMLO19* obtained by Sanger in seven apple genotypes. Colored columns correspond to SNPs present in the FruitBreedomics dataset and confirmed by re-sequencing.

CADMA-HRM screenings and validation

A total of 159 apple genotypes were screened with CADMA-HRM for the presence of insertion T-1201 (Table S1). Twenty-seven genotypes were sampled in the orchard of Wädenswil (Switzerland): twelve were PM resistant, six susceptible and nine intermediate (Table S1 - Wädenswil). Fifty genotypes were from the collection present at FEM (Trento province, Italy), selected because their level of PM resistance/susceptibility was known (Swensen, 2006) (Table S1 - FEM). An additional group of 52 genotypes was collected in FEM: three were genotypes included in the FruitBreedomics dataset ('Patte de Loup', 'Fuji' and 'Young America'), 13 were accessions of wild *Malus* species and the other 37 were either commonly used in breeding, commercially relevant or selected because their level of PM resistance/susceptibility was known (Table S1 – FEM2). Twenty-seven genotypes were collected from the orchards of Laimburg Research Center (Bolzano province, Italy) (Table S1 - Laimburg). Twelve of the genotypes considered for HRM were included in the FruitBreedomics dataset: 'McIntosh', 'Mela Rozza', 'Patte de Loup', 'Jonathan', 'Braeburn', 'Durello di Forlì', 'Renetta Grigia di Torriana', 'Young America', 'Antonovka', 'Delicious', 'Fuji' and 'Golden Delicious'.

CADMA-HRM discriminated the presence or absence of insertion T-1201 (Fig. 4). The melting profile of 'McIntosh' was the same as 'Young America' and 'Patte de

Loup' (Fig. 4 in red), but different from the profile of 'Golden Delicious' (Fig. 4 in green). The profile of 'Mela Rozza' was different, supporting the heterozygosity of the insertion in this genotype (Fig. 4 in blue). 'Golden Delicious', 'McIntosh' and 'Mela Rozza' were selected as reference genotypes for further analyses.

The HRM screening of 159 genotypes, including the three references, indicated that the insertion was present in 110 of them, homozygous in 60 and heterozygous in 50 (Fig. S2B, D, E, F). A further analysis on two homozygous genotypes ('Pomme Douce' and 45223, both from Wädenswil), showed that the insertion was not present in 'Pomme Douce' and that 45223 was heterozygous (Figure S3C). Thus, the genotypes containing the insertion were 109, 58 homozygous and 51 heterozygous.

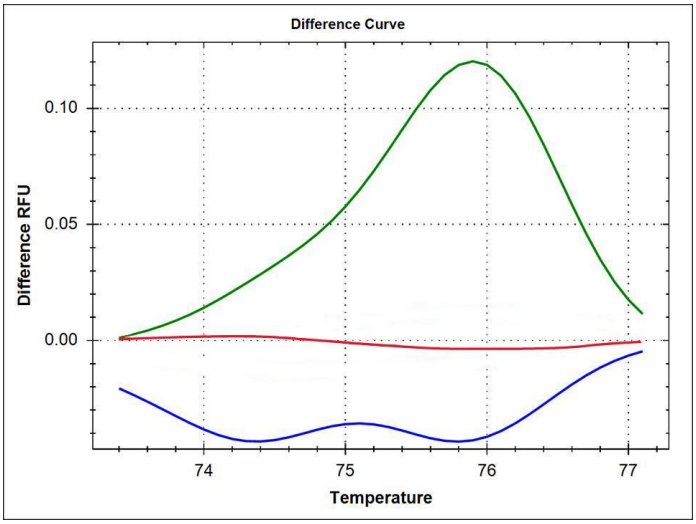

Figure 4. CADMA-HRM melting profiles of the three apple genotypes used as reference for further analysis. The red curve indicates homozygous insertion ('McIntosh'), blue heterozygous ('Mela Rozza') and green absence of insertion ('Golden Delicious').

To validate the HRM results, 18 genotypes were re-sequenced. Seven of them were PM susceptible ('Idared', 'Crimson Crisp', 'Cortland', 'Cox's Orange Pippin', 'Renetta Grigia di Torriana', 'Jonamac' and 'Calville Blanc'), five were resistant ('Jackii', 'Fuji',

‘Delicious’, ‘Durello di Forlì’ and ‘Florina’) and for the other six (‘Clivia’, ‘Baujade’, ‘Sonya’, ‘Sieboldii MA4’, ‘Reinette Sik’ and ‘Antonovka’) the phenotype was not known. Fifteen of the genotypes were homozygous and three heterozygous (‘Antonovka’, ‘Durello di Forlì’ and ‘Renetta Grigia di Torriana’). The sequencing confirmed that 10 of the 15 genotypes homozygous for the insertion were so, whereas the other five (‘Sonya’, ‘Clivia’, ‘Delicious’, ‘Baujade’ and ‘Calville Blanc’) were heterozygous (Fig. 6 and S3). ‘Durello di Forlì’ and ‘Renetta Grigia di Torriana’ were confirmed to be heterozygous, whereas ‘Antonovka’ did not carry the insertion. Among the six PM susceptible genotypes supposedly homozygous for the insertion, four were actually carrying it in homozygosity, namely ‘Cortland’, ‘Jonamac’, ‘Cox’s Orange Pippin’ and ‘Idared, whereas “Crimson Crisp’ and ‘Calville Blanc’ were heterozygous (Fig. 6 and S3). This second re-sequencing showed that insertion T-1201 is almost always linked to the three SNPs G-1181-A, T-1188-C and C-1205-T. The only exception was the *Malus baccata* genotype ‘Jackii’ that, among the three SNPs linked to insertion T-1201, carried only C-1205-T (Fig. 6). None of the re-sequenced genotypes carried deletion G-1181, the mutation that in ‘Pepino Jaune’ resulted in the regain of the reading frame.

As further validation, a fragment of *MdMLO19* from the heterozygous genotype ‘Durello di Forlì’ was cloned in a plasmid and sequenced. Of the eight *E. coli* colonies sequenced, four carried insertion T-1201 and the three SNPs linked to the insertion, whereas the other four colonies did not carry the insertion (Fig. S4), confirming the heterozygosity of ‘Durello di Forlì’ and the ability of the primers designed for HRM to discriminate between homozygosity and heterozygosity.

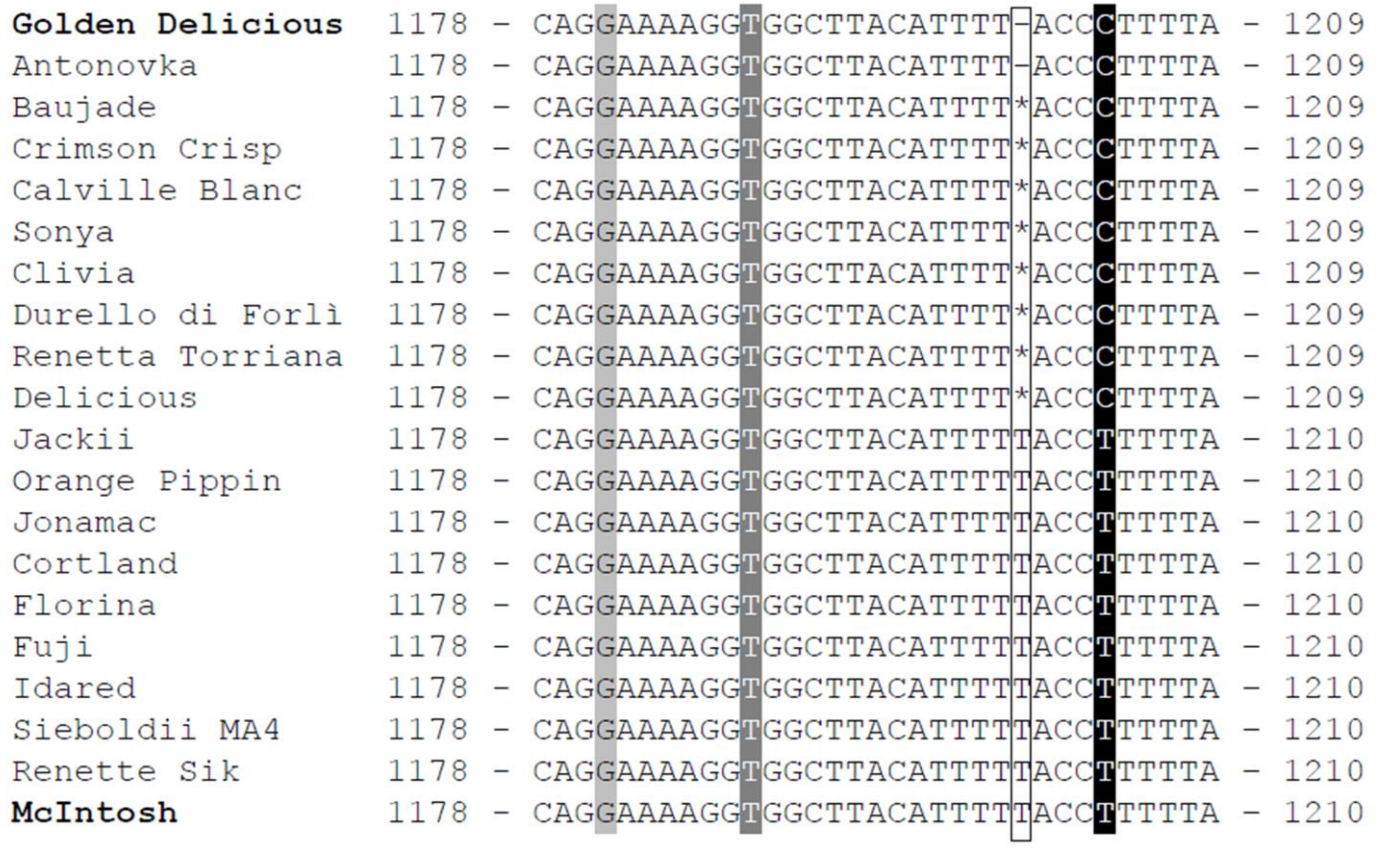

Figure 5. Sequences of a fragment of *MdMLO19* obtained by Sanger in 18 apple genotypes and two references ('Golden Delicious' and 'McIntosh'). Colored columns correspond to SNPs present in the FruitBreedomics data and confirmed by re-sequencing. The asterisks indicate the genotypes heterozygous for insertion T-1201.

The final count was 108 genotypes carrying the insertion, 53 homozygous and 55 heterozygous. The insertion was absent in 46 genotypes (Fig. S2B, D, E, F and 5), whereas five others had unique melting profiles (Fig. S2D in orange). All the genotypes tested by HRM or included in FruitBreedomics were divided in three categories according to the presence or absence of the insertion (Fig. 5). Of the 12 FruitBreedomics genotypes tested by HRM, four were homozygous for the insertion, five heterozygous and three did not carry it (Fig. 5). The CCA analysis, aimed at linking the presence or absence of insertion T-1201 to resistance or susceptibility to PM, showed a correlation between the presence of the homozygous insertion and resistance to PM, as well as between susceptibility and the heterozygosity or absence of the insertion (Fig. 7). The Kruskall-Wallis test was carried out on the 144 genotypes for which phenotypic data were available and it showed that the disease severity was significantly lower for genotypes containing the homozygous insertion (Fig. 8).

DISCUSSION

The screening of the FruitBreedomics re-sequencing dataset returned 678 SNPs in five *MLO* genes. Not surprisingly, SNP distribution was not balanced between introns and exons: the fewer SNPs numbers in exons can be explained by positive selection against non-advantageous mutations, whereas mutations in the introns are to a large extent neutral and subjected to random fixation (Kimura, 1977). The same holds for the predominance of silent and conservative mutations in exons over non-conservative and nonsense ones. None of the 127 SNPs found in exons affected the 30 amino acids identified by Elliott *et al.* (2005) as fundamental for the S-genes activity of *MLOs.* The case of *MdMLO5* deserves a comment: only six SNPs were detected in exons, suggesting that the gene is under and intense stabilizing selection. However, since *MdMLO5* is not targeted by *P. leucotricha* (Chapter 2 and 3), selection due to PM should not favor the fixation of new mutations. The opposite situation was observed

No insertion

Hilleri	82600
Begolden	101995
Baumanns Renette	Süsser Apfel
Hansen's Baccata	Goldrush
45362	Kardinal
Freedom	Braeburn*
Greensleves	Topaz
Yellow Transparent	Reanda
Liberty	Q71
Gärteler	Vrenech
Kaiser Alexander	Citronenapfel
Golden Delicious *#	Geneva
65404	GMAL1461
75630	KAZ95 05 01
99TU 08 02 1 4 2 3	KAZ95 18 10 2 12 3 3
Antonovka Kamenichka	KAZ95 18 10 2 13 1 3
CH97 05 06	Elstar
Crimson Gold	Ambrosia
Gala	Gäsdonker Reinette
CH97 06 07 China	Landsberger Renette
Roter Jonathan	Pomme Douce
45365	
Rosmarina	(Busiard)*#
Renoir	Antonovka*#

Heterozygous insertion

Sentinel	Douce
Wellington	105628
9-AR2T196	Pomme de Normandie
A723_6	1006322
TSR33T239	82643
Winterbananeapfel	Zimtapfel
Fuji Red Spot	TSR34T15
Harberts Reinette	TSR33T239
Reinette du Canada	105480
45228	45223
B45	Prinz Albert
GMAL 2473	Delicious*#
Warbler	Durello di Forlì*#
Rubinette	
Gravenstein	CH97 05 11 Chiana
J34	Grenoble Reinette
Minister Hammerstein	Renetta Grigia INFEL
Brügler	Honeycrisp
81144	Pink Lady
Granny Smith	Dulmener Rosen
99TU 08 02 GMAL	Crimson Crisp#
99TU 08 02 Turkey	Renetta Grigia di Torriana*#
Jonathan*	Calville Blanc#
Rosmarina Rossa	Ledermannsreinette
Osnarbrucker Reinette	Reinette brune
Mela Rozza*#	Renewa
Malus silvestrii	Stark Earliest
(Alfred Jolibois)*#	Clivia#
(Priscilla)*	Baujade#
(Abbondanza)*	Sonya#
(Filippa)*	

Homozygous insertion

McIntosh*#	Jackii
Dolgo	Winter Winesap
Floribunda	Winesap Spur
Wijck McIntosh	Turley Winesap
Marshall McIntosh	Stayman Winesap
Early Red McIntosh	Ruby
Spartan	Remo C
Okanoma	Starkrimson
Harrold Red Delicious	
Britegold	Pinova
Murray	Reinette Blanche
Viking	Reinette Champagne
Prima	Reinetta Grise
Patte de Loup*#	Royal Gala
Enterprise	Rus 98 04 03
Ingrid Marie	Toringo
James Grieve	Telamon
Dayton	Fiesta
Rubinola	Redfree
Nova Easygro	Empire
Evereste	Florina
Geneva Early	Niagara
Beacon	Fuji*#
Jonamac#	Cortland#
Idared#	Cox's Orange Pippin#
GMAL2948	Young America*#
Sieboldii MA4#	Renette Sik#
(Kronprins)*	

- Very resistant
- Resistant
- Susceptible
- Very susceptible
- Unknown

* Fruitbreedomics genotypes
\# sequenced by sanger
Bold: reference for HRM

() not included in HRM
Red: discrepancies between analyses

Figure 6. List of apple genotypes characterized by the presence or absence of insertion T-1201. The color code indicates the resistance/susceptibility of the genotypes. The five genotypes with peculiar HRM profiles were not included because it was not possible to assign them to one of the three genotypic states considered. The 19 genotypes from FruitBreedomics are indicated with an asterisk. Six of them are in brackets because they were not tested by HRM. The three genotypes in bold were used as reference for the HRM. Genotypes indicated by the hash (#) were re-sequenced by Sanger. The genotypes in red are those for which discrepancies between analyses were observed: between HRM and FruitBreedomics data ('Antonovka', 'Renetta Grigia di Torriana', 'Durello di Forlì', 'Fuji' and 'Delicious'), between re-sequencing and FruitBreedomics data ('Busiard') and between HRM and re-sequencing ('Crimson Crisp', 'Calville Blanc', 'Sonya', 'Baujade' and 'Clivia').

for *MdMLO19*, the gene with the highest number of SNPs and the only one where nonsense mutations were found; this indicates that selection favored the fixation of mutations. Three factors may contribute: first, *MdMLO19* is the primary target of *P. leucotricha,* suggesting that the observed high mutation rate maybe the result of the co-evolution of host and pathogen; the second is that in apple *MdMLO19* cause susceptibility to PM (Chapter 3), a situation where disruptive mutations result in resistance to the pathogen. The third factor is that *MdMLO19* paralog, *MdMLO11* (Pessina *et al.,* 2014), because of its metabolic redundant activity, supports a loss-of-function of *MdMLO19* without drastically reducing the fitness of the plant.

Two SNPs of *MdMLO19* were selected for detailed investigation. The insertion of a thymine in position 1201 caused a frameshift mutation resulting in an early stop codon located 15-17 bp downstream of the insertion. As a result, the insertion causes the translation of a 405 aa protein instead of the 590 aa of the regular protein (Fig. 2). The loss of 185 aa alone would probably compromise the function of MdMLO19; moreover, the C-terminal MLO region carries a calmodulin-binding domain which absence reduces by 50% the capacity of MLO to negatively regulate defense against PM (Kim *et*

al., 2002). It is reasonable to assume that the truncated MdMLO19 is a non-functional or partially functional protein. Considering that the knock-down of *MdMLO19* results in PM resistance (Chapter 3), the homozygosity of insertion T-1201 should also support PM resistance. The second interesting SNP found in *MdMLO19* was a nucleotide substitution ending in a nonsense mutation, specifically G-1176-A,

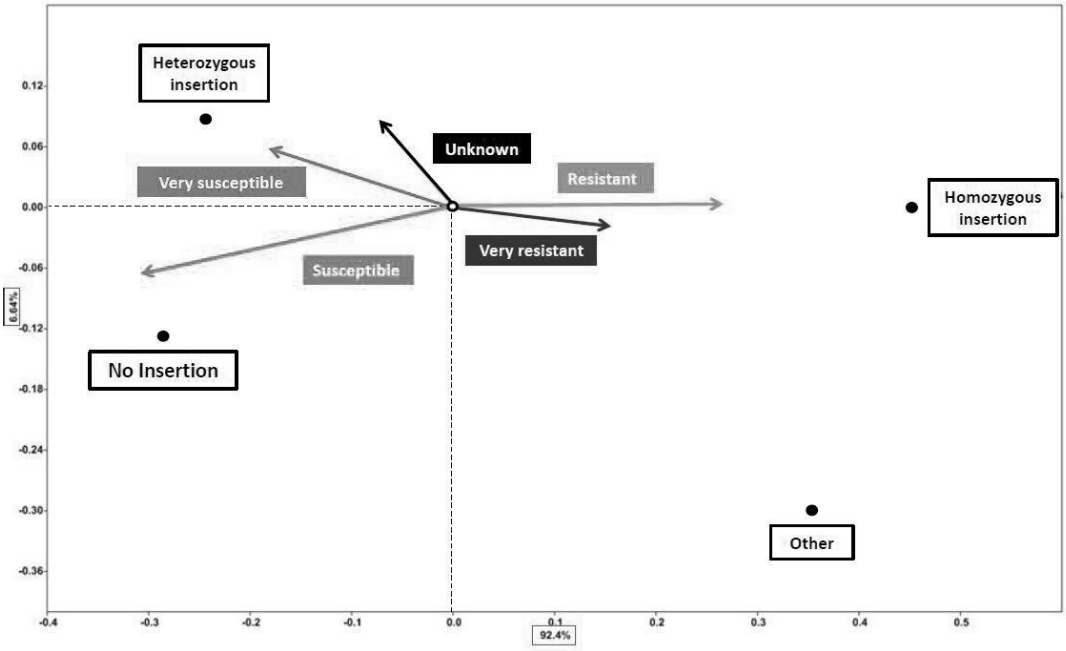

Figure 7. Canonical correspondence analysis (CCA) ordination biplot representing genotypes aggregation and phenotypical variables. The four genetic compositions in exams are: no insertion, heterozygous insertion, homozygous insertion and other (colored boxes). The six phenotypes considered are: very resistant, resistant, moderately susceptible, susceptible, very susceptible and unknown (solid arrows). The horizontal and vertical axes explained 92.4% and 6.64% of the variation, respectively.

present in only one of the 63 FruitBreedomics genotypes. The early stop codon would result in a 391 amino acids protein, for which the same argument brought for insertion T-1201 is valid. However, sequencing showed that mutation G-1176-A was an artifact and no further investigations were carried out.

The main purpose of our study was the analysis of the frequency of mutations in *MLO* genes when a representative sample of apple germplasm is considered. In this respect, however, FruitBreedomics data needed to be validated before further analyses. Thus, insertion T-1201 had to be confirmed by sequencing. Three more SNPs (G-1181-A, T-1188-C and C-1205-T) were found to be always associated to

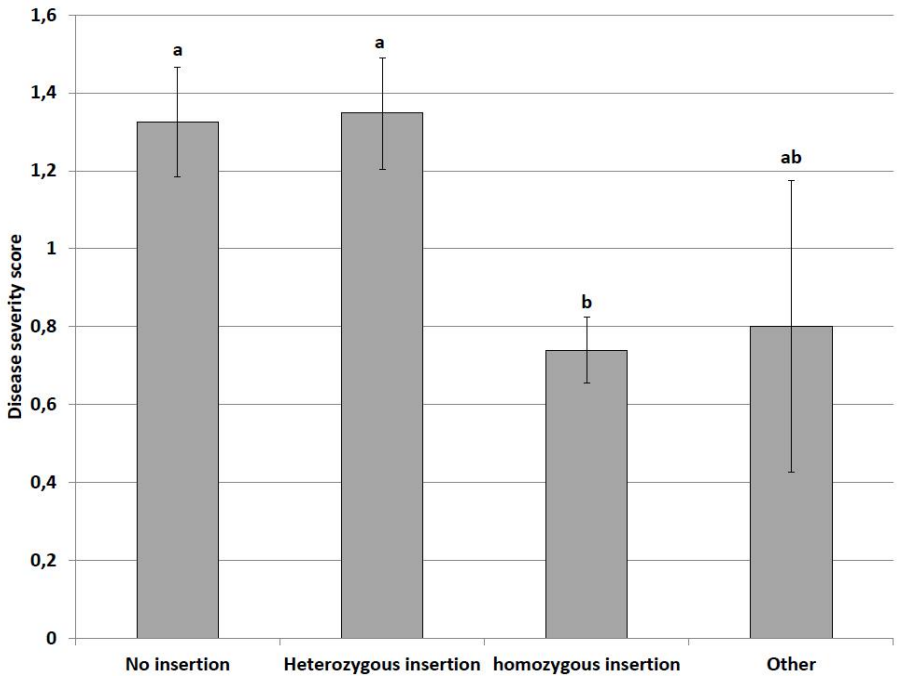

Figure 8: Average disease severity score for the four genetic groups. Error bars show the standard error of the mean. The letter code indicates the statistically significant differences, according to Kruskall-Wallis test (P=0.05).

insertion T-1201, suggesting that the insertion is carried only by a specific haplotype. Considering that the FruitBreedomics dataset includes the genome sequences of the 14 genotypes from which the large majority of European apple varieties originated (Evans *et al.,* 2010; Bianco *et al.,* 2014), it was interesting that three of them contained insertion T-1201, namely 'McIntosh', 'Priscilla' and 'Jonathan'. It is reasonable to think that the allele present in the lineages of these genotypes subsequently spread through their extensive use in breeding.

The HRM analysis was carried out on 159 genotypes, of which 'Golden Delicious', 'McIntosh' and 'Mela Rozza' were used as references. 'Golden Delicious' does not carry insertion T-1201, whereas 'McIntosh' is homozygous and 'Mela Rozza' heterozygous. The insertion was present in 108 genotypes, heterozygous in 55 and homozygous in 53. Five genotypes returned a unique melting profile. Among them, the case of 'Arkansas' was explained by its triploidy. Forty-five of the genotypes carrying the homozygous insertion were resistant or very resistant to PM, whereas 'Cortland', 'Jonamac', 'Idared' and 'Cox's Orange Pippin' were susceptible. For four other genotypes no phenotypic informations were available. To explain why four genotypes carrying an homozygous loss-of-function mutation of a PM S-gene were susceptible to the disease, we hypothesized that they could carry other mutations that prevented the formation of the early stop codon. However, the re-sequencing did not reveal anything that could have caused a regain of the reading frame. It cannot be excluded that other mutations in different parts of the sequence could do that, but it does not seem likely. The only mutation found in the FruitBreedomics data that could cause a regain of the reading frame was deletion G-1181 in 'Pepino Jaune', and the re-sequencing showed that it was not present in any of the considered genotypes. Further investigations are necessary to elucidate this point. However, it should be noted that, with the exception of 'Idared', the information about the phenotypes of the susceptible homozygous genotypes come from

empirical observations and not from properly designed experiments, therefore they should be validated. Furthermore, the DNA samples were not always collected from the same plants for which the observation of the phenotype was carried out, thus the possibility of mislabeling, a problem seen quite often in experimental orchards, cannot be excluded. Despite of this, the data point at the existence of a link between the presence of the homozygous insertion and PM resistance. This was supported by two statistical tests: CCA analysis, which showed a link between the homozygosity of T-1201 and PM resistance, and Kruskall-Wallis, which indicated that genotypes carrying the homozygous insertion have a significantly lower disease severity score. The high frequency of insertion T-1201 in the apple germplasm could help to explain those cases of PM resistance in genotypes known not to carry any R-gene active against PM. However, if deletion G-1181 is present together with insertion T-1201, as in 'Pepino Jaune', the early stop codon is not formed. This is why genotypes carrying insertion T-1201 should be tested also for deletion G-1181, before classifying them as carriers of a non-functional *MLO* allele.

Discrepancies between the HRM results and the FruitBreedomics data have been noted. According to HRM, 'Fuji' and 'Delicious' should be homozygous for insertion T-1201 and 'Antonovka', 'Durello di Forlì' and 'Renetta Grigia di Torriana' heterozygous. On the contrary, according to the FruitBreedomics data, 'Delicious', 'Fuji', 'Durello di Forlì', 'Renetta Grigia di Torriana' and 'Antonovka' should not carry the insertion. The re-sequencing of these genotypes confirmed that 'Fuji' was homozygous for the insertion and 'Durello di Forlì' and 'Renetta Grigia di Torriana' were heterozygous. Conversely, 'Antonovka' did not carry the insertion and 'Delicious' was heterozygous. Three out of five sequencing confirmed the HRM ('Fuji', 'Durello di Forlì' and 'Renetta Grigia di Torriana'), one confirmed the FruitBreedomics data ('Antonovka) and for 'Delicious', the three analysis gave three different results. In general, the HRM looks more reliable than the FruitBreedomics data, but sequencing is the best option to be

absolutely sure about the presence/absence of a mutation. This was also supported by the putative nonsense mutation G-1176-A, found in the FruitBreedomics dataset but not confirmed by sequencing. Further discrepancies were noted between HRM and re-sequencing: 'Sonya', 'Clivia', 'Crimson Crisp', 'Baujade' and 'Calville Blanc', according to HRM, should have been homozygous for the insertion, whereas the sequencing showed that they were heterozygous. Discrepancies like these open the discussion on the degree of reliability of CADMA-HRM, or, more correctly, on the reliability of the primer pair in use, which could not always be able to properly discriminate between heterozygosity and homozygosity. The adoption of HRM, nonetheless, is cost-effective when planning to screen a large number of genotypes to subsequently choose a smaller sample for further investigations. Furthermore, even accepting the possibility of CADMA-HRM errors, the key finding of our investigation remains valid: insertion T-1201 is common among PM-resistant apple genotypes.

In some cases, the knock-out of *MLO* genes is associated to pleiotropic phenotypes that limit the use of non-functional *MLO* alleles, such as reduced grain yield and early senescence-like leaf chlorosis in barley (Jørgensen, 1992), stunted growth and increased susceptibility to necrotroph pathogens in Arabidopsis (Consonni *et al.,* 2006), and reduced plant size in pepper (Zheng *et al.,* 2013). On the contrary, fitness costs associated to the knock-out of tomato *SlMLO1* have not been reported (Bai *et al.,* 2008). The absence of fitness costs seems also the case of apple, where the knock-down of *MdMLO19* did not generate any evident phenotypic modification (Chapter 3). On this basis, it is credible that the advantage in terms of disease resistance of the *MdMLO19* loss-of-function, might have favored its spreading in apple germplasm, thus explaining the high, and unexpected, frequency of the insertion T-1201.

To date, loss-of-function of *MdMLO19* is the third finding of a natural loss-of-function in an *MLO* gene, after those of barley *mlo-11* (Piffanelli *et al.,* 2004) and tomato *ol-2* (Bai *et al.,* 2008). Barley has been extensively studied for PM resistance, with around

4100 accessions tested, and the frequency of spontaneous *mlo* mutations was found to vary between 0.2 and 0.6% (Jørgensen, 1992). The scenario in apple has two noteworthy differences, compared to barley: the first concerns the frequency of the insertion T-1201, which is high in apple germplasm. The second instance relates to the presence of insertion T-1201 in apple genotypes commonly used in breeding, whereas spontaneous *HvMLO* mutants of barley had to be searched among landraces (Jørgensen, 1992). An estimate of the frequency of insertion T-1201 can be based on FruitBreedomics data and re-sequencing results. If only genotypes homozygous for the insertion are considered, six FruitBreedomics genotypes have this genetic state. Out of these, only 'McIntosh', 'Patte de Loup', 'Young America' and 'Kronprins' should be considered because 'Pepino Jaune' does not carry an early stop codon, whereas in 'Busiard' the presence of T-1201 insertion was not confirmed by Sanger sequencing. To these four genotypes, 'Fuji' should be added, as the sequencing revealed the presence of the homozygous insertion. Five genotypes out of 63 correspond to a frequency of 7.9%, a value between 13 and 40 times higher than in barley. If also heterozygosity is considered, the total number of genotypes carrying the insertion is 14, meaning a frequency of 22.2%, a value between 37 and 111 times higher than in barley. The genotypes included in FruitBreedomics have been selected to represent as best as possible the diversity of apple germplasm (Dr. R. Velasco FEM, personal communication), irrespective of their PM-resistance. This supports the inference that the genotypic frequencies here reported are a good estimate of the frequency of the insertion T-1201 in apple germplasm.

CONCLUSIONS

MdMLO19 is an S-gene of apple which knock-down results in reduction of susceptibility to PM. The screening of the FruitBreedomics re-sequencing dataset revealed the presence of insertion T-1201 in *MdMLO19* that caused the formation of an early stop

codon, most likely resulting in a non-functional MLO protein. The HRM analysis showed that the insertion is present in a high number of genotypes and homozygous in 54 of them. All these genotypes are resistant to PM, with the exception of 'Cortland', 'Cox's Orange Pippin', 'Idared' and 'Jonamac'. Assuming that the insertion results in a non-functional *MdMLO19* allele, the estimate of its frequency appeared much higher than for natural *mlo* mutants of barley.

Alleles of *MdMLO19* carrying insertion T-1201 may represent a valuable source of durable PM-resistance in apple. The diffusion in apple germplasm of resistance due to insertion T-1201 is unmatched in other species and deserves further studies. Moreover, the screening of germplasms of other species might provide more informations on the important and yet poorly studied aspect of the frequency of spontaneous *mlo* mutants.

Our results showed how re-sequencing genotypes present in datasets like FruitBreedomics' one are powerful tools to study the natural diversity of the germplasm of a species and how they can lead to the discovery of valuable alleles to integrate in breeding programs. Furthermore, the screening of re-sequencing data could allow identifying candidates *MLO* S-genes: the presence of homozygous nonsense mutations in specific *MLO* genes of PM resistant genotypes would be an important indication that the gene might act as an S-gene. Finally, this approach could be extended to other diseases and other S-genes.

AKNOWLEDGMENTS

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AUTHOR'S CONTRIBUTION

SP analysed the SNPs found in the five target genes, carried out the statistical analysis on the PM scoring data collected in Wädenswil, designed the HRM primers, carried out

the CADMA-HRM analysis and wrote the major part of the manuscript. LP assisted in all the steps concerning HRM and the interpretation of data, carried out the CCA and contributed to the revision of the manuscript. LB screened the FruitBreedomics re-sequencing dataset, returned the list of SNPs and revised the manuscript. JG carried out the PM scoring on the genotypes from the orchard in Wädenswil and revised the manuscript. RGFV contributed to the experimental design and revised the manuscript. HJS contributed to the experimental design and revised the manuscript. FS contributed to the experimental design and revised the manuscript. RV contributed to the experimental design and revised the manuscript. MM contributed to the experimental design and was the main reviser of the manuscript.

SUPPLEMENTARY MATERIALS

Table S1: Genotypes collected in Wädenswil considered in CADMA-HRM screening

Genotype (ID)	Score – class*	Species	Insertion	Origin
Douce (1013960)	1.00 – VR	/	Heterozygous	Wädenswil
Unknown (105628)	1.00 – VR	/	Heterozygous	Wädenswil
Pomme de Normandie (1003912)	1.00 – VR	/	Heterozygous	Wädenswil
Unknown (1006322)	1.00 – VR	/	Heterozygous	Wädenswil
Unknown (45362)	1.00 – VR	/	Absent	Wädenswil
Kardinal (47512)	1.17 – VR	/	Absent	Wädenswil
Unknown (82600)	1.50 – VR	/	Absent	Wädenswil
Unknown (101995)	1.50 – VR	/	Absent	Wädenswil
Unknown (82643)	1.50 - VR	/	Heterozygous	Wädenswil
Zimtapfel (66407)	1.67 – VR	/	Heterozygous	Wädenswil
Süsser Apfel (72162)	2.17 – VR	/	Absent	Wädenswil
Vrenech (1000319)	2.67 – R	/	Absent	Wädenswil
Unknown (105480)	3.17 – R	/	Heterozygous	Wädenswil
Unknown (45223)	3.83 – R	/	Heterozygous	Wädenswil
Warbler (105812)	4.00 – R	/	Heterozygous	Wädenswil
Unknown (45228)	4.17 – R	/	Heterozygous	Wädenswil
Gärteler (1009329)	4.33 – R	/	Absent	Wädenswil
Citronenapfel (59119)	5.00 – R	/	Absent	Wädenswil
Brügler (64159)	5.33 – S	/	Heterozygous	Wädenswil
Unknown (81144)	5.33 – S	/	Heterozygous	Wädenswil
Unknown (75630)	5.67 – S	/	Absent	Wädenswil
Gäsdonker Reinette (55949)	6.50 – S	/	Absent	Wädenswil
Unknown (65404)	6.83 – S	/	Absent	Wädenswil
Reinette brune (1018173)	7.17 – VS	/	Heterozygous	Wädenswil
Ledermannsreinette (61342)	7.17 – VS	/	Heterozygous	Wädenswil
Pomme Douce (1019894)	7.17 – VS	/	Absent	Wädenswil
Unknown (45365)	7.83 – VS	/	Absent	Wädenswil
Dolgo	VR**	Hybrid	Homozygous	FEM
Floribunda	VR**	*Malus floribunda*	Homozygous	FEM
Britegold	R**	*Malus domestica*	Homozygous	FEM
Wijck McIntosh	VR**	*Malus domestica*	Homozygous	FEM
Marshall McIntosh	VR**	*Malus domestica*	Homozygous	FEM
Early Red McIntosh	VR**	*Malus domestica*	Homozygous	FEM
Murray	R**	*Malus domestica*	Homozygous	FEM
Viking	R**	Hybrid	Homozygous	FEM
Sentinel	VR**	Hybrid	Heterozygous	FEM
Arkansas	R**	*Malus domestica*	/	FEM

Hilleri	VR**	*Malus floribunda*	Absent	FEM
Prima	R**	Hybrid	Homozygous	FEM
Delicious	R**	*Malus domestica*	Heterozygous	FEM
Enterprise	R**	Hybrid	Homozygous	FEM
Freedom	R**	*Malus domestica*	Absent	FEM
Begolden	VR**	*Malus domestica*	Absent	FEM
Ingrid Marie	R**	*Malus domestica*	Homozygous	FEM
James Grieve	R**	*Malus domestica*	Homozygous	FEM
Spartan	VR**	*Malus domestica*	Homozygous	FEM
Dayton	R**	*Malus domestica*	Homozygous	FEM
Jonafree	S**	*Malus domestica*	/	FEM
Rubinola	R**	*Malus domestica*	Homozygous	FEM
Fuji Red Spot	R**	*Malus domestica*	Heterozygous	FEM
Nova Easygro	R**	*Malus domestica*	Homozygous	FEM
Evereste	R**	*Malus domestica*	Homozygous	FEM
Bountiful	VR**	*Malus domestica*	/	FEM
Wellington	VR**	*Malus domestica*	Heterozygous	FEM
Akane	R**	*Malus domestica*	/	FEM
Geneva Early	R**	*Malus domestica*	Homozygous	FEM
Greensleves	R**	*Malus domestica*	Absent	FEM
Beacon	R**	*Malus domestica*	Homozygous	FEM
Yellow Transparent	R**	*Malus domestica*	Absent	FEM
Niagara	R**	*Malus domestica*	Homozygous	FEM
Redfree	R**	*Malus domestica*	Homozygous	FEM
Okanoma	VR**	*Malus domestica*	Homozygous	FEM
Harrold Red Delicious	VR**	*Malus domestica*	Homozygous	FEM
Winter Winesap	VR**	*Malus domestica*	Homozygous	FEM
Winesap Spur	VR**	*Malus domestica*	Homozygous	FEM
Turley Winesap	VR**	*Malus domestica*	Homozygous	FEM
Stayman Winesap	VR**	*Malus domestica*	Homozygous	FEM
Ruby	VR**	*Malus domestica*	Homozygous	FEM
Remo C	VR**	*Malus domestica*	Homozygous	FEM
Double Red Stayman	VR**	*Malus domestica*	/	FEM
Liberty	R**	*Malus domestica*	Absent	FEM
Reanda	R**	*Malus domestica*	Absent	FEM
Braeburn	R**	*Malus domestica*	Absent	FEM
Starkrimson	VS**	*Malus domestica*	Homozygous	FEM
Jonamac	S**	*Malus domestica*	Homozygous	FEM
Calville Blanc	VS**	*Malus domestica*	Heterozygous	FEM
Topaz	R**	*Malus domestica*	Absent	FEM
Elstar	S°	*Malus domestica*	Absent	FEM2
Pink Lady	S°	*Malus domestica*	Heterozygous	FEM2
Crimson Crisp	S°	*Malus domestica*	Heterozygous	FEM2
Gala	S°	*Malus domestica*	Absent	FEM2

Fiesta	R°	*Malus domestica*	Homozygous	FEM2
Cox's Orange Pippin	S°	*Malus domestica*	Homozygous	FEM2
Honeycrisp	S°	*Malus domestica*	Heterozygous	FEM2
Fuji	R°	*Malus domestica*	Homozygous	FEM2
Goldrush	VR°	*Malus domestica*	Absent	FEM2
Empire	R°	*Malus domestica*	Homozygous	FEM2
Florina	R°	*Malus domestica*	Homozygous	FEM2
Jonathan	S°	*Malus domestica*	Heterozygous	FEM2
Patte de Loup	R#	*Malus domestica*	Homozygous	FEM2
Cortland	S#	*Malus domestica*	Homozygous	FEM2
Renetta Bianca	R#	*Malus domestica*	Homozygous	FEM2
Renetta Champagne	R#	*Malus domestica*	Homozygous	FEM2
Renetta Grigia	R#	*Malus domestica*	Homozygous	FEM2
Royal Gala	R#	*Malus domestica*	Homozygous	FEM2
Rus 98 04 03	R#	*Malus orientalis*	Homozygous	FEM2
Toringo	R#	*Malus toringo*	Homozygous	FEM2
Telamon	R#	*Malus domestica*	Homozygous	FEM2
Pinova	R#	*Malus domestica*	Homozygous	FEM2
99TU 08 02 1 4 2 3	S#	*Malus orientalis*	Absent	FEM2
Antonovka Kamenichka	S#	*Malus domestica*	Absent	FEM2
CH97 05 06	S#	*Malus orientalis*	Absent	FEM2
CH97 06 07 China	S#	*Malus zhaojiaoensis*	Absent	FEM2
Crimson Gold	S#	*Malus domestica*	Absent	FEM2
Geneva	S#	*Malus domestica*	Absent	FEM2
GMAL1461	S#	*Malus orientalis*	Absent	FEM2
KAZ95 05 01	S#	*Malus sieversii*	Absent	FEM2
KAZ95 18 10 2 12 3 3	S#	*Malus sieversii*	Absent	FEM2
KAZ95 18 10 2 13 1 3	S#	*Malus sieversii*	Absent	FEM2
99TU 08 02 GMAL	S#	*Malus orientalis*	Heterozygous	FEM2
99TU 08 02 Turkey	S#	*Malus orientalis*	Heterozygous	FEM2
CH97 05 11 Chiana	S#	*Malus prattii*	Heterozygous	FEM2
Grenoble Reinette	S#	*Malus domestica*	Heterozygous	FEM2
Renetta Grigia di Torriana	S#	*Malus domestica*	Heterozygous	FEM2
Renetta Grigia INFEL	S#	*Malus domestica*	Heterozygous	FEM2
Sonya	/	*Malus domestica*	Heterozygous	FEM2
GMAL2948	/	*Malus coronaria*	Homozygous	FEM2
Clivia	/	*Malus domestica*	Heterozygous	FEM2
Baujade	/	*Malus domestica*	Heterozygous	FEM2
Rubinette	/	*Malus domestica*	Heterozygous	FEM2
Stark Earliest	/	*Malus domestica*	Heterozygous	FEM2
Rosmarina	/	*Malus domestica*	Absent	FEM2
Sieboldii MA4	/	*Malus domestica*	Homozygous	FEM2
Young America	/	*Malus domestica*	Homozygous	FEM2
Renewa	/	*Malus domestica*	Heterozygous	FEM2

Renoir	/	*Malus domestica*	Absent	FEM2
Antonovka	/	*Malus domestica*	Absent	FEM2
Malus silvestri	/	*Malus silvestri*	Heterozygous	FEM2
Reinette Sik	/	*Malus domestica*	Homozygous	FEM2
Granny Smith	1.75 – S	*Malus domestica*	Heterozygous	Laimburg
Reinette du Canada	1.43 – R	*Malus domestica*	Heterozygous	Laimburg
Jackii	1 – VR	*Malus baccata*	Homozygous	Laimburg
9-AR2T196	1.17 – VR	*Malus domestica*	Heterozygous	Laimburg
A723_6	1.25 – VR	*Malus domestica*	Heterozygous	Laimburg
B45	1.46 – R	Hybrid	Heterozygous	Laimburg
Dulmener Rosen	1.54 – S	*Malus domestica*	Heterozygous	Laimburg
Durello di Forlì	1.38 – R	*Malus domestica*	Heterozygous	Laimburg
GMAL 2473	1.38 – R	*Malus domestica*	Heterozygous	Laimburg
Gravenstein	1.58 – S	*Malus domestica*	Heterozygous	Laimburg
J34	1.63 – S	Hybrid	Heterozygous	Laimburg
Minister Hammerstein	1.59 – S	*Malus domestica*	Heterozygous	Laimburg
Osnarbrucker Reinette	1.92 – VS	*Malus domestica*	Heterozygous	Laimburg
Roter Jonathan	1.88 – VS	*Malus domestica*	Absent	Laimburg
Prinz Albert	1.46 – R	*Malus domestica*	Heterozygous	Laimburg
Rosmarina Rossa	1.88 – VS	*Malus domestica*	Heterozygous	Laimburg
TSR33T239	1.29 – R	*Malus domestica*	Heterozygous	Laimburg
TSR34T15	1.42 – R	*Malus domestica*	Heterozygous	Laimburg
Winterbananeapfel	1.23 – VR	*Malus domestica*	Heterozygous	Laimburg
Idared	1.66 – S	*Malus domestica*	Homozygous	Laimburg
Harberts Reinette	1.33 – R	*Malus domestica*	Heterozygous	Laimburg
Ambrosia	1.54 – S	*Malus domestica*	Absent	Laimburg
Baumanns Reinette	1.17 – VR	*Malus domestica*	Absent	Laimburg
Hansen's Baccata	1.13 – VR	*Malus baccata*	Absent	Laimburg
Kaiser Alexander	1.46 – R	*Malus domestica*	Absent	Laimburg
Landsberger Reinette	1.71 – S	*Malus domestica*	Absent	Laimburg
Q71	1.38 – R	*Malus domestica*	Absent	Laimburg
Golden Delicious	S**	*Malus domestica*	Absent	Reference
McIntosh	VR**	*Malus domestica*	Homozygous	Reference
Mela Rozza	/	*Malus domestica*	Heterozygous	Reference

*VR: very resistant; R: resistant; S: susceptible; VS: very susceptible

** Swensen (2006)

° http://www.orangepippin.com/apples

Direct observation

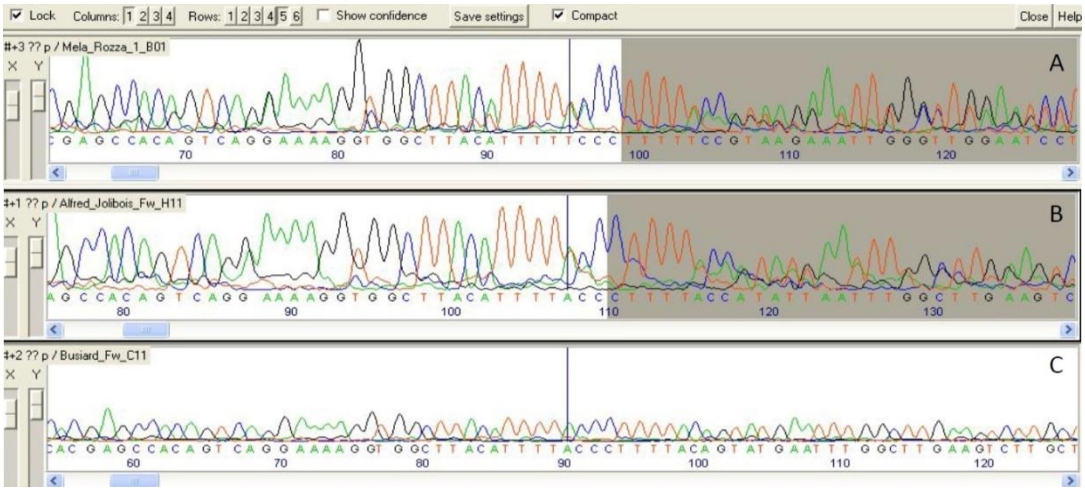


Figure S1. Electropherograms of the re-sequencing of 'Mela Rozza', 'Alfred Jolibois' and 'Busiard' as shown by the Staden package software. Adenines, thymines, cytosine and guanines are indicated by green, red, blue and black peaks, respectively. The blue bars indicate position 1201. The grey-background indicates areas of low quality sequencing. In electropherograms A and B the adenine peak in position 1201 overlaps with the thymine peak, whereas in electropherograms C there is no overlapping. This indicates that samples A and B are heterozygous for insertion T-1201, whereas sample C is homozygous.

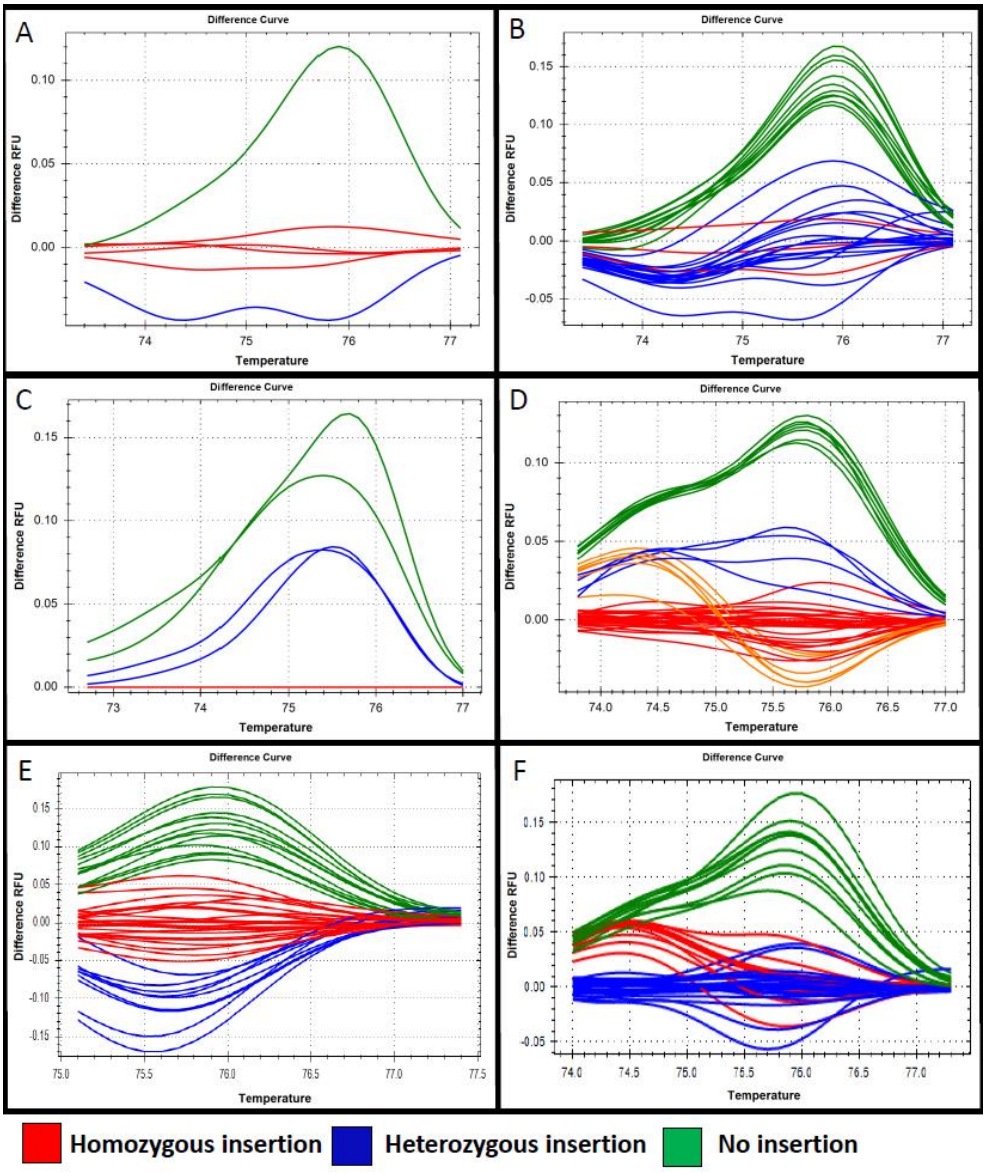
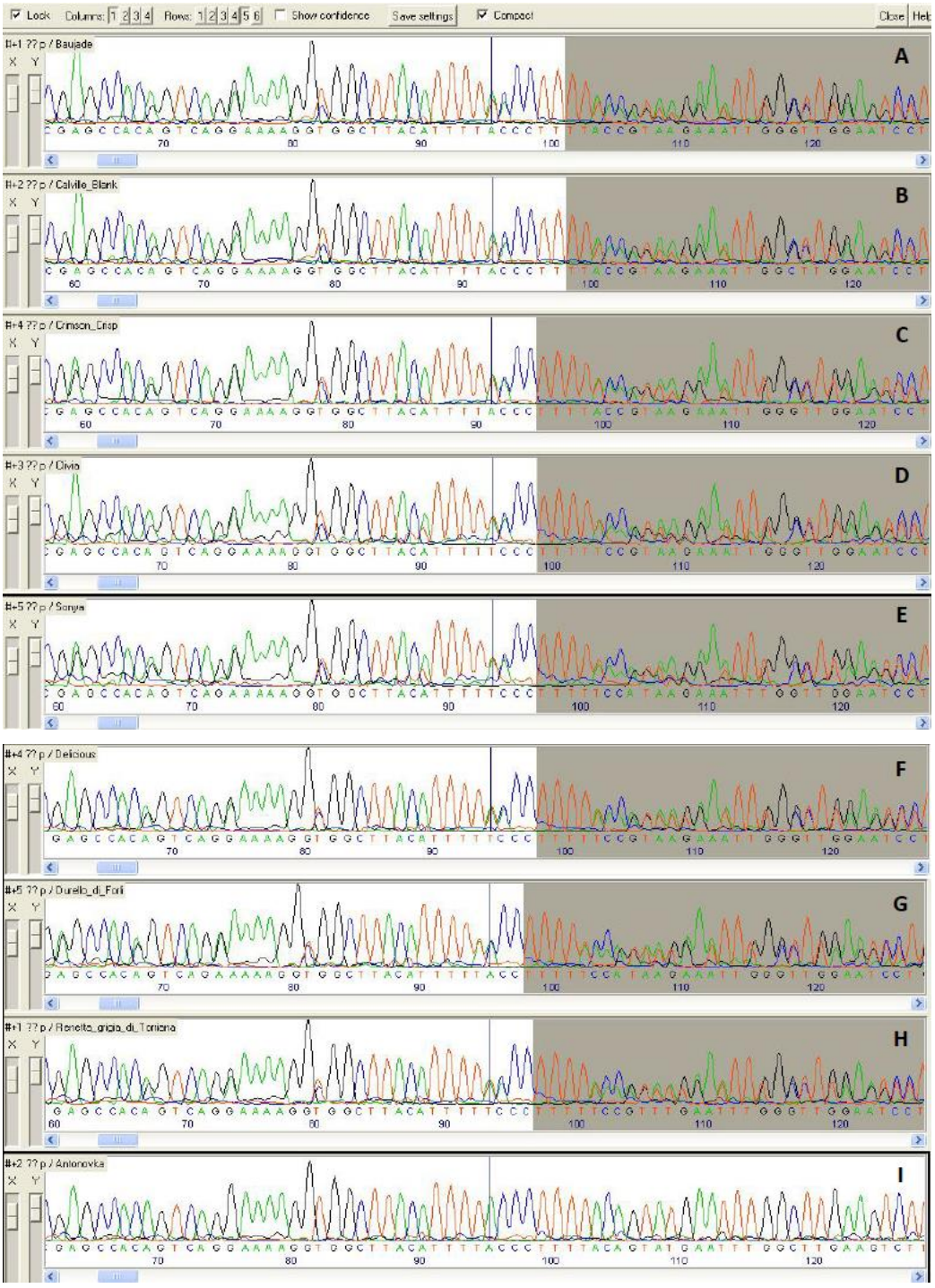
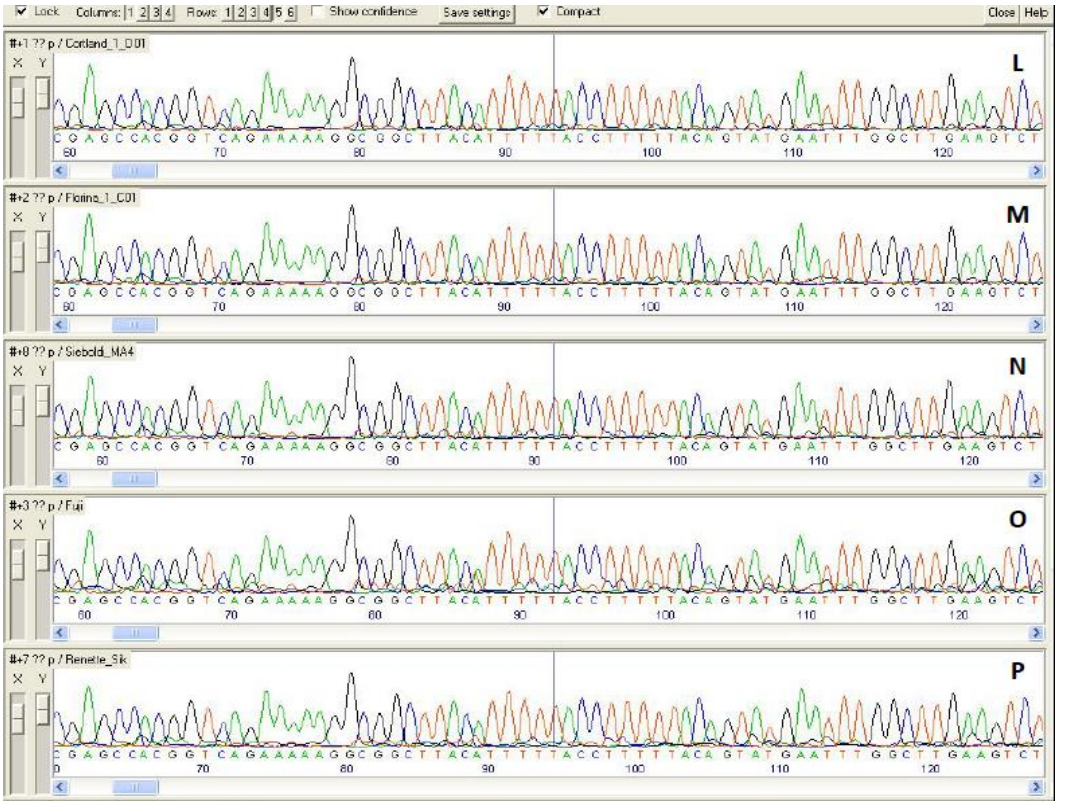


Figure S2. melting profiles of 159 apple genotypes. The red curves are the homozygous insertion, blue heterozygous and green absence of insertion. Orange curves are different melting profiles. (A) Profiles of six genotypes from FruitBreedomics, including the three references 'Mela Rozza', 'Golden Delicious' and 'McIntosh'. (B) Profiles of 27 genotypes from Wädenswil. (C) Profiles repeated for two Wädenswil genotypes and for three reference genotypes. (D) Profiles of the first group of 50 genotypes collected In FEM. (E) Profiles of the second group of 52 genotypes collected in FEM. (F) Profiles of 27 genotypes collected in Laimburg.


Lock
Columns: 1 2 3 4
Rows: 1 2 3 4 5 6
Show confidence
Save settings
Compact
Close
Help
#+1 ?? p / Baujade
A
#+2 ?? p / Calville_Blank
B
#+4 ?? p / Crimson_Crisp
C
#+3 ?? p / Civia
D
#+5 ?? p / Sonya
E
#+4 ?? p / Delicious
F
#+5 ?? p / Durello_di_Forlì
G
#+1 ?? p / Renetta_grigia_di_Torriana
H
#+2 ?? p / Antonovka
I


Lock
Columns: 1 2 3 4
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Show confidence
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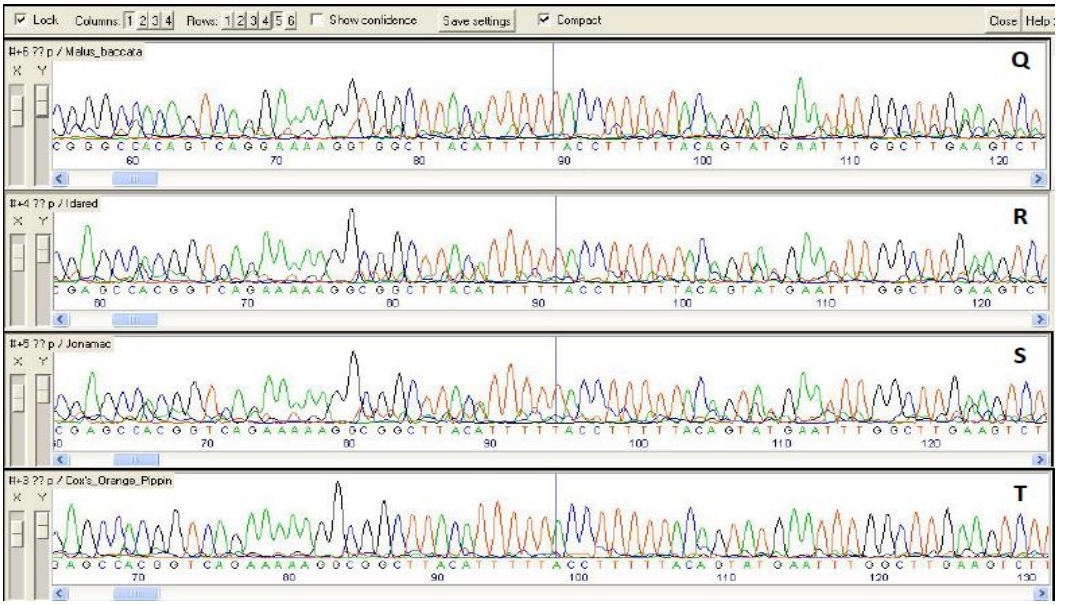
#+1 ?? p / Cortland_1_D01
L
#+2 ?? p / Florina_1_C01
M
#+8 ?? p / Siebold_MA4
N
#+3 ?? p / Fuji
O
#+7 ?? p / Renette_Sik
P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Columns: 1 2 3 4
Rows: 1 2 3 4 5 6
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#+6 ?? p / Malus_baccata
Q
#+4 ?? p / Idared
R
#+5 ?? p / Jonamac
S
#+3 ?? p / Cox's_Orange_Pippin
T

Figure S3: Electropherograms of the re-sequencing of 18 genotypes as shown by Staden package software. Adenines, thymines, cytosine and guanines are indicated by green, red, blue and black peaks, respectively. The blue bars indicate position 1201. The grey-background indicates areas of low quality sequencing. In electropherograms from A to H, the adenine peak in position 1201 overlaps with the thymine peak, whereas in electropherograms from L to T there is no overlapping. Electropherograms I show the absence of insertion in 'Antonovka'. This indicates that samples from A to E are heterozygous for insertion T-1201, whereas samples from F to O are homozygous.

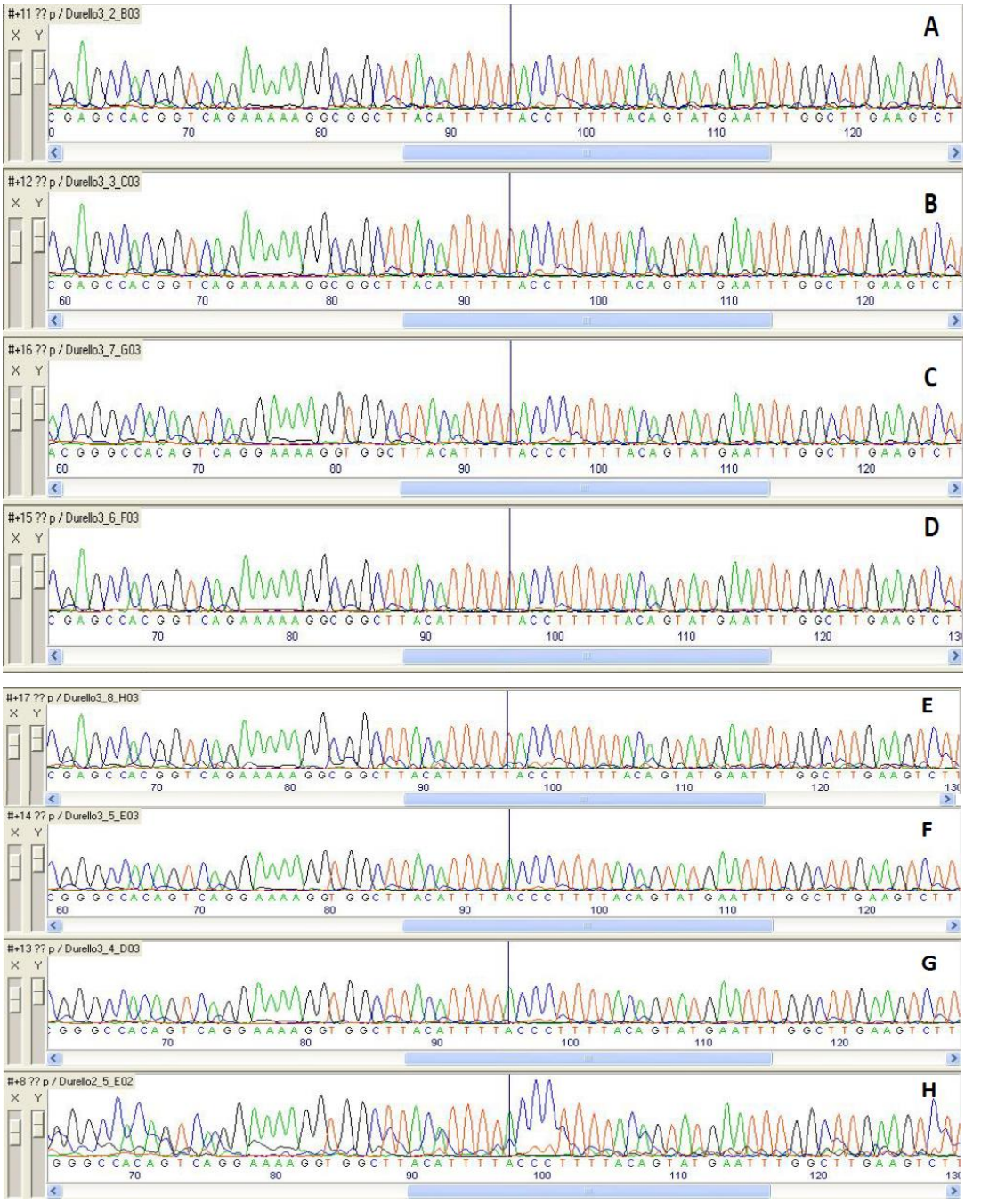
#+11 ?? p / Durello3_2_B03
A
#+12 ?? p / Durello3_3_C03
B
#+16 ?? p / Durello3_7_G03
C
#+15 ?? p / Durello3_6_F03
D
#+17 ?? p / Durello3_8_H03
E
#+14 ?? p / Durello3_5_E03
F
#+13 ?? p / Durello3_4_D03
G
#+8 ?? p / Durello2_5_E02
H

Figure S4: Electropherograms as shown by the Staden package software of the re-sequencing of 'Durello di Forlì' cloned into a plasmid. Adenines, thymines, cytosine and guanines are indicated by green, red, blue and black peaks, respectively. The blue bars indicate position 1201. The grey background indicates areas of low quality OF sequencing. In electropherograms A, B, D and E there is the insertion of a T in position 1201, whereas in electropherograms C, F, G and H in position 1201 there is an A.

CHAPTER 5

MLO genes Knock-down reduces susceptibility to powdery mildew in grapevine (*Vitis vinifera*)

Stefano Pessina, Luisa Lenzi, Michele Perrazzolli, Manuela Campa, Lorenza Dalla Costa, Simona Urso, Giampiero Valè, Francesco Salamini, Riccardo Velasco, Mickael Malnoy

ABSTRACT

Erysiphe necator is the causal agent of powdery mildew (PM), one of the most destructive diseases of grapevine. Powdery mildew is controlled by sulphur-based and synthetic fungicides, which every year are dispersed in the environment. This is why PM resistant varieties should become a priority for sustainable grapevine and wine production. Resistance can be achieved by knocking-out susceptibility S-genes, such as those residing at genetic loci known as *MLO* (*Mildew Locus O*). All *MLO* S-genes of dicots belong to the phylogenetic clade V, including grapevine genes *VvMLO7*, *11, 13*, which are up-regulated during PM infection, and *VvMLO6,* which is not up-regulated. Before adopting a gene editing approach to knock-out candidate S-genes, the evidence that loss-of-function of *MLO* genes can reduce PM susceptibility is necessary. This paper reports the knock-down through RNA interference of *VvMLO6*, *7*, *11* and *13*. Knock-down of *VvMLO6*, *11* and *13*, alone or combined, did not decrease PM severity, whereas the knock-down of *VvMLO7,* alone or in combination with *VvMLO6* and *VvMLO11,* reduces severity up to 77%. Cell wall appositions (papillae), a response to PM attack, were present in both resistant and susceptible lines, but were larger in resistant lines. Thirteen genes involved in defense were less up-regulated in infected *mlo* plants, highlighting the early *mlo*-dependent disruption of PM invasion.

INTRODUCTION

Grapevine orchards are treated with an impressive amount of chemical compounds, particularly fungicides, to prevent yield losses due to fungal pathogens. In France, Italy, Spain and Germany, between 1992 and 2003, 73% of the fungicides were used for grapevine protection, a crop that covers only 8% of the agricultural land in those countries (EUROSTAT, 2007).

Worlwide, grapevine powdery mildew (PM), caused by the fungus *Erysiphe necator*, is a destructive disease (Feechan *et al.,* 2011). *E. necator* Schw. (syn. *Uncinula necator* (Schw.) Burr.) is an obligate biotroph infecting all green tissues of grapevine and resulting in significant losses in yield and berry quality. Symptoms are a white or grey powder covering both leaf surfaces, and, after infection, the fruits show shriveling or cracking (Wilcox, 2013). The quality of the fruit is severely damaged, due to increased acidity and decreased anthocyanin and sugar content (Calonnec *et al.,* 2004).

Powdery mildew can be controlled by frequent applications of fungicides, particularly those based on sulphur. However, due to the ecological drawbacks of fungicides (Wightwick *et al.,* 2010), the relative high costs (up to 20% of total grapevine production expenses; Fuller *et al.,* 2014), and to the rapid appearance of resistant strains of the pathogen (Baudoin *et al.,* 2008; Dufour *et al.,* 2011, Kunova *et al.,* 2015) because of its adaptive gene copy number variation (Jones *et al.,* 2014), new alternatives to chemical treatments should be adopted. Resistant varieties are one of the best options. The use of PM-resistant cultivars could reduce "Chardonnay" production costs in California by 720 $/ha, with a significant reduction of fungicide usage (Fuller *et al*., 2014).

Vitis vinifera is susceptible to PM (Gadoury *et al.* 2003), whereas North American *Vitis* species, due to their co-evolution with *E. necator,* have variable degrees of resistance to the pathogen (Fung *et al.,* 2008). Their resistances have been transferred to *V. vinifera* but the acceptance of resistant hybrids by producers and consumers has been very limited because of attachment to traditions and lower quality of resulting wine (Fuller *et al.,* 2014).

A strategy to create crops resistant to diseases is based on the exploitation of R-genes that encode proteins that recognize pathogen effectors and trigger defense response (Pavan *et al.,* 2010; Dodds and Rathjen, 2010), such as the *Vitis REN* and

RUN genes (Qiu *et al*., 2015). Resistance is manifested as localized hypersensitive response at the site of attempted infection (Bari and Jones, 2009). However, R-genes are frequently overcome by mutations of the pathogen (Parlevliet *et al.,* 1993). An alternative approach is based on susceptibility genes (S-genes), which loss-of-function results in recessively inherited resistance (Pavan *et al.,* 2010). Knock-out of S-genes may, however, induce pleiotropic phenotypes in the plant (Pavan *et al.* 2011; Van Schie and Takken, 2014).

A typical class of S-genes is represented by the *Mildew Locus O* (*MLO*) genetic factors which, when inactivated, results in recessive *mlo* resistance, as discovered in barley (Jørgensen, 1992). *MLO* genes are largely conserved across the plant kingdom and their loss-of-function resulted in PM resistance in *Arabidopsis* (Consonni *et al.,* 2006), pea (Pavan *et al.,* 2011), tomato (Bai *et al.,* 2008), wheat (Wang *et al.,* 2014), and pepper (Zheng *et al.,* 2013). Of the seven phylogenetic clades in which the *MLO* family is divided (Acevedo-Garcia *et al.,* 2014; Pessina *et al.,* 2014), only two include S-genes: clade IV with all monocot S-genes (Panstruga *et al.,* 2005; Reinstädler *et al.,* 2010) and clade V with all dicot S-genes (Consonni *et al.,* 2006; Bai *et al.,* 2008; Feechan *et al*., 2008; Winterhagen *et al.,* 2008). Not all members of clades IV and V are S-genes, but candidates can be identified during early stages of PM infection because of their increased expression, as documented in tomato (Bai *et al.,* 2008), barley (Piffanelli *et al.,* 2002), pepper (Zheng *et al.,* 2013), grapevine (Feechan *et al*., 2008; Winterhagen *et al.,* 2008) and apple (Pessina *et al.,* 2014). In grapevine, of four clade V *MLO* genes, three (*VvMLO7*, *VvMLO11* and *VvMLO13)*, are up-regulated early after PM infection, whereas *VvMLO6,* the fourth*,* is not transcriptionally responsive to the pathogen (Feechan *et al.,* 2008; Winterhagen *et al.,* 2008).

MLO are membrane proteins with seven trans-membrane domains involved in a variety of physiological processes in different tissues (Devoto *et al.,* 1999). Their

proposed function is the negative regulation of vesicle-associated and actin-dependent defense pathways at the site of PM penetration (Panstruga *et al.,* 2005). The secretory vesicle traffic controls pathogen penetration, allowing the formation of cell wall appositions called papillae (Miklis *et al.,* 2007; Feechan *et al.,* 2011), which are associated with *mlo* resistance (Consonni *et al.,* 2006; Aist and Bushnell, 1991).

The development of DNA editing tools is rapidly changing plant genetics and biotechnology, due to the possibility of inducing mutations in specific genes (Lozano and Cutler, 2014; Gaj *et al.,* 2013; Puchta and Fauser, 2014). Before adopting a gene editing approach to knock-out candidate S-genes, the evidence that loss-of-function of *MLO* genes can reduce PM susceptibility is necessary. This paper reports the knock-down through RNA interference of *VvMLO6*, *7*, *11* and *13* and its effect on PM infection in grapevine.

MATERIALS AND METHODS

Constructs

300-600 bp fragments of genes *VvMLO6*, *VvMLO7*, *VvMLO11* and *VvMLO13* were amplified (Table S1) and cloned in pENTR/SD- TOPO (Invitrogen). After sequence control, the fragments were inserted in the RNAi Gateway vector pK7GWIWG2D(II) (Karimi et al. 2002; http://www.psb.ugent.be/), as in Urso *et al.* (2013). After sequencing both strands, the constructs were inserted in *Agrobacterium tumefaciens* strain GV3101, as in Zottini *et al.* (2008). *A. tumefaciens*-transformed cells were tested by PCR for the presence of constructs, using primers annealing to the 35S promoter (5'- CGCACAATCCCACTATCCTT – 3') and the *MLO* fragment (Table S1).

Plant material, gene transfer and screening of regenerated plant

Plant material of the grapevine cultivar Long-Cluster Brachetto was cultivated *in vitro* as described by Dalla Costa *et al.* (2014). Somatic embryos were used for gene transfer. Gene transfer, regeneration and selection of transgenic plants were performed as in Dalla Costa *et al.* (2014). Five different gene transfers were carried out: four aimed to silence the four *MLO* target genes, and the fifth was a control consisting of the empty vector (pK2WG7). DNA was extracted from *in vitro* leaf tissue (Phytopure kit, GE Healthcare, UK). Integration was proven using the primers described above. Transformed *in vitro* grown lines were moved to a woody plant (WP) medium (McCown and Lloyd, 1981) in growth chamber at 20-24°C and transferred in fresh medium once a month.

Greenhouse acclimation

Plants were first acclimated to greenhouse conditions in a growth chamber at 25°C, 16 hours day / 8 hours night, humidity 70±5%. One-month old-plants with at least two main roots 3 cm long were transferred in a 250 ml plastic cup containing wet autoclaved turf (Tercomposti Spa, Brescia, Italy) and sealed with parafilm, to preserve humidity. Every seven days, holes were made in the parafilm cover to progressively reduce air humidity and promote the formation of the foliar cuticle. After three weeks, parafilm was completely removed and, after one more week, the plants were transplanted in 1 L pots kept in the greenhouse at 25 °C, 16 hours day / 8 hours night, humidity 70±5%.

***Erysiphe necator* inoculation and disease severity assessment**

Originally, the PM inoculum was isolated in northern Italy (Trentino region) from leaves of an untreated vineyard. Subsequent reproduction of the inoculum was carried out infecting the *V. vinifera* cultivar Pinot Noir, under greenhouse

conditions. Transformed plants were dry inoculated onto target leaves gently brushing them with infected young leaves carrying fresh sporulating mycelium (Blaich *et al.,* 1989). Inoculated plants were incubated in a greenhouse at 25±1 °C, relative humidity of 100% for 6h to promote fungal penetration, and then kept at 25±1 °C, relative humidity of 70±10 % until the last symptom's evaluation. Disease severity was assessed on all leaves at 14, 22 and 30 days post inoculation (dpi), following the standard guidelines of the European and Mediterranean Plant Protection Organization (EPPO, 1998). Disease severity was expressed as the average percentage (intervals of 5%) in each plant of the adaxial leaf area covered by PM mycelia. Two inoculation experiments were carried out. In each experiment, three to nine biological replicates (plants) per line were analyzed in a randomized complete block design. Disease severity was calculated as (disease severity in control plants - disease severity in inoculated plants)/disease severity in control plants and expressed as percentage. To analyze all time points together, the area under disease progress curve (AUDPC) was considered as a quantitative summary of disease intensity over time (Campbell and Madden, 1990; Madden *et al.,* 2007).

The number of *E. necator* conidia produced from infected leaves was assessed as in Angeli *et al.* (2012). Three leaves were collected from each replicate at 30 dpi and four disks of 0.8 cm diameter for each leaf were cut, for a total of 12 disks per replicate. Leaf disks were transferred to 50 mL tubes containing 5 mL distilled water with 0.01% tween. Tubes were vortexed for one min and the concentration of conidia per ml was determined by a haemocytometer count. The values obtained were converted in conidia per square centimeter (cm^2) of grapevine leaf.

***Plasmopara viticola* inoculation and disease severity assessment**

A *P. viticola* population was isolated in 2014 in Northern Italy (Trentino region) from an untreated vineyard. Subsequent reproduction of the inoculum was carried out

infecting the *V. vinifera* cultivar Pinot Noir, under greenhouse conditions (Perazzoli *et al.,* 2011). Fresh sporangia were obtained by placing plants with oil spots symptoms in the dark at 99-100% RH overnight. Sporangia were then collected by washing the abaxial surfaces, carrying freshly sporulating lesions, with distilled water at 4°C. The concentration of the inoculum was adjusted to $2*10^5$ sporangia/mL by. Abaxial surfaces of all leaves of each tested plant were sprayed with the inoculum suspension of *P. viticola*. Inoculated plants were incubated overnight in the dark at 25°C with 99-100% RH, and then maintained under controlled greenhouse conditions at 25±1°C and RH 70±10%. Six days after inoculation, plants were incubated overnight in darkness at 25°C with 99-100% RH to allow downy mildew sporulation. Severity of downy mildew was visually assessed on all leaves of each plant, according to standard guidelines of the European and Mediterranean Plant Protection Organization (EPPO, 2001). For each leaf, disease severity was expressed as the percentage (intervals of 5%) of abaxial leaf area covered by white sporulation of *P. viticola.* A mean value was calculated for each plant and six to nine replicates (plants) for each line were analyzed in two independent randomized complete block design experiments.

Histological analysis

Two inoculated leaves were collected from three replicates of each transgenic and control line at 3, 10 and 21 dpi for hyphae visualization and histological analyses. Leaves were treated as described by Vanacker *et al.* (2000) with the following modifications: small pieces of leaf with the adaxial surface up were laid on filter paper moistened with ethanol:glacial acetic acid (3:1, v/v) until the chlorophyll was removed. After transfer to water soaked filter paper for 2 h, they were mounted on microscope slides and a drop of aniline blue (0.1% [w/v] in lactoglycerol) was pipetted on their surface. Hyphae were visualized using the bright field illumination

of a Leica LMD6500 microscope (Leica Microsystems, Wetzlar, Germany). For the detection of papillae, leaves were cleared in ethanol:glacial acetic acid (3:1, v/v) to remove chlorophyll, and equilibrated overnight in lactic acid, glycerol and water (1:1:1). Papillae were visualized using the LMD filter of a Leica LMD6500 microscope.

RNA extraction and gene expression analysis

A first gene expression analysis of transgenic plants was carried out on *in vitro* grown lines, to identify genotypes with reduced expression of target genes. Three biological replicates were collected from each line. The second analysis was carried out on acclimated transgenic plants, with leaf samples collected before inoculation, 24 hours and 10 days post PM inoculation, the time of the last two samplings corresponding to the up-regulation of *MLO* genes after infection (Feechan *et al.,* 2008; Winterhagen *et al.,* 2008). Five biological replicates were collected from each line. For each line at each time point, the third and fifth half leaves from the top were collected, frozen in liquid nitrogen and stored at -80°C.

Total RNA was extracted with the Spectrum^{tm} Plant Total RNA kit (Sigma-Aldrich). Following a treatment with the DNAse I (Sigma-Aldrich), the RNA was reverse transcribed using the SuperScript III reverse transcriptase (Invitrogen, Life Technologies, Waltham, USA).

qPCR amplification (SYBR Green Supermix, Bio-Rad, Hercules, USA) was carried out in a 15-µL volume (primers in Table S2) and the results recorded by a CFX96 Touch Real-Time PCR detection system (Bio-Rad, Hercules, USA), run by CFX Manager software. The software applies comparative quantification with an adaptive baseline. Each sample was run in two technical replicates with the following parameters: 95°C 3 min, 40 cycles of 95°C 10 sec and 55°C 30 sec, with a final step at 95°C 10 sec. Primers for genes *VvMLO6, VvMLO1*1 and *VvMLO13* were taken

from Winterhagen *et al.* (2008), while for *VvMLO7* they were specifically designed (Table S2). Primers for *VvWRKY19*, *VvWRKY27, VvWRKY48* and *VvWRKY52* were taken from Guo *et al.* (2014), for *VvEDS1* from Gao *et al.* (2014) and for *VvPR1*, *VvPR6* and *VvLOX9* from Dufour *et al.* (2013). The new primer pairs were designed with the NCBI Primer Designing Tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table S2). cDNA samples diluted 10, 100, 1000 and 10000 times were used to test calculate the efficiency of the primers pairs and the size of the PCR products was confirmed by agarose gel electrophoresis. Presence of a specific final dissociation curve was determined after every qPCR run with progressive increments of temperature from 65°C to 95°C (0.5°C each step, 5 sec).

Reference genes were, as reported for grapevine (Reid *et al.*, 2006), *Elongation Factor 1α*, *GAPDH* and *Actin*. Reference genes stability was assessed with GeNorm (medgen.ugent.be/~jvdesomp/genorm/): the three genes had M-values lower than 0.5, well below the threshold of 1.5 considered sufficient for stability (Ling and Salvaterra, 2011; Van Hiel *et al*., 2009; Strube *et al.,* 2008).

Threshold cycles (Ct) were converted to relative expression following Hellemans *et al.* (2007) and based on the average Ct of two technical replicates. For *MLO* genes the reference Ct was the average of all samples; for other genes, the control EVB at T=0 was adopted.

Statistical analyses

Disease severity. Data were analyzed with the Statistica 9 software (StatSoft, Tulsa, USA) and the package SPSS (IBM, Armonk, USA). The smallest statistical unit considered was a plant. Severity values of all leaves were averaged, resulting in the value considered in further analyses. Normal distributions (Kolmogorov-Smirnov and Shapiro-Wilk tests $P > 0.05$) were validated for variances homogeneity (Levene's test, $P > 0.05$) and subsequently used for one-way ANOVA with Tukey's

post-hoc test ($P < 0.05$) at each time point. Data were transformed in arcsin(x) to meet the pre-requisites of ANOVA. In case of non-homogeneous variances, the Games-Howell's post-hoc test was used.

In some cases, data from two experiments were pooled and the ANOVA applied independently for each time point (14, 22 and 30 dpi). AUDPC data were treated as for severity data. Conidia counts were analyzed with the Kruskall-Wallis test ($P < 0.05$).

qPCR data analysis. Values of relative expression were expressed in logarithms (Pessina *et al*., 2014) to obtain normal distributions and homogeneity of variances of the residues, as assessed with Shapiro-Wilk ($P \leq 0.05$) and Levene ($P \leq 0.05$). Homoscedastic data were analyzed with Tukey's test ($P < 0.05$) and non-homoscedastic with Games-Howell test ($P < 0.05$) using the statistical package SPSS (IBM).

Expression data from two experiments were analyzed independently and pooled. Differences were revealed by one-way ANOVA with Tukey post-hoc test ($P < 0.05$). In addition, a two-way ANOVA with Tukey post-hoc test ($P < 0.05$), considered at the same time the effects of the transgenic line and of the time point. For the gene expression characterization of TLB4, Fisher post-hoc test was used.

Correlations. Correlations were investigated with two-tailed Pearson's correlation test. Correlations considered were between disease severity and amount of conidia at 30 dpi, and between disease severity at 14 dpi and relative expression of *MLO* genes at 10 dpi. In both cases, all data, severity and relative expression were expressed as arcsin.

RESULTS

Gene transfer, selection and acclimation of *MLO* transgenic lines

A total of five gene transfers were carried out. Four were aimed to knock-down (KD) specific *MLO* genes (i = KD-*VvMLO6,* ii = KD-*VvMLO7,* iii = KD-*VvMLO11,* iv = KD-

VvMLO13), the fifth to insert an empty vector. Thirty-four regenerated lines were obtained, with 26 of them confirmed to contain the insert (Table S3). The result of the PCR analysis of six lines is shown in Fig. S1. Twenty-six transgenic lines were propagated *in vitro* and tested for the silencing of *MLO* genes with qPCR. This was evident for three lines out of eight from gene transfer (iii) (KD-*VvMLO11*), and three out of nine from gene transfer (iv) (KD-*VvMLO13*). Gene transfers (i) (KD-*VvMLO6*) and (ii) (KD-*VvMLO7*) resulted in a small number of regenerated lines that showed no reduction of expression (Table S3). Regenerated lines were also tested for off-target silencing, showing that the RNAi fragments targeted other clade V *MLO* genes. Six lines with various combinations of silenced genes were selected and indicated with acronyms TLB1 (Transgenic Line of Brachetto) to TLB6 (Table S3). Lines from TLB1 to 3 came from gene transfer (iii) (KD-*VvMLO11*), lines from TLB4 to TLB6 from gene transfer (iv) (KD-*VvMLO13*) (Table S3). The control was the EVB line (Empty Vector Brachetto). In addition, TLB7, a regenerated line with no reduction of expression, was also included. All lines, including the control, will be referred in the text as "transgenic lines". Lines from TLB1 to 7 are further indicated as "RNAi lines" and from TLB1 to 6 "*mlo* lines".

The survival rate of plants to the acclimation process was around 85%. Under greenhouse conditions, the transgenic plants showed normal growth and no pleiotropic phenotypes.

Powdery mildew and downy mildew resistance of transgenic lines

Two independent experiments of PM inoculation were carried out on the RNAi lines TLB1 to 7, and the transgenic control EVB. Three *mlo* lines, TLB4, 5 and 6, showed a reduction of *E. necator* infection higher than 60% at 30 dpi (Fig. 1; Table 1). The disease reduction of TLB6 decreased with the progression of the infection (Table 1).

TLB2, 3, and 7 had a level of susceptibility to PM comparable to EVB (Figs. 1 and S2). The leaf phenotypes in Fig. 1 visualize the differences between the different lines. All the *mlo* lines showed fewer conidia at 30 dpi compared to EVB and the decrease was statistically significant for TLB4, TLB5 and TLB6 (Fig. S3). Compared to EVB plants, TLB4, 5 and 6 had, respectively a reduction of 93%, 95% and 72%. Conidia counts and disease severity were, as expected, correlated ($R = 0.58$; $P \leq 0.01$). The reduction of conidia in TLB 4, 5 and 6 (93%, 95% and 72%) was higher than the reduction of PM symptoms (68.4%, 76.6% and 65.1%), indicating that the leaf diseased area had a lower concentration of conidia in TLB 4, 5 and 6 compared to EVB.

Line TLB4 was characterized by histological analysis, demonstrating a reduced progression of PM infection compared to EVB (Fig. 2). In EVB, conidiophores appeared at 10 dpi and at 21 dpi they were present all over the leaf surface (Fig. 2A), while on TLB4 leaves they were visible in a limited number only at 21 dpi (Fig. 2B). Formation of papillae was observed at 3 dpi in TLB4 and EVB (Fig. 3). The papilla of EVB had defined edges and it was present only in correspondence of the infection site of *E. necator* (Fig. 3A and B), while those of TLB4 were diffuse, larger and present also outside of the site of infection of the fungus (Fig. 3C and D).

An experiment was designed to test the cross-reaction of *mlo* lines to fungal pathogens different from PM. Three *mlo* lines (TLB1, 3 and 4) and the EVB control were inoculated with the downy mildew causal agent *Plasmopora viticola*. None of the plants were resistant and all plants showed statistically comparable levels of susceptibility to the pathogen (Fig. S4).

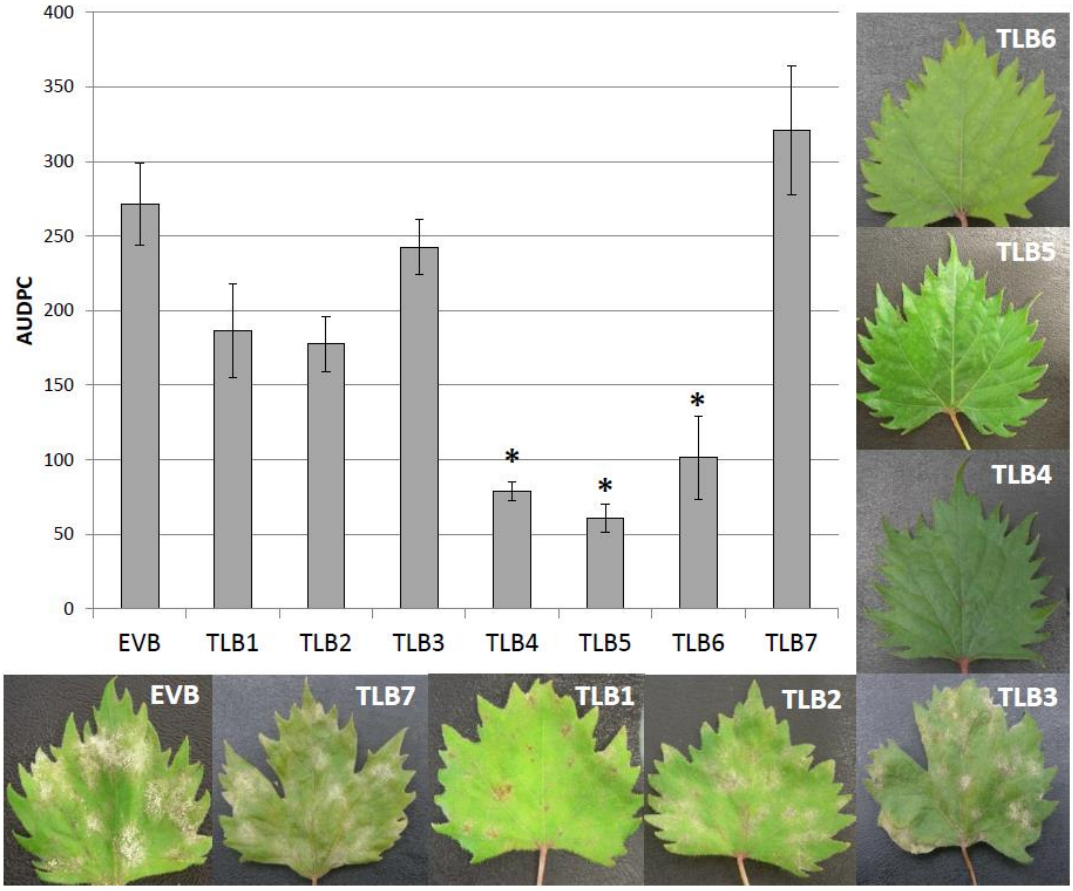

Figure 1. Area under disease progress curve (AUDPC) of grapevines inoculated with *Erysiphe necator* in control (EVB) and transgenic lines (TLB1, 2, 3, 4, 5, 6 and 7). The average scores of AUDPC (from 8-19 biological replicates) from two experiments are reported. Error bars show standard error of the mean. The asterisks indicates statistically significant differences respect to the control line EVB, according to Tukey or Games-Howell post-hoc test (P = 0.05). The representative leaves reproduced here were collected 30 days after inoculation.

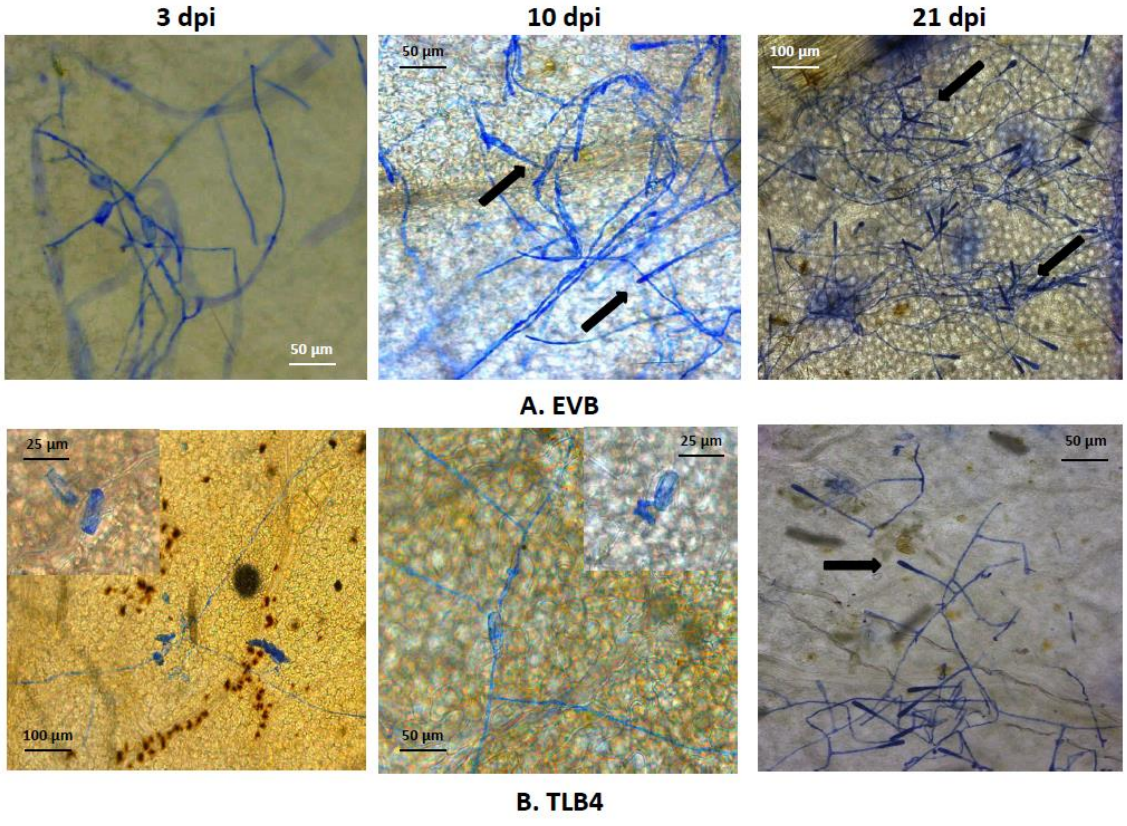

Figure 2: Germination of *Erysiphe necator* conidia in the control line EVB (A) and in the resistant transgenic line TLB4 (B). Microscopy images of infected leaves were taken at 3, 10 and 21 days post inoculation (dpi) with powdery mildew. Insert at high magnification highlighs the germination of an *E. necator* conidia at 3 and 10 dpi.

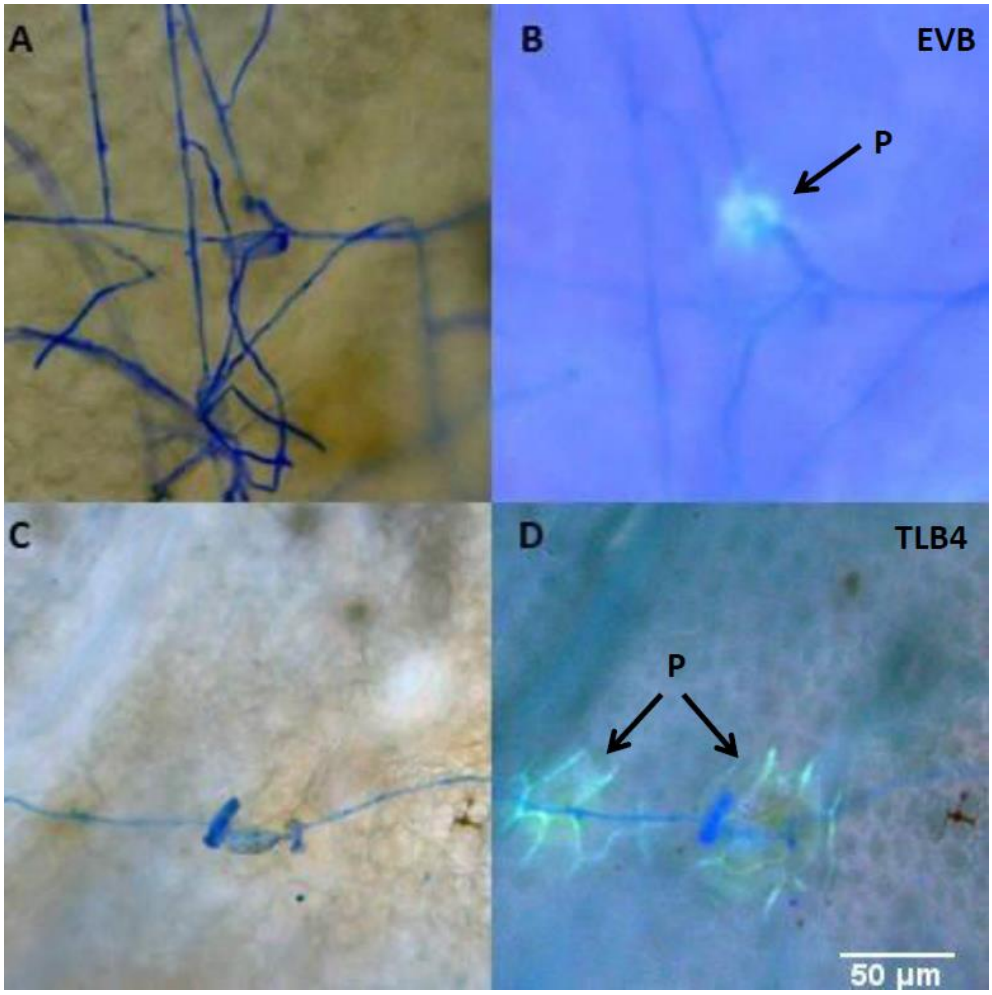

Figure 3. Formation of papillae in the control line EVB (A, B) and in the resistant transgenic line TLB4 (C, D). Microscopy images were taken with bright field (A, C) and fluorescence (B, D) microscope at three days post inoculation (dpi). The arrows indicate the papillae (P). The scale bar is the same for the four images.

Table 1. Disease reduction of seven RNAi lines transformed with *MLO* knock-down constructs.

	Gene transfer	Number of plants	Disease reduction %*			Average reduction (%)
			14 dpi	22 dpi	30 dpi	
TLB1	iii	8	22.8	32.3	34.3	29.8
TLB2	iii	15	49.2	37.2	23.8	36.8
TLB3	iii	15	17.9	14.8	2.0	11.6
TLB4	iv	19	60.8	71.7	72.8	68.4
TLB5	iv	14	76.7	79.1	74.0	76.6
TLB6	iv	11	71.8	63.1	60.3	65.1
TLB7	iii	13	-8.0[#]	-21.5[#]	-21.2[#]	-16.9[#]

* Line EVB was the control (12 replicates). Disease reduction was calculated as Disease severity of EVB – disease severity of the transgenic line divided by disease severity of EVB × 100.

[#] The negative values of TLB7 indicate higher level of infection compared to EVB

Expression of *MLO* genes in the *MLO* transgenic lines and correlation with disease severity

The lines TLB1 to TLB6 and the EVB control were considered in a gene expression analysis. The results concerned four clade V *MLO* genes and supported the off-target cross-silencing noted *in vitro,* showing, in addition, some variability among samples of different time points (Fig. 4). Lines TLB1, 2 and 3, all resulting from transformation (iii) (KD-*VvMLO11*), as expected had the target gene *VvMLO11* silenced. TLB1 showed also knock-down of *VvMLO13* and TLB3 of *VvMLO6* (Table 2). Lines TLB4, 5 and 6 derived from transformation (iv) (KD-*VvMLO13*) showed more off-target silencing: in TLB4 and 6, all four clade V *MLO* genes were, to some degree, significantly silenced, whereas in TLB5 genes *VvMLO6, 7* and *11* were silenced (Table 2).A statistically significant (P = 0.05) positive Pearson's correlation was found between the relative expression of *VvMLO7* and the severity of PM symptoms, but not for the other three *MLO* genes. The Pearson correlation

coefficient for *VvMLO7* was, however, only of 0.27, meaning that the correlation, although significant, was weak.

Table 2. Relative level of expression (RE%)[#] of VvMLO6, 7, 11 and 13 in transgenic lines TBL1 to 6

	VvMLO6	VvMLO7	VvMLO11	VvMLO13
TLB1	67	72	25**	49**
TLB2	79	94	40**	156
TLB3	71*	93	27**	69
TLB4	38**	49**	34**	33**
TLB5	35**	55**	50**	88
TLB6	42**	53**	55**	45**

[#] Each RE% value is the average of time points 0, 1 and 10 dpi and of two experiments. RE% = (RE of control EVB / RE of *mlo* line)*100.

*, ** : statistically significant difference at P=0.05 and P=0.01 respectively, according to the Tukey post-hoc test.

Gene expression analysis of the *mlo* line TLB4The expression profile at three time points of 13 genes not belonging to MLO family and modulated by PM infection, was carried out for the resistant line TLB4 compared to the EVB (Fig. 5 and Table S4). The reasons to choose TLB4 over the other resistant lines were that in this line all four *MLO* clade V genes were knocked-down and the knock-down was more intense than TLB5 and 6. In EVB, in general the genes tested were up-regulated, particularly at 10 dpi. In TLB4, fewer genes were up-regulated and, when so, increase of expression was limited in terms of fold-change (Fig. 5 and Table S4). Moreover, three genes were down-regulated in TLB4 after inoculation, namely *VvPR6* (*pathogenesis related*) at 1 dpi and *VvNPF3.2* (*nitrate transporter/peptide transporter family*) and *VvALS1* (*acetolactate synthase*)) at 10 dpi. It is noteworthy that, before the inoculation, there were no differences in expression between TLB4 and the control EVB (Fig. 5 and Table S4).

DISCUSSION

Loss-of-function mutations of *MLO* genes reduce susceptibility to PM in barley (Büschges *et al*., 1997), *Arabidopsis* (Consonni *et al.,* 2006), pea (Pavan *et al.,* 2011), tomato (Bai *et al.,* 2008), wheat (Wang *et al.,* 2014), and pepper (Zheng *et al.,* 2013). Because in dicots not all Clade V MLO S-genes are implicated in PM susceptibility (Consonni *et al.,* 2006; Bai *et al.,* 2008; Feechan *et al*., 2008; Winterhagen *et al.,* 2008), the aim of this work was to identify which of the clade V *MLO* genes of grapevine has a role in PM susceptibility, and can thus be inactivated to develop resistant genotypes. Out of 26 transgenic lines, six from gene transfers (iii) (KD-*VvMLO11*) and (iv) (KD-*VvMLO13*) supported significant gene knock-down. In the regenerated lines obtained from gene transfers (i) (KD-*VvMLO6*) and (ii) (KD-*VvMLO7*), reduction of expression was not evident. It cannot be excluded that this was due to the short RNAi fragments present in the constructs (Preuss and Pikaard, 2003). The detection of off-target silencing in five of the six mentioned lines was expected, as clade V *MLO* genes have high levels of sequence identity (36-60%, 46% on average; Feechan *et al.,* 2008; Winterhagen *et al.,* 2008). To find a balance between specificity (short RNAi fragments) and effectiveness (long RNAi fragments) is particularly difficult in gene families with high sequence similarity (Zhao *et al*., 2005). Since the aim was studying the effect of the knock-down of four *MLO* genes similar to each other, long RNAi fragments were chosen, so that off-target silencing was not only expected, but also desired.

Knock-out and knock-down of *MLO* genes may induce pleiotropic phenotypes, like necrotic spots on leaves and reduced grain yield in barley (Jørgensen, 1992), slow growth in *Arabidopsis* (Consonni *et al.,* 2006) and reduced plant size in pepper (Zheng *et al.,* 2013). In grapevine, no pleiotropic phenotypes were observed under the experimental conditions adopted.

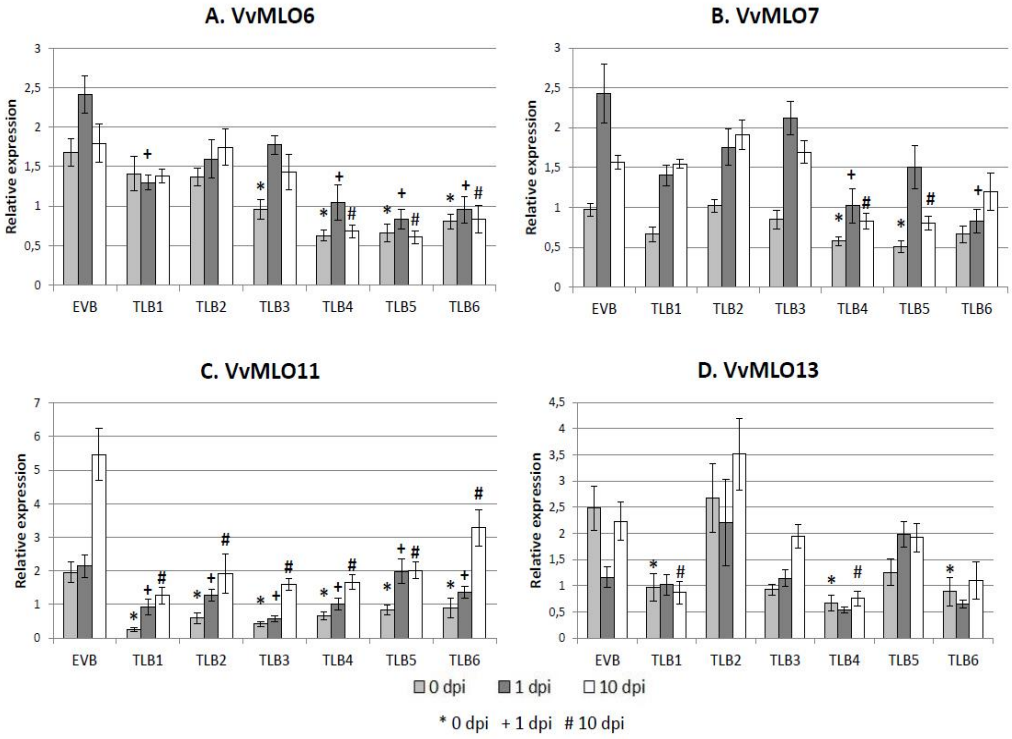

Figure 4. Gene expression of four grapevine *MLO* genes in the *mlo* lines TLB1, 2, 3, 4, 5, and 6 and in the control EVB, following inoculation with *Erysiphe necator*. Expression of *VvMLO6* (A), *VvMLO7* (B), *VvMLO11* (C) and *VvMLO13* (D) was analyzed before (0 dpi; light grey), one (dark grey), and ten (white) days post inoculation. The mean scores of five to nine plants pooled from the two experiments are reported for each line. Error bars show standard error of the mean. For each time point, symbols highlight significant differences respect to the control, according to Tukey or Games-Howell post-hoc test (P = 0.05): * for 0 dpi, + for 1 dpi and # for 10 dpi.

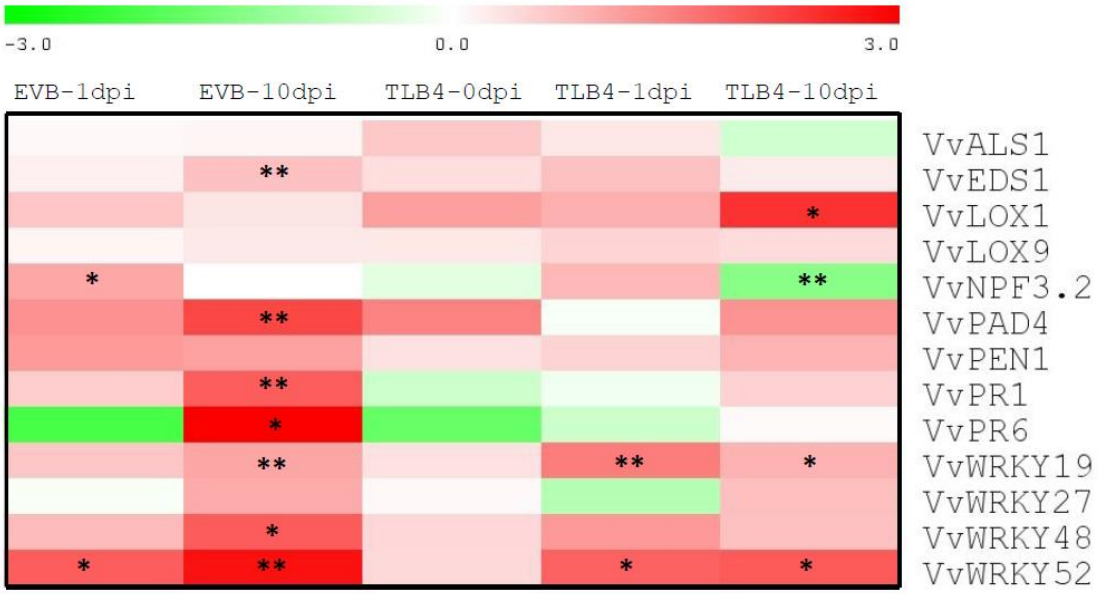

Figure 5. Relative expression of 13 grapevine genes at three time points in the control line EVB and in the resistant line TLB4. The color scale indicates the expression values relative to the control EVB at 0 dpi, used as reference for data normalization. The asterisks highlight statistically significant differences according to Fisher post-hoc test. One and two asterisks indicate significance at P=0.05 and P=0.01, respectively. The image was prepared with the Multiexperiment Viewer software with the Log2 of relative expression data.

Lines TLB4, 5 and 6, which showed clear resistance to PM, allowed studying the link between resistance and the expression of specific *MLO* genes. *VvMLO11* expression was significantly reduced in susceptible and resistant *mlo* lines: it is concluded that its knock-down was not directly linked to grapevine susceptibility to PM. *VvMLO6* was significantly silenced in the resistant lines TLB4, 5 and 6 and in the susceptible line TLB3. Like for *VvMLO11*, the knock-down of *VvMLO6* in both susceptible and resistant lines indicates that this should not be a S-gene. Similarly to *VvMLO6*, was

significantly silenced in the resistant lines TLB4, 5 and 6 and in the susceptible line TLB3. Like for *VvMLO11*, the knock-down of *VvMLO6* in both susceptible and resistant lines indicates that this should not be a S-gene. Similarly to *VvMLO6*, *VvMLO13* was knocked-down in the resistant lines TLB4 and 6, but also in the susceptible line TLB1. *VvMLO7* was knocked-down only in the three resistant lines TLB4, 5 and 6; the conclusion is that *VvMLO7* represents the main candidate for causing PM susceptibility in *V. vinifera*. The significant positive correlation between the relative expression of *VvMLO7* and the disease severity in the MLO transgenic lines stimulates the conclusion that either site directed mutagenesis or searching for natural non-functional alleles may be used in breeding programs to obtain PM resistant genotypes. It was, however, noted that *VvMLO7* was always knocked-down together with other two or three *MLO* genes. Also in *Arabidopsis* the contemporary knock-out of three *MLO* genes is necessary to obtain complete resistance: knock-out of *AtMLO2* results in a moderate level of resistance, whereas knock-out of *AtMLO*6 and *AtMLO1*2, alone or combined, does not decrease the intensity of the infection. When *AtMLO2* is knocked-out together with *AtMLO6* or *AtMLO12,* the level of resistance rises, to become complete when the three genes are knocked-out together (Consonni *et al*., 2006). In grapevine, *VvMLO7* seemed to act like *AtMLO2* of *Arabidopsis.* Two candidates for an additive and synergistic role in PM susceptibility in grapevine are *VvMLO6* and *VvMLO11*, since their expression was significantly reduced in all three resistant lines. In *Arabidopsis,* the knock-out of three *MLO* genes induces complete resistance (Consonni *et al*., 2006), a situation not observed in grapevine, in agreement with the incomplete silencing of *MLO* genes obtained by the RNAi approach. A complementation test, carried out in *Arabidopsis mlo* triple mutant*,* showed that *VvMLO11* and *VvMLO13* induce susceptibility to PM, whereas *VvMLO7* has only a partial effect and *VvMLO6* has no effect at all (Feechan *et al.,* 2013b). However, single and double *VvMLO11* and

VvMLO13 knock-down mutants of *V. vinifera* obtained by RNAi, did not show significant reduction of PM penetration (Qiu *et al*., 2015). Accordingly, our data indicated *VvMLO7* as the main S-gene of grapevine, with a putative additive effect provided by *VvMLO11* and *VvMLO6.* The role of *VvMLO6* would be particularly surprising, as it was not up-regulated during PM infection (Feechan *et al.,* 2008; Winterhagen *et al*., 2008). Conversely, *VvMLO13*, which knock-down was expected to provide a significant effect on PM susceptibility, turned out to be ineffective. However, it should be considered that Feechan *et al*. (2013b) operated in a heterologous system (*Arabidopsis)* not reproducing with fidelity the PM infection of grapevine plants.

The precise mechanism through which the reduction of *MLO* genes expression ends up in resistance to PM pathogens is not completely clear. Resistance seems linked to secretory vesicles traffic (Miklis *et al.,* 2007; Feechan *et al*., 2011) and to the formation of cell wall appositions called papillae (Consonni *et al.*, 2006). These structures consists of a callose matrix enriched in proteins and autofluorogenic phenolics compounds (Vanacker *et al*., 2000), and their formation depends on endomembrane transport (Hückelhoven, 2014). The results shown in this paper indicate that all transgenic lines accumulate autofluorogenic materials over-imposed to the papilla structure, although shape and dimensions of papillae were different in resistant and susceptible lines. It is known that the defense response based on papillae differs between resistant and susceptible genotypes in timing of formation, composition and size (Chowdhury *et al.*, 2014; Hückelhoven, 2014; Lyngkjær *et al.* 2000). Rapid formation of papillae in *mlo* resistant barley (Lyngkjær *et al.* 2000) and increased size (Stolzenburg *et al.*, 1984) correlate with *mlo* resistance. In grapevine, papilla formation is restricted to the site of infection in control plants, whereas it is diffused in the resistant line TLB4. Chowdhury *et al*. (2014) showed that the difference between effective and non-effective papillae is

due to the higher concentration of callose, cellulose and arabinoxylan of the effective ones. This suggests that, in the case of grapevine, different types of fluorescence could reflect differences in the composition of the papillae. The MLO protein has been proposed to be a negative regulator of vesicle-associated and actin-dependent defense pathways at the site of attempted PM penetration (Panstruga, 2005). Furthermore, Miklis *et al*. (2007) proposed that, once MLO proteins are under the control of the fungus, actin filaments serve the purpose of supplying nutrients for the growing hyphae through vesicular transport. It is like if the pathogen is able to control the transport of material to the cell-wall, with the purpose of changing the composition of the papillae and turning them from effective to non-effective.

The formation of papillae is not the only process instigated by the activity of *MLO* genes. To understand the effect of *MLO* knock-down on other genes involved in plant-pathogen interaction, the expression of 13 genes known to be differentially expressed after PM inoculation was analyzed. In the resistant line TLB4, the knock-down of *MLO* genes did not affect the level of expression of the 13 genes in absence of PM infection. Under *E. necator* infection (Guo *et al*., 2014), transcription factors *VvWRKY19*, *VvWRKY48* and *VvWRKY52* are up-regulated: the same genes appeared up-regulated in EVB in our experiments, but they were so at a much lower level in TLB4. *VvNPF3.2,* a nitrite/nitrate transporter up-regulated in grapevine infected with *E. necator* (Pike *et al*., 2014), was down-regulated in TLB4 at 10 dpi, indicating that in this line only a severe infection elicits *VvNPF3.2* up-regulation. *VvEDS1* (*enhanced disease susceptibility*) and *VvPAD4* (*phytoalexin deficient*) are grapevine defense genes involved in the salicylic acid (SA) pathway (Gao *et al*., 2014). SA activates pathogenesis related genes and induces disease resistance (Ward *et al.,* 1991). Both genes were up-regulated in the control line EVB at 10 dpi (*VvPAD4* also at 1 dpi). This may indicate that only a heavy *E. necator* infection triggers the plant

defense depending on SA. *VvEDS1* was not up-regulated in TLB4, whereas *VvPAD4* was up-regulated only at 10 dpi, like if the level of PM infection was insufficient to activate the reaction of the plant. Up-regulation in the control and no up-regulation in TLB4 were also observed for both *VvPR1* and *VvPR6*, pathogenesis-related genes involved in plant defense and known to be up-regulated in PM infected grapevine leaves pre-treated with an SA analogue (Dufour *et al*., 2013). *VvLOX1* encodes a lipoxygenase and is the homologous to *Arabidopsis AtLOX2*, which is up-regulated in plants infected with PM spores (Lorek*,* 2012). Surprisingly, this gene was up-regulated in TLB4 at 10 dpi, but not in EVB. A second lipoxygenase, *VvLOX9*, did not show in the grapevine lines considered any change in expression, despite being known to be involved in plant defense (Dufour *et al.,* 2013). *VvPEN1* (penetration) encodes for a SNARE protein homologous to *Arabidopsis AtPEN*1 and barley *ROR2,* which have important roles in PM penetration resistance (Collins *et al*., 2003). *VvPEN1* when expressed in a heterologous system (*Arabidopsis*) is known to co-localize with *VvMLO11* at sites of attempted PM penetration (Feechan *et al.,* 20013b). However, infection with *E. necator* did not cause any change of its expression. *VvALS1* is the homologous of a tomato acetolactate synthase, a key enzyme in the biosynthesis of the amino acids valine, leucine and isoluecine, and involved in PM resistance (Gao D. *et al.,* 2014). Silencing of *SlALS1* in *Ol-1 tomato* compromises its resistance to PM, suggesting that amino acid homeostasis is an important process connected to PM resistance (Gao D. *et al.,* 2014). The complete lack of transcriptional change indicated that the function of this gene in grapevine does not depend on the transcript level.

The knock-out of *MLO* genes increased susceptibility to other pathogens in barley (Jarosch *et al.,* 1999; Kumar *et al.,* 2001) and *Arabidopsis* (Consonni *et al.,* 2006). The infection with *P. viticola*, an obligate biotroph fungus like *E. necator*, revealed that the knock-down of *MLO* genes did not change the susceptibility of grapevine

to downy mildew, supporting the conclusion that MLOs S-genes are specific for *E. necator* and are not involved in the plant interaction with *P. viticola*.

CONCLUSIONS

The knock-down of *MLO* genes substantially reduces PM susceptibility of *Vitis vinifera*. The reduction of expression of *VvMLO7* was the main factor involved in resistance, but the additive effects of *VvMLO6* and *VvMLO11* knock-down further contribute in reducing PM severity. Absolute resistance was not observed, as expected based on the incomplete silencing of *MLO* genes via RNAi. In *mlo* lines, no pleiotropic phenotypes were detected under greenhouse conditions. This work provides crucial information that can be used in breeding grapevine varieties resistant to *E. necator*. The tagging via genome editing of the *MLO* genes identified in this paper, particularly of *VvMLO7,* should result in knock-out mutants highly resistant to PM. Alternatively, the search in *V. vinifera* and in wild species of non-functional *MLO* alleles, particularly of *VvMLO7*, should contribute to the creation of durable resistance.

AKNOWLEDGMENT

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ABBREVIATIONS

AUDPC: area under disease progress curve

dpi: days post inoculation

EVB: empty vector Brachetto

PM: powdery mildew

RE: relative expression

TLB1-7= Transgenic Line Brachetto 1-7

SUPPLEMENTARY MATERIALS

Table S1. Primers used to amplify *MLO* genes fragments for the RNAi constructs.

Gene[#]	*Alternative name*°	*Genoscope ID**	*Primer Forward*	*Primer Reverse*	*Amplicon Lenght*
VvMLO6	*VvMLO13*	*GSVIVT0002450600*	CACCTGCTTACAGTATTACAAACTCCC	TTTCCCTTGCATACCTAAAC	327 bp
VvMLO7	*VvMLO17*	*GSVIVT00018219001*	CACCGACAATTTTTAACGAGAGAGT	ATCTCATGTTGGGTTCGGATT	369 bp
VvMLO11	*VvMLO3*	*GSVIVT00023170001*	CACCTCACTTATGCTACTGGGGTT	ATCAACTTTGGGAACTGATCTGAC	504 bp
VvMLO13	*VvMLO4*	*GSVIVT00024507001*	CACCGAGCTAATGTTGCTAGGGTT	AAATTTTGCATGGCTTTGAG	627 bp

[#] *MLO* genes nomenclature proposed by Winterhagen *et al.* (2008).

° MLO genes nomenclature proposed by Feechan *et al.* (2008).

* Sequences available at http://www.genoscope.cns.fr

Table S2 (Part I). Primers used to amplify MLO genes fragments for the qPCR analysis.

Name	Genoscope ID*	Forward (‘5 – ‘3)	Reverse (‘5 – ‘3)	Class	Reference
EF1α	GSVIVT01025147001	GAACTGGGTGCTTGATAGGC	AACCAAAATATCCGGAGTAAAAGA		Reid *et al.* (2006)
GAPDH	GSVIVT01007521001	TTCTCGTTGAGGGCTATTCCA	CCACAGACTTCATCGGTGACA		Reid *et al.* (2006)
Actin	GSVIVT01026580001	TCCTTGCCTTGCGTCATCTAT	CACCAATCACTCTCCTGCTACAA		Reid *et al.* (2006)
VvMLO6	GSVIVT01025160001	GTGCAGTTATGTGACACTCCC	ACACACCATCCGAGTGC	*MLO* gene	Winterhagen *et al.* (2008)
VvMLO7	GSVIVT01016304001	CTTTCTTCGCATGGAGCACG	GAGCCCATCTGTGTCACCAA	*MLO* gene	/
VvMLO11	GSVIVT01025653001	GCACCCCCTTACATGGC	TCTGGACCAGGATTTCTATGATG	*MLO* gene	Winterhagen *et al.* (2008)
VvMLO13	GSVIVT01025162001	CTGGTGACACAGATGGGTTC	CTACTTGACATGGGTGTGGC	*MLO* gene	Winterhagen *et al.* (2008)
VvWRKY19	GSVIVT01000752001	GGGGAGGCTGTGGTTAGGTT	GTTTGGCATTTGGCTTGTCT	Transcription factor	Guo *et al.* (2014)
VvWRKY27	GSVIVT01030174001	CTTGGATCAGAATCACCCCTAA	GCCGTGGTATGTGGTTTTGTA	Transcription factor	Guo *et al.* (2014)
VvWRKY48	GSVIVT01027069001	CAAGATTTCAAGGACCAAGCAG	AGTATGCCTTCCTCGGTATGT	Transcription factor	Guo *et al.* (2014)
VvWRKY52	GSVIVT01028718001	CCTCTTGATGATGGGTTTAGTT	GTCTTCCACGGTAGGTGATTT	Transcription factor	Guo *et al.* (2014)
VvALS1	GSVIVT01033088001	CCGTGCATACCGAGCATTTG	AGGCCGGTTCTGTATGTTGG	Acetolactate synthase	/
VvEDS1	GSVIVT01007836001	AGGGTTTTATATTGTTATCTCAAGGC	GGAAGAAAATATCTTATTACTACATA ATGTTTC	Lipase-like protein	Gao *et al.* (2014)
VvLOX9	GSVIVT01000084001	GACAAGAAGGACGAGCCTTG	CATAAGGGTACTGCCCGAAA	lipoxygenase	Dufour *et al.* (2013)

* Sequences available at http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/

Table S2 (part II). Primers used to amplify MLO genes fragments for the qPCR analysis.

Name	Genoscope ID*	Forward (‘5 – ‘3)	Reverse (‘5 – ‘3)	Class	Reference
VvLOX1	GSVIVT01032029001	ATCAATGCTCTTGCTCGGGA	CCAGAGCTGGTCATAGGCAG	lipoxygenase	/
VvPAD4	GSVIVT01007860001	ACGATTGCACTGGTAAGCCA	CGACTCCGTCATCGCCTAAA	Lipase-like protein	/
VvPEN1	GSVIVT01022593001	CTTCGCAAGAAGCTCAGGGA	TGCTCTTGGATCGCCTTCTG	syntaxin	/
VvPR1	GSVIVT01037005001	CCCAGAACTCTCCACAGGAC	GCAGCTACAGTGTCGTTCCA	Pathogenesis related	Dufour *et al.* (2013)
VvPR6	GSVIVT01018137001	ACGAAAACGGCATCGTAATC	TCTTACTGGGGCACCATTTC	Pathogenesis related	Dufour *et al.* (2013)
VvNPF3.2	GSVIVT01025795001	TCGTCACATCAGCACAGCTT	ATCTGCGAGCCAATGGAACA	NO_2^-/ NO_3^- transporter	/

* Sequences available at http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/

Table S3. Summary of gene transfers results

Gene transfer	Knock-down construct	Regenerant lines	Confirmed transgenic	Selected
i	*VvMLO6*	1	1	/
ii	*VvMLO7*	3	1	/
iii	*VvMLO11*	13	10	TLB1, TLB2, TLB3, TLB7
iv	*VvMLO13*	13	11	TLB4, TLB5, TLB6
Empty Vector	/	7	6	EVB

Table S4. Relative expression of 13 grapevine genes at three time points in EVB and in TLB4

	0 dpi EVB	1 dpi EVB	10 dpi EVB	0 dpi TLB4	1 dpi TLB4	10 dpi TLB4
VvALS1	1.00	1.06	1.09	1.58	1.22	0.69
VvEDS1	1.00	1.13	1.67	1.32	1.66	1.19
VvLOX1	1.06	1.60	1.25	2.20	1.93	5.40
VvLOX9	1.12	1.09	1.20	1.22	1.42	1.34
VvNPF3.2	1.02	2.06	0.99	0.79	1.79	0.38
VvPAD4	1.29	2.46	4.51	2.78	0.94	2.42
VvPEN1	1.13	2.29	2.16	1.28	1.43	1.84
VvPR1	1.06	1.51	3.75	0.67	0.89	1.45
VvPR6	1.07	0.22	12.08	0.29	0.66	1.04
VvWRKY19	1.02	1.58	2.07	1.27	2.91	1.88
VvWRKY27	1.27	0.94	1.99	1.06	0.55	1.69
VvWRKY48	1.21	1.74	3.78	1.38	2.31	1.67
VvWRKY52	1.21	3.76	7.00	1.37	3.61	3.84

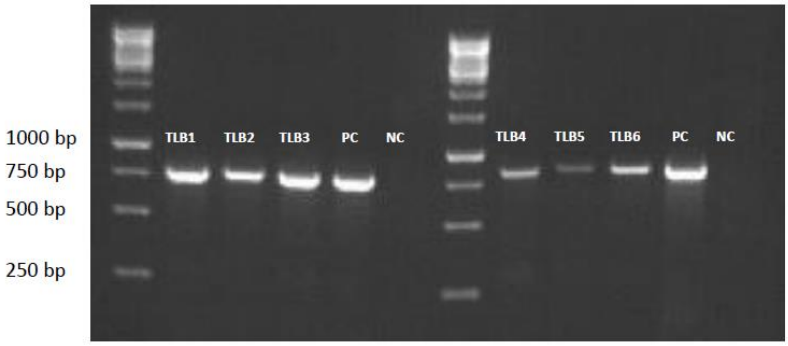

Figure S1. Presence of the construct in the transgenic lines selected for powdery mildew inoculation. The positive control (PC) was a colony PCR on the *Agrobacterium tumefaciens* strain used for gene transfer. The negative control (NC) was the DNA of wild-type ''Long-Cluster Brachetto''. The expected fragments length were 714 bp for lines TLB1 to 3 (210 for the 35s promoter and 504 bp for the insert), and 837 bp for lines TLB4 to 6 (210 for the 35s promoter and 627 bp for the insert).

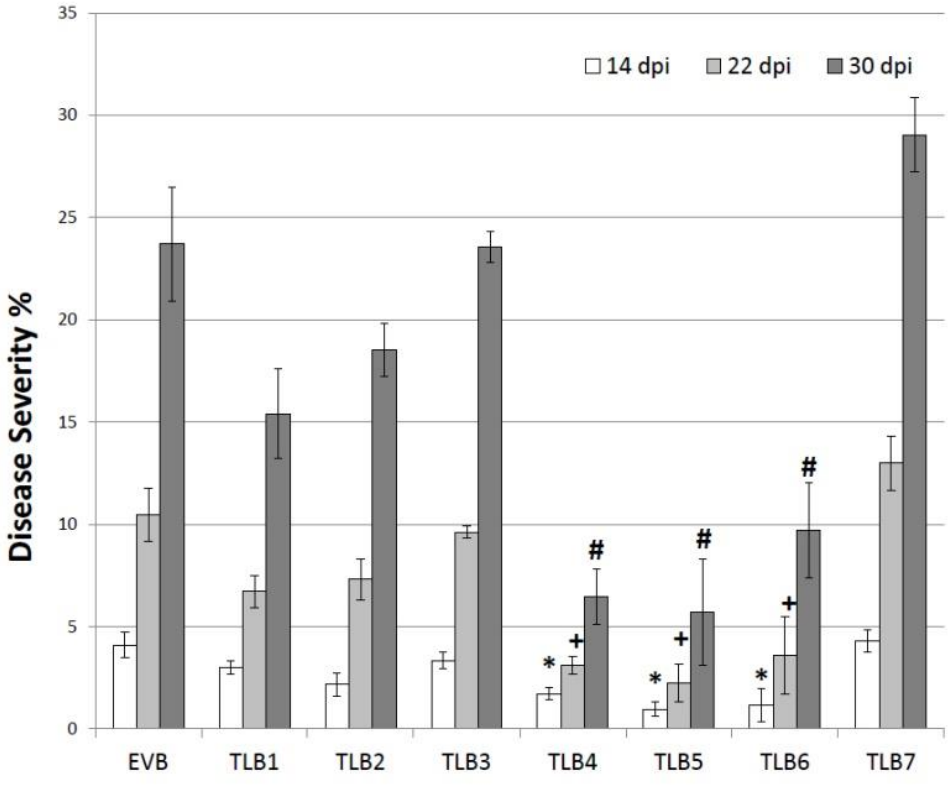

Figure S2. Disease severity at three time point of grapevine transgenic lines inoculated with *Erysiphe necator*. The mean scores of powdery mildew severity were calculated on 8-19 biological replicates from two experiments. Error bars show standard error of the mean. For each time point, symbols highlight significant differences respect to the control EVB, according to Tukey or Games-Howell post-hoc test (P = 0.05): * for 0 dpi, + for 1 dpi and # for 10 dpi.

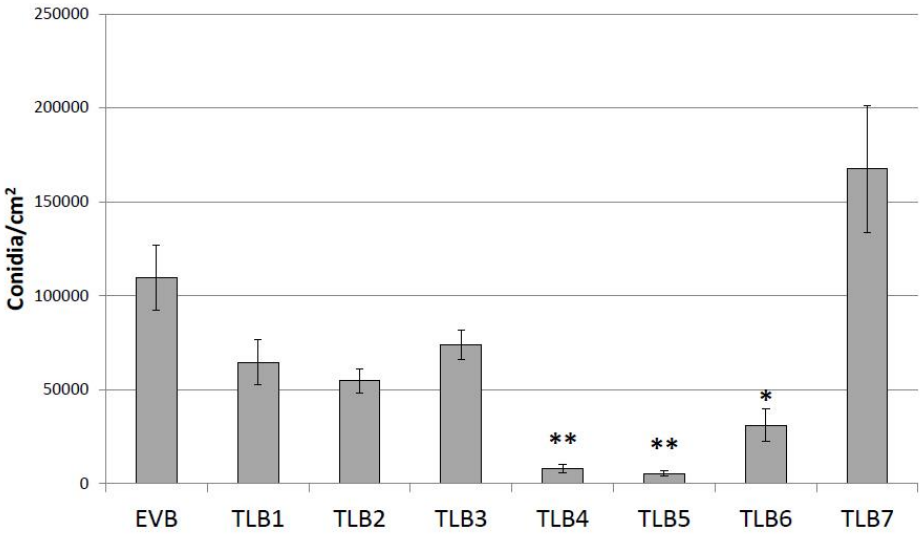

Figure S3. Number of conidia per leaf surface (cm^2) of grapevines inoculated with *Erysiphe necator* at 30 dpi. Control (EVB) and transgenic lines (TLB1, TLB2, TLB3, TLB4, TLB5, TLB6 and TLB7). The mean values of conidia counts of 8-19 biological replicates from two independent experiments are reported. Error bars show standard error of the mean. One (P = 0.05) and two (P = 0.01) asterisks highlight statistically significant differences compared to line EVB, according to Kruskall-Wallis test.

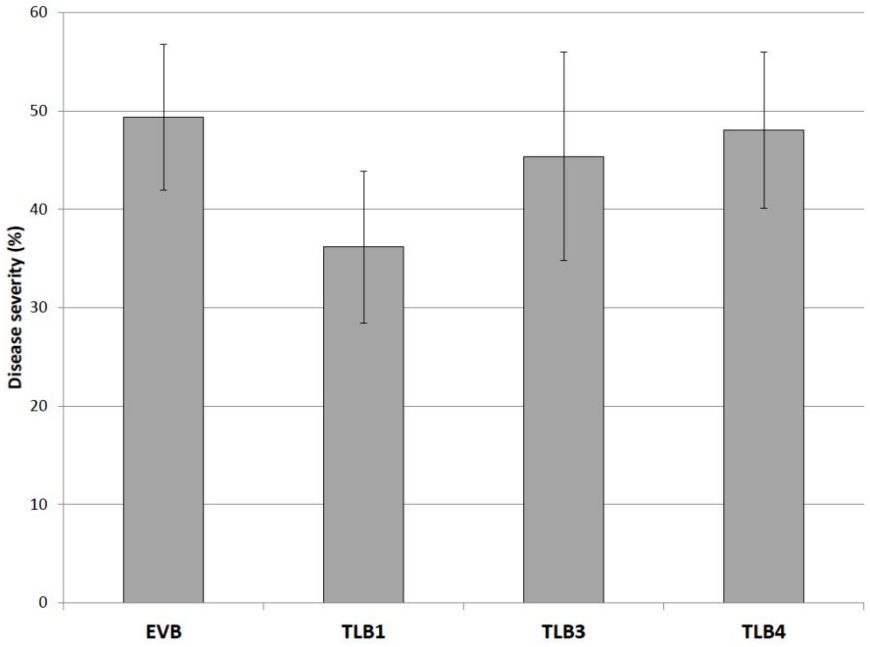

Figure S4. Disease severity a 7 dpi of grapevine transgenic lines inoculated with *Plasmopora viticola*. The mean scores of downy mildew severity were calculated on 6-9 biological replicates from two experiments. Error bars show standard error of the mean. Tukey post-hoc test (P = 0.05) revealed non-significant differences among the grapevine lines.

CHAPTER 6

General Discussion

The *mlo* resistance

Powdery mildew (PM) is a major plant disease affecting thousands of species worldwide. The main symptoms of PM are grey or white spots on the upper surface of the leaves, but blossoms and fruits can be infected as well (Glawe, 2008). Apple and grapevine are both susceptible to PM and they require a huge amount of chemicals for disease control. Resistance genes (R-genes) are available, but most of them lose their efficacy in a few years. However, PM resistance can also be granted by loss-of-function of specific *MLO* genes (*mlo* resistance). This particular kind of resistance was discovered in Germany in 1942, when a PM resistant barley line was obtained by random mutagenesis (Jørgensen, 1992). Successfully used in barley breeding for decades, *mlo* resistance is still effective nowadays. The most appealing characteristics of *mlo* resistance are durability and broad-spectrum efficacy (Jørgensen, 1992), two extremly valuable traits for both economic and environmental reasons. For a long time *mlo* resistance was considered unique for barley (Jørgensen, 1992), but it was subsequently discovered or obtained by various means in other plant species (Consonni *et al.,* 2006; Bai *et al.,* 2008; Pavan *et al.,* 2011; Zheng *et al.,* 2013; Wang *et al.,* 2014). This resistance is the result of the loss-of-function of specific member of the large *MLO* gene family, which act as S-genes. These S-genes codes for negative regulator of vesicle-associated and actin-dependent defense pathways at the site of attempted PM penetration and their function is harnessed by PM-pathogens to shut down plant defense (Panstruga*,* 2005).

The *mlo* resistance is a remarkable trait with clear applications in breeding. The introgression of durable and broad-spectrum resistance in cultivated species would allow a significant decrease in the amount of fungicides applied to control PM, resulting in huge benefits for the environment, the workers and the growers (Yoder, 2000; Fuller *et al.,* 2014). Furthermore, there is a growing scientifc interest in the

complex topic of plant-pathogen interaction and the study of *MLO* genes can help to shed some light on it.

Apple and grapevine are two important fruit crops and they require a big amount of fungicides for PM control. *MLO* genes of grapevine have been studied in recent years (Feechan *et al.,* 2008; Winterhagen *et al*., 2008; Feechan *et al.,* 2013b), but the responsible gene(s) for PM-susceptibility were not identified. No studies have been ever carried out on apple *MLO* genes, therefore we started retrieving the family members from the apple genome and proceeded with their functional characterization.

Characterization of MLO genes in Rosaceae

The Rosaceae family comprises some of the most important plant species for humans: ornamental plants like rose and fruit plants like apple, pear, peach, apricot, strawberry and cherry. PM affects many rosaceae species, including the widely cultivated apple, strawberry and peach (Boesewinkel 1979; Xiao *et al.*, 2001; Foulongne *et al*., 2003; Turecheck, 2004).

The screening of the peach (*Prunus persica*) and woodland strawberry (*Fragaria vesca*) genomes returned 19 and 18 *MLO* homologs, respectively (Chapter 2). Previous genome-wide studies on dicotyledonous species resulted in comparable outcomes: 15 *MLO* homologs in *Arabidopsis thaliana* (Devoto *et al.,* 2003), 17 in grapevine (Feechan *et al.,* 2008; Winterhagen *et al*., 2008), 13 in cucumber (Schouten *et al.,* 2014) and 17 in tomato (Chen *et al.,* 2014). Conversely, the screening of apple (*Malus domestica*) genome returned 21 genes, which is lower than expected: the *Pyrae* tribe went through a recent genome duplication (Velasco *et al.*, 2010). Therefore the expected number of apple MLO homologous was around 30. The *MLO* homologues of apple, peach and woodland strawberry are scattered in different chromosomes, consistently with what observed in other

species (Devoto *et al.,* 2003; Feechan *et al.,* 2008; Winterhagen *et al*.,2008; Chen *et al.,* 2014; Schouten *et al.,* 2014). Nonetheless, some clusters were found, such as *PpMLO3-PpMLO8-PpMLO18, PpMLO12-PpMLO16, PpMLO1-PpMLO14, FvMLO3-FvMLO4, FvMLO6-FvMLO7* and *MdMLO2-MdMLO3-MdMLO8*. This suggests that the prevailing evolutionary mechanism for the Rosaceae *MLO* genes was segmental duplication, although the clusters are probably the result of tandem duplications (Chapter 2).

The phylogenetic analysis led to the identification of one, or possibly two, further clade(s), depending on the clustering method adopted (UPGMA or Neighbor-Joining). However, the results of other studies support the presence of one extra clade only, named clade VII (Zhou *et al.,* 2013; Acevedo-Garcia *et al.,* 2014; Chen *et al.,* 2014). The presence in clade VII of an homologue from tomato (SlMLO2 - Chen *et al.,* 2014) and one from cucumber (CsMlO11 - Zhou *et al.,* 2013), rules out the possibility that the new clade is specific for Rosaceae, as initially hypothesized (Chapter 2). Furthermore, the phylogenetic analysis revealed the presence of apple, strawberry, peach and apricot homologs in clade V, which is the clade containing all the dicots MLO homologs with a role in susceptibility (Bai *et al.,* 2008; Consonni *et al.,* 2006; Pavan *et al.,* 2011; Zheng *et al.,* 2013). It has to be noted that, to our knowledge, there is no phylogenetic tree that includes all MLO homologues retrieved so far. The possible outcomes of this kind of analyses are interesting, such as the grouping of clades that are now divided or the division of clades that are now united.

Identification of candidate S-genes in apple

If it is true that all S-genes of dicots belong to Clade V, it is not true that all members of clade V are S-genes. The simple phylogenetic analysis is not enough to identify S-genes, although it allows narrowing down the number of candidates. Conversely,

gene expression analysis is considered a more reliable system to identify S-genes. *MLO* S-genes are up-regulated upon PM-fungi inoculation, as documented in tomato (Bai *et al.,* 2008), barley (Piffanelli *et al.,* 2002), pepper (Zheng *et al.,* 2013) and grapevine (Feechan *et al*., 2008; Winterhagen *et al.,* 2008), although very little is known about the mechanism behind this transcriptional response. In apple, three genes (*MdMLO11, MdMLO18* and *MdMLO19*) were significantly up-regulated in three cultivars ('Golden Delicious', 'Gala' and 'Braeburn') between 4 and 8 hours from the inoculation. An average 2-fold increase of expression was observed, with three peaks of 4-fold increase. This up-regulation intensity is comparable to what observed in grapevine by Winterhagen *et al.* (2008). Conversely, Feechan *et al.* (2008) detected a much higher fold-change (12-40 fold). Both mentioned works were carried out in the grapevine cv. Cabernet Sauvignon, but Feechan *et al.* (2008) inoculated detached leaves, whereas Winterhagen *et al*. (2008) inoculated whole plants. In both studies the same inoculation system was used, i.e. dry-brushing the target leaves with infected ones. Despite the fact that this method does not really allow to quantify the spores contained in the inoculum, the inocula of the two studies were probably comparable. Therefore this factor cannot explain the different *MLO* genes up-regulation observed in the two studies. The up-regulation of *MLO* genes might be less intense in whole plants rather than detached leaves.

MdMLO11 and *MdMLO19* encode for proteins falling in clade V, thus making them obvious candidates for causing PM susceptibility. These two genes are paralogs: they resides on chromosomes 4 and 12 respectively, both generated from the duplication of a chromosome in the ancestor of apple (Velasco *et al*., 2010), and they have high sequence identity and similarity (88% identity at nucleotide level, 93% similarity and 86% identity at amino acids level). Peach *PpMLO3*, apricot *PaMLO3* and woodland strawberry *FvMLO4* are orthologs of *MdMLO19*. Since

orthologs often maintain similar functions during evolution, the expression of these genes might also be responsive to PM-fungi.

MdMLO18, the third up-regulated gene upon *Podosphaera leucotricha* inoculation, encodes a protein grouping in clade VII. This is not the first case of an up-regulated gene outside clade V, as seen in tomato (Appiano et *al.,* unpublished), but there are no reports of S-genes outside clade V in dicots. Peach *PpMLO9* and woodland strawberry *FvMLO15* are likely orthologs of *MdMLO18*, so they should also be considered as putative PM-responsive genes.

Apple clade V contains two more genes, *MdMLO5* and *MdMLO7*, which showed no up-regulation upon inoculation. Accordingly, some clade V *MLO* genes of grape (Feechan *et al*., 2008; Winterhagen *et al*., 2008) and tomato (Appiano *et al.,* unpublished) were also not up-regulated upon inoculation. These apple non-responsive genes were not considered candidates S-genes.

Validation of the role of candidate S-genes of apple

Based on the available apple genome sequence, there are 21 MLO genes in *M. domestica*, some of which might be involved in the interaction with the PM causing agent *P. leucotricha*. Chapter 3 describes our study on the role of three apple *MLO* genes in PM susceptibility: *MdMLO11* and *MdMLO19,* which both belong to clade V, and *MdMLO18*, which belongs to clade VII. To date, there are no reports of *MLO* genes outside clade V acting as S-genes in dicots. However, clade VII appears to be basal to both clade IV and clade V (see phylogenetic tree presented in Chapter 2), and thus might have contained ancestral proteins which later on evolved into PM susceptibility factors. With the intention of unravelling a possible role for clade VII in the interaction between the host and PM-fungi, *MdMLO18* was studied by using it in a complementation test in *A. thaliana Atmlo2/6/12* mutant, but it failed to complement. This confirms the large amount of evidence that only genes

belonging to clade V can act as S-genes in dicots (Consonni *et al.,* 2006; Bai *et al.,* 2008; Humphry *et al.,* 2011; Pavan *et al.,* 2011; Zheng *et al.,* 2013; Acevedo-Garcia *et al.,* 2014). However, it should be noted that the complementation results obtained in *A. thaliana* for grapevine *MLO* genes (Feechan *et al.,* 2013b) were not confirmed in *V. vinifera* plants (Chapter 5), therefore the role of *MdMLO18* should be further studied through knock-down or knock-out in *M. domestica* plants. We obtained one apple transgenic line where *MdMLO18* is knocked-down, but we did not have time to test it. This will be done in the future.

MdMLO11 and *MdMLO19* were knocked-down in apple to study their putative role in PM susceptibility. *MdMLO19* expression was reduced in both resistant lines, suggesting that it was the gene responsible for susceptibility. The knock-down of *MdMLO11,* either alone or combined with *MdMLO19*, did not result in any additional reduction of infection. The knock-down of the other two apple genes clustering in clade V, *MdMLO5* and *7,* was not studied because they were not up-regulated upon *P. leucotricha* infection (Chapter 2). However, our work in grapevine (Chapter 5) revealed a putative role for *VvMLO6*, a gene that is also non-responsive to PM inoculation, therefore, the possibility of a role in susceptibility for *MdMLO5* and *MdMLO7* cannot be ruled out entirely and should be further investigated.

The expression of 17 genes known to be involved in plant-pathogen interaction was analysed, in order to better understand the effect of the knock-down of *MLO* genes. In absence of infection, five genes involved in a variety of processes were down-regulated in TG11+19 compared to 'Gala'. Three of them were defense genes, suggesting that TG11+19 defense is moderately inhibited under normal conditions. However, TG11+19 was also the most responsive line upon *P. leucotricha* inoculation, with 13 up-regulated genes out of 17 at 24 hpi. Only three of these genes were still up-regulated at 10 dpi, suggesting that the transcriptional response of the plant is more intense in early stages of pathogenesis. TG19 showed less

transcriptional changes in absence of infection, as only one gene was down-regulated. The response of the line was moderate also upon inoculation: four genes were up-regulated at 24 hpi and two of them were still up-regulated at 10 dpi. This limited transcriptional response might be due to the moderate infection on the leaves of TG19, not sufficient to trigger the up-regulation of specific genes. In 'Gala', five genes were up-regulated at 24 hpi and only two were still up-regulated at 10 dpi. The gene expression analysis at 24 hpi and 10 dpi showed that the response to *P. leucotricha* inoculation is similar between control and *mlo* lines, as only few significant differences were detected. The conclusions are: 1) not surprisingly, the knock-down of two *MLO* genes in TG11+19 caused more changes in the expression of other genes compared to the knock-down of one single *MLO* gene in TG19. 2) The effect of *P. leucotricha* inoculation is moderate at 10 dpi. 3) The most intense transcriptional response happens in the early stages of pathogenesis, in agreement with several studies that showed that transcriptional response of *MLO* genes is concentrated in the first hours post-inoculation (Chapter 2; Piffanelli *et al.,* 2002; Bai *et al.,* 2008; Feechan *et al.,* 2008; Winterhagen *et al.,* 2008; Zheng *et al.,* 2013). 4) Of the 17 genes considered, only *MdGST* showed a similar pattern of down-regulation in the two transgenic lines. Three other genes were down-regulated in both TG19 and TG11+19, but only at specific time points, namely *MdBSI3* at 0 hpi and *MdVSP1* and *MdPR2* at 10 dpi. All these genes are involved in defense.

Allele mining of apple *MLO* genes

The screening of five *MLO* genes of apple (*MdMLO5, MdMLO7, MdMLO11, MdMLO18* and *MdMLO19*) in the Fruitbreedomics re-sequencing dataset (www.fruitbreedomics.com) led to the identification of 678 SNPs, 127 of which were located in the exons. Silent and conservative mutations were predominant over non-conservative and nonsense ones. This can be explained by positive

selection that prevents the inheritance and spreading of non-advantageous mutations, whereas neutral mutations are subjected to random fixation (Kimura, 1977). Two of the five genes considered were particularly interesting for opposite reasons: *MdMLO5* was the gene with the lowest number of SNPs, whereas *MdMLO19* was the gene with the highest number of SNPs and the only one where a mutation causing an early stop codon was found. The case of *MdMLO5* suggests that the gene is under an intense stabilizing selection and since *MdMLO5* is not targeted by *P. leucotricha* (Chapter 2 and 3), PM selection pressure should not favor the fixation of new mutations. Three factors may contribute to the higher number of mutations in *MdMLO19*: 1) it is the primary target of *P. leucotricha,* suggesting that the co-evolution of the host and the pathogen might be the reason of the high mutation rate; 2) *MdMLO19* causes susceptibility to PM and its loss-of-function resulted in a reduction of susceptibility (Chapter 3), a situation where disruptive mutations result in resistance to the pathogen; 3) the activity of *MdMLO11*, the paralog of *MdMLO19* (Chapter 2), supports the loss-of-function of *MdMLO19* without drastically reducing the fitness of the plant because *MdMLO11* may partially accomplish the metabolic function of *MdMLO19*.

The only mutation causing the formation of an early stop codon was found in *MdMLO19*: the insertion of a thymine in position 1201 causes a frameshift that results in an early stop codon located 15-17 bp after the insertion. The total length of the CDS of *MdMLO19* is 1773 bp, meaning that the insertion causes the translation of an incomplete protein of 405 amino acids instead of the regular 590. The loss of 185 aa alone would probably compromise the function of MdMLO19, but the C-terminal is also the region of MLO proteins that carry the calmodulin-binding domain, which loss has been demonstrated to halve the ability of HvMLO to negatively regulate defense against barley PM (Kim *et al.,* 2002). It is reasonable to assume that the truncated MdMLO19 is a non-functional or partially functional

protein. To grant resistance to PM, both the allele of *MdMLO19* would have to carry the insertion.

To date, the natural mutation causing the loss-of-function of *MdMLO19* would be the third example of a natural *mlo* mutant leading to PM resistance, after barley *mlo-11* (Piffanelli *et al.,* 2004) and tomato *ol-2* (Bai *et al.,* 2008). The germplasm of barley has been extensively studied for PM resistance, with around 4100 accessions tested, and the frequency of spontaneous *mlo* mutations was found to vary between 0.2 and 0.6% (Jørgensen, 1992). The Fruitbreedomics data indicated a much higher frequency in apple: 22.2% for the insertion and 7.9% when in homozygosity. Compared to the frequency of barley natural mlo mutation, the frequency of apple is 37-111 times higher, 13-40 times when considering homozygosity only. The genotypes included in the Fruitbreedomics dataset have been selected to represent as best as possible the diversity present in apple germplasm (Dr. R. Velasco, FEM; personal communication), therefore the frequencies here calculated are a reasonable estimation of the real frequency of insertion T-1201 in the germplasm of apple. It was noteworthy that, among the genotypes carrying insertion T-1201, there were 'McIntosh' and 'Fuji' (homozygous), and 'Jonathan' and 'Delicious' (heterozygous). These four genotypes belong to the 14 cultivars from which the majority of European apple descend (Evans *et al.,* 2010; Bianco *et al.,* 2014). The loss-of-function of *MLO* genes is often associated with pleiotropic phenotypes, although this is not the case of apple, where the knock-down of *MdMLO19* did not result in any evident pleiotropic phenotype (Chapter 3). Therefore, the advantage connected to the loss-of-function of *MdMLO19* might have favored its spreading, explaining the high frequency of insertion T-1201.

High resolution melting (HRM) was chosen to assess the frequency of insertion T-1201 of *MdMLO19* in apple germplasm. The insertion was present in 108 of th 159

genotypes considered, heterozygous in 55 of them and homozygous in 53. All the homozygous genotypes were resistant to PM, except for four susceptible and four unknown genotypes. The re-sequencing of the susceptible genotypes showed that they do not carry any other mutation that could cause the regain of the reading frame. Possible explanations for the susceptibility of these four genotypes are given in Chapter 4.

Our data suggested the existence of a link between the presence of the homozygous insertion and resistance to PM and two statistical analyses supported this conclusion: CCA analysis, which showed a link between the homozygosity of T-1201 and PM resistance, and Kruskall-Wallis, which indicated that genotypes carrying the homozygous insertion have a significantly lower disease severity score. The high frequency of insertion T-1201 could explain the PM-resistance observed in apple genotypes known to not carry any functional R-gene to PM. It has to be noted that some discrepancies between the Fruitbreedomics data, the HRM and the re-sequencing were observed. However, HRM is still a cost-effective system to screen a big number of samples and narrow the field for further investigations. Furthermore, some degree of uncertainty does not compromise the key finding that insertion T-1201 is common among resistant genotypes.

Alleles of *MdMLO19* carrying insertion T-1201 might represent a valuable source of durable PM-resistance in apple. The marker we developed can be used to screen a larger collection of genotypes and assess the frequency of insertion T-1201. On a wider perspective, we have shown that re-sequencing projects like Fruitbreedomics are a powerful tool to study the natural diversity in the germplasm of a species and they can lead to the discovery of valuable alleles to integrate in breeding programs.

Validation of the role of candidate S-genes of grapevine

In Chapter 5 we studied the role of clade V *MLO* genes of grapevine in PM susceptibility. The knock-down of *VvMLO6*, *VvMLO11* and *VvMLO13*, alone or combined, did not result in any significant reduction of susceptibility to PM, whereas the knock-down of *VvMLO7* was detected only in the resistant *mlo* lines, leading to the conclusion that it is the main candidate for causing PM susceptibility in *Vitis vinifera*. However, it was noted that *VvMLO7* was always knocked-down together with two or three other *MLO* genes. In *A. thaliana*, the simultaneous knock-out of three *MLO* genes is necessary to obtain complete resistance. *AtMLO2* is the main susceptibility factor, whereas *AtMLO*6 and *AtMLO1*2 have an additive role, meaning that they contribute to reduced PM-susceptibility only when they are knocked-out together with *AtMLO2* (Consonni *et al*., 2006). In grapevine, *VvMLO7* seemed to act like *AtMLO2,* whereas the two candidates for an additive role were *VvMLO6* and *VvMLO11*, since their expression was significantly reduced in all three resistant lines.

The role of *VvMLO6* in susceptibility was not proven with absolute certainty, but this finding was still completely unexpected. If the role of *VvMLO6* will be confirmed, it partially questions the reliability of the gene expression analysis as a system to identify *MLO* S-genes and, on a wider perspective, it suggests that more attention should be given to the possible role of non-responsive clade V *MLO* genes in PM susceptibility. Our findings also question the validity of the complementation test in *A. thaliana Atmlo2/6/12* mutant: Feechan *et al.* (2013b) deducted from their experiments in *A. thaliana* that *VvMLO11* and *VvMLO13* were the responsible genes for susceptibility to PM in grapevine, whereas *VvMLO7* had only a partial effect and *VvMLO6* no effect at all. This is very different from what we observed and another study in *V. vinifera* confirmed that *VvMLO11* and *VvMLO13* are not relevant for PM susceptibility, as both single and double knock-down mutants of these two genes

do not show reduction of PM penetration rate (Qiu *et al.,* 2015). *A. thaliana* complementation is a powerful tool to collect preliminary information, but it cannot substitute *in planta* studies and in some cases, like the one just described, it can even result in misleading information.

The expression of 13 genes known to be differentially expressed after PM inoculation was analyzed in order to understand the effect of *MLO* genes knock-down on other genes involved in plant-pathogen interaction. The knock-down of *MLO* genes in the resistant line TLB4 did not affect the expression of the 13 target genes in absence of infection. However, several differences between the control EVB and TLB4 were noted upon *E. necator* inoculation, like the number of induced genes: seven in the control and only three in TLB4. Only two genes showed a similar pattern in EVB and TLB4, namely the transcription factors *VvWRKY19* and *VvWRKY52*. These differences could be explained by the lower infection present on TLB4, not sufficient to trigger the transcriptional response of the plant. The up-regulation was more intense for both lines at 10 dpi rather than 1 dpi.

Knock-down of *MLO* genes in apple and grapevine: differences and similarities

The knock-down of *MLO* genes resulted in resistance to PM in both *M. domestica* and *V. vinifera.* However, some differences between the two plant species were noted and they are here discussed.

In both apple and grapevine, the approach chosen to knock-down *MLO* genes was by using RNAi. However, the design fragments for RNAi was carried out differently: in apple, which was chronologically the first system we studied, we used short fragments of 150 bp or less, whereas in grapevine we used long fragments of 300-600 bp. Short fragments often do not work, in fact significant silencing was detected in only three apple lines out of 52 (5.8%), whereas the longer fragments caused significant gene knock-down in six grapevine lines out of 26 (23.1%). However, short

fragments are more specific and, accordingly, no off-target knock-down was detected in apple. Conversely, five of the six knock-down grapevine lines showed some off-target effects. Surprisingly, the length of the RNAi fragments did not affect the intensity of the knock-down, but only its frequency. As a matter of fact, the average reduction of expression in grapevine *mlo* lines was around 56%, whereas it was around 84% for apple *mlo* lines.

Among the tested *MLO* genes, the most important PM susceptibility factors were two, one in apple (*MdMLO19*) and one in grapevine (*VvMLO7*). However, complete resistance in grapevine seemed to require the silencing of two additional genes (*VvMLO6* and *11*), whereas no other gene besides *MdMLO19* had any influence on apple susceptibility to PM.

MLO genes are negative regulators of defense and their knock-out was expected to cause pleiotropic phenotypes. However, no differences with the control were observed in neither of the two species. Pleiotropic phenotypes are known to be particularly severe in non-optimal conditions (Jørgensen, 1992), but in some cases they are visible also in absence of any stress. For instance, we noticed that the growth of *A. thaliana Atmlo2/6/12* mutant is often slower than the growth of Col-0 plants, whereas Zheng *et al.* (2013) observed reduced size of pepper *mlo* plants compared to wild-type plants growing in identical conditions. Nothing comparable was observed in apple or grapevine, despite the fact that grapevine line TLB4 had four *MLO* genes knocked-down and apple line TG11+19 had two. It is also important to consider that *MLO* genes were not completely silenced in our plants and this could be the reason why there were no visible pleiotropic phenotypes. Growing apple and grapevine *mlo* plants under different conditions could allow to discover which of these conditions can cause the appearance of negative phenotypes connected to *MLO* genes knock-down. This, in turn, could help to understand the function of *MLO* genes. However, it should not be assumed that the knock-down of

MLO genes in apple and grapevine necessarily causes pleiotropic phenotypes. In fact, the knock-out of *SlMLO1* in tomato does not have any known consequences for the fitness of the plant, and this could therefore also be true for apple and grapevine. Accordingly, apple genotypes carrying homozygous non-functional alleles of *MdMLO19*, such as 'Fuji' and 'McIntosh', are cultivated and traded on a wide scale, suggesting that disruptive mutations of apple *MLO* genes do not have severe negative effects on the agricultural value of the plant as graft.

Resistance granted by the knock-down of *MLO* genes is based on the formation of cell wall appositions called papillae that constitute a mechanic barrier for the fungal invader (Consonni *et al.*, 2006). Their formation depends on actin-dependent endomembrane transport (Hückelhoven, 2014). Papillae are always formed in case of pathogen attack, but they are not always effective. Three characteristics are hypothesized to determine the efficacy of papillae: timing of formation (Hückelhoven, 2014), composition (Chowdhury *et al.*, 2014) and size (Lyngkjær *et al.* 2000). The composition seems particularly important, with effective papillae having an higher concentration of callose, cellulose and arabinoxylan compared to non-effective ones (Chowdhury *et al.*, 2014). In our observations in apple and grapevine, the same difference was noted between papillae of susceptible and resistant *mlo* plants: non-effective papillae formed in control lines of apple and grapevine emitted a more intense fluorescence compared to effective papillae of *mlo* lines and they had more defined edges. Furthermore, papillae of *mlo* lines were less defined and bigger. The results in the two species support each other and suggest that the size of the papilla is important to stop fungal penetration. Moreover, the difference in fluorescence could reflect a difference in the composition of the papillae.

MLO proteins have been proposed to be negative regulators of vesicle-associated and actin-dependent defense pathways at the site of attempted PM penetration

(Panstruga, 2005). Furthermore, Miklis *et al*. (2007) proposed that PM-fungi can control MLO proteins in order to supply nutrients to the growing hyphae through vesicular transport. Our hypothesis is that the pathogen is able to control the transport of material to the cell-wall, with the purpose of changing the composition of the papillae from effective to non-effective.

The transcriptional analysis of 13 genes of grapevine and 17 of apple showed an important difference between the two species. Despite grapevine line TLB4 had four *MLO* genes knocked-down, there was no difference of expression in comparison with the control in absence of infection. Conversely, apple line TG11+19, which had two *MLO* genes knocked-down, had five genes down-regulated, three of which involved in defense. Therefore, the effect of *MLO* genes knock-down was more intense in apple rather than grapevine. Another significant difference was noted at 24 hpi: the inoculation with *E. necator* triggered an intense transcriptional response in the grapevine control EVB but not in the *mlo* resistant line TLB4, whereas the inoculation of apple with *P. leucotricha* had almost the opposite effect, with the control 'Gala' showing a moderate transcriptional response and line TG11+19 having most of the genes up-regulated.

Of the 13 genes of grapevine and 17 of apple, the expression of nine of them was tested in both the species. There was no resemblance in the expression patterns of these genes in the two species (Table 1). Further studies are necessary to confirm and explain the different response of apple and grapevine to the knock-down of *MLO* genes. It should be considered that *E. necator* and *P. leucotricha* are different species that belong to different genus (Glawe, 2008), therefore the different reaction of the hosts can be due to differences in the pathogens. Furthermore, the co-evolution between the host and the pathogen plays an important role and it does not necessarily go in the same direction in different species.

Why do plants have *MLO* S-genes?

The fact that S-genes haven't been excluded by evolution despite being responsible for susceptibility to pathogens suggests that they have important physiological functions for the plant and the fitness cost associated to their loss-of-function would be too high (Pavan *et al*., 2010). The observation that silent mutations and mutations in introns are more abundant than mutations leading to a change in the protein indicates also that selection preserve the function of *MLO* genes (Chapter 4).

However, this is valid for *MLO* genes that do not act as S-genes. The case of apple *MdMLO19* shows that the scenario is different for S-genes. More mutations, including disruptive ones, were found in *MdMLO19* compared to other *MLO* genes , suggesting that the loss of PM susceptibility balances the fitness cost connected to the loss-of-function of the gene.

Nonetheless, functional *MLO* S-genes are still predominant in most of the species. However, It should be considered where the plant and the pathogen originated: if they originated in distant areas and came in contact only recently, it is possible that the plant simply didn't have the time to adapt to the pathogen. This is the case of *E. necator* and European grapevine, which came in contact only around 1850. Furthermore, it is likely that S-genes are lost only in areas where the damages caused by the pathogen are more severe than the negative phenotypes resulting from their loss-of-function.

Another important consideration that is often overlooked is that an evolutionary disadvantage is not always an agronomical disadvantage. For instance, if the loss-of-function of an *MLO* gene leads to abnormal roots development (Chen *et al.,* 2009), that would not be a problem for grafted species like apple and grapevine. Similarly, if the loss-of-function of an *MLO* gene causes male sterility (Kessler *et al*, 2010), for an obligate cross pollinator

	Gene	*VvEDS1*	*SlALS1*		*AtLOX1*	*VvNPF3.2*		*VvPAD4*	*VvPR1*	*VvWRKY52*
	Grapevine homologue	***VvEDS1***	***VvALS1***		***VvLOX1***	***VvNPF3.2***		***VvPAD4***	***VvPR1***	***VvWRKY52***
	Apple homologue	*MdEDS1*	*MdALS1*	*MdALS2*	*MdLOX*	*MdNPF3.1*	*MdNPF3.2*	*MdPAD1*	*MdPR1*	*MdWRKY30*
0 hpi	Grape control	-	-	-	-	-	-	-	-	-
	Grape TLB4	-	-	-	-	-	-	-	-	-
	Apple control	-	-	-	-	-	-	-	-	-
	Apple TG19	-	-	-	Down	-	-	-	-	-
	Apple TG11+19	-	-	-	Down	-	Down	-	-	-
24 hpi	Grape control	-	-	-	-	-	-	-	-	-
	Grape TLB4	-	-	-	-	-	-	-	-	-
	Apple control	-	-	-	-	-	-	-	-	-
	Apple TG19	-	-	-	-	-	-	-	-	-
	Apple TG11+19	-	-	-	-	Up	-	-	-	-
10 dpi	Grape control	-	-	-	-	-	-	-	-	-
	Grape TLB4	-	-	-	Up	Down	Down	Down	Down	-
	Apple control	-	-	-	-	-	-	-	-	-
	Apple TG19	Up	Up	Down	-	-	-	-	-	-
	Apple TG11+19	-	-	Down	-	-	-	-	-	-

Table 1. Expression pattern of nine genes tested in both apple and grapevine. The row 'Gene' indicates the gene that was used to retrieve the sequences of its homologues of apple and grapevine. 'Up' means that the gene is up-regulated at the particular time point in comparison with the control line, whereas 'down' means that the gene is down-regulated. The hyphen indicates no significant difference compared to the control. The up and down-regulations are statistically significant according to Tukey or Games-Howell post hoc tests (P=0.05).

species like apple that would not be an issue either, as long as good pollinators are present in the orchard.

However, the question that gives the title to this paragraph is not entirely correct. The error is the assumption that there are no species where loss-of-function mutations in *MLO* genes are common. In Chapter 4 we showed that this is not the case of apple, although our study was not definitve and further confirmations are required. So far, the only species where an exhaustive analysis of the germplasm was carried out is barley. The estimated frequency of apple natural *mlo* mutants resistant to PM was around 13-40 times higher than the frequency of natural *mlo* mutants of barley (Jørgensen, 1992). This indicates that *mlo* mutants might be more common than anticipated. A natural *mlo* mutant was found also in tomato (Bai *et al.,* 2008), but the germplasm of the species was not screened, therefore it is not known if there are other *mlo* mutants of tomato growing somewhere in the world. Apple is the first dicot species tested, but further studies on the germplasm of other species could reveal that natural *mlo* mutants exist and are not rare. The questions we should ask are: how common are natural loss-of-function mutations of *MLO* genes? Is there a difference in the frequency of *mlo* mutations between monocot and dicot species? Are *mlo* mutations present also in wild species or only/mostly in commercial varieties? If so, why? These questions can be addressed through the analysis of the germplasm of the species of interest. In this light, re-sequencing projects are particularly valuable.

Future perspectives

The results presented here were obtained with the contribution of several people in four years of work. As often happens in science, every question that was answered resulted in one or more new questions. Some of these questions are worth to be answered in the future.

For both apple and grapevine, the role of some clade V *MLO* genes was not completely unravelled in this thesis. In Chapter 5 we showed that *VvMLO7* is the main S-genes of grapevine and speculated that *VvMLO6* and *11* may have an additional, synergistic role in susceptibility. However, we did not obtain any transgenic line where *VvMLO7* was silenced alone, therefore we were not able to prove whether this gene is the sole responsible for PM susceptibility in grapevine. The single knock-out or knock-down of *VvMLO7* would answer the question: if *Vvmlo7* plants were as resistant as *Vvmlo6/7/11* plants, it would mean that *VvMLO6* and *11* do not have any role in susceptibility. On the other hand, if *Vvmlo7* plants were less resistant than *Vvmlo6/7/11* plants, it would be necessary to generate also the double mutants *Vvmlo6/7* and *Vvmlo7/11*. If a role in susceptibility will be confirmed for *VvMLO6*, the knock-out or knock-down of apple *MdMLO5* and *7* should also be carried out: like *VvMLO6*, these two genes are not up-regulated upon PM inoculation, therefore their possible role in susceptibility should be considered. All the *mlo* apple and grapevine lines obtained so far resulted from RNAi, therefore both gene knock-down and resistance were not complete. The generation of knock-out lines would allow to test if complete resistance can be achieved with the target genes identified and if complete silencing causes pleiotropic phenotypes.

Apple *MdMLO18* was the only gene outside clade V considered in this thesis. The complementation in *A. thaliana Atmlo2/6/12* mutant suggested that this gene does not have a role in susceptibility, according to the many evidences that only clade V

genes can act as S-genes. However, complementation in *A. thaliana* proved to be unreliable in grapevine (Feechan *et al.,* 2013b; Chapter 5), therefore, to rule out the possible role of *MdMLO18* with absolute certainty, the gene should be knocked-out or knocked-down in apple plants. We obtained an apple transgenic line where *MdMLO18* is knocked-down, but we did not have time to test it. This will be done in the future. Even if *MdMLO18* does not have any role in susceptibility, its knock-down could produce new information on the metabolic role of this particular *MLO* gene in the plant.

Knock-out of *MLO* genes is often associated with pleiotropic phenotypes. We did not observe any obvious one, neither in apple nor grapevine, but we did not carry out extensive tests on this aspect. To definitively exclude this possibility *mlo* plants could be grown under different stresses, in order to observe if they show any pleiotropic effects.

The screening of apple germplasm resulted in the discovery of insertion T-1201 in *MdMLO19*, a mutation that causes the formation of an early stop codon. Based on the position of the early stop codon, we assumed that the resulting protein is non-functional. However, to confirm that genotypes carrying the homozygous insertion are actual *mlo* mutants, this assumption must be demonstrated. Furthermore, the frequency of insertion T-1201 should be assessed in a wider collection of apple genotypes.

The screening of apple germplasm returned interesting results and showed the potential of re-sequencing. So far, the diffusion of putative *mlo* mutants of apple is unmatched in other species, but this is mostly due to the fact that there is only another study of this kind, carried out in barley. The screening of the germplasm of any species is a promising perspective that could allow finding *mlo* mutations potentially useful for breeding. When re-sequencing data of other species will be available, it will be important to consider the presence of pseudogenes, particularly

in resistant genotypes, as pseudogenes of genes involved in susceptibility could explain resistance to PM. Furthermore, the screening of re-sequencing data could allow identifying candidates *MLO* S-genes: the presence of homozygous nonsense mutations in specific *MLO* genes of PM resistant genotypes would be an important indication that the gene might act as an S-gene.

The study of the composition of secondary phenolic metabolites of *mlo* apple plants, showed that four compounds were differentially accumulated in comparison with the control. The meaning of this different accumulation is unknown. A putative role in defense could be assessed by spraying the leaves of wild-type apple plants with the four compounds, and subsequently inoculate them with *P. leucotricha* and observe any variation in PM susceptibility.

Conclusions

The issue of the role of *MLO* genes in susceptibility to PM in apple and grapevine was addressed in this thesis. All genes causing susceptibility to PM belong to clade V and are up-regulated in the early stages of PM pathogenesis. We were therefore able to identify three candidate S-genes of apple, which were studied together with the three candidate genes of grapevine known from literature. Two of these genes, *MdMLO19* of apple and *VvMLO7* of grapevine, were the major genes responsible for PM susceptibility. Both belonging to clade V, they are similar to each other (identity 55.6%, similarity 76.3%), but they are not homologues. In grapevine, also *VvMLO6* and *11* might have an additive role, but further experiments are required to confirm it.

The Fruitbreedomics dataset was screened and among the sequences of 63 apple cultivars, 127 SNPs were found in the exons of the five MLO genes of apple considered (the four clade V genes, plus *MdMLO18*). Insertion T-1201, a mutation causing an early stop codon, was found in *MdMLO19*, the gene responsible for

susceptibility to PM in apple. This insertion was common in homozygosity in PM-resistant genotypes, including 'McIntosh', a PM-resistant cultivar commonly used in breeding. The frequency of the insertion was calculated based on the Fruitbreedomics data and it was around ten times higher than the frequency of natural *mlo* mutations of barley.

In this thesis we demonstrated that *MLO* genes can be used to obtain durable, broad-spectrum resistance in fruit crops and that natural *mlo* mutations might be more common in some species than previously anticipated.

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Summary

Powdery mildew (PM) is a major fungal disease that threatens thousands of plant species. PM is caused by *Podosphaera leucotricha* in apple and *Erysiphe necator* in grapevine. Powdery mildew is controlled by frequent applications of fungicides, having negative effects on the environment, and leading to additional costs for growers. To reduce the amount of chemicals required to control this pathogen, the development of resistant apple and grapevine varieties should become a priority.

PM pathogenesis is associated with up-regulation of specific MLO genes during early stages of infection, causing down-regulation of plant defense pathways. These up-regulated genes are responsible for PM susceptibility (S-genes) and their knock-out causes durable and broad-spectrum resistance. All *MLO* S-genes of dicots belong to the phylogenetic clade V. In grapevine, four genes belong to clade V. *VvMLO7*, *11* and *13* are up-regulated during PM infection, while *VvMLO6* is not.

Chapter 2 reports the genome-wide characterization and sequence analysis of the MLO gene family in apple, peach and woodland strawberry, and the isolation of apricot MLO homologs. Twenty-one homologues were found in apple, 19 in peach and 17 in woodland strawberry. Evolutionary relationships between MLO homologs were studied and syntenic blocks constructed. Candidate genes for causing PM susceptibility were inferred by phylogenetic relationships with functionally characterized MLO genes and, in apple, by monitoring their expression following inoculation with the PM causal pathogen *P. leucotricha.* In apple, clade V genes *MdMLO11* and *19* were up-regulated, whereas the two other members of clade V, *MdMLO5* and *7,* were not up-regulated. The clade VII gene *MdMLO18* was also up-regulated upon *P. leucotricha* infection.

Chapter 3 reports the knock-down, through RNA interference, of *MdMLO11* and *19*, as well as complementation of the mutant phenotype by expression of the *MdMLO18* gene in the *Arabidopsis thaliana* triple *mlo* mutant *Atmlo2/6/12*. The

knock-down of *MdMLO19* resulted in a reduction of PM disease severity up to 75%, whereas the knock-down of *MdMLO11,* alone or combined with *MdMLO19,* did not cause any reduction or additional reduction of susceptibility compared to *MdMLO19* alone. Complementation by *MdMLO18* did not restore susceptibility. Cell wall appositions (papillae), a response to PM infection, were found in both susceptible plants and PM resistant plants where MdMLO19 was knocked-down, but were larger in resistant lines. The expression analysis of 17 genes related to plant defense, and quantification of phenolic metabolites in resistant lines revealed line-specific changes compared to the control.

Chapter 4 evaluates the presence of non-functional alleles of the *MdMLO19* S-gene in apple germplasm. The screening of the re-sequencing data of 63 apple genotypes led to the identification of 627 SNP in five *MLO* genes (*MdMLO5, MdMLO7, MdMLO11, MdMLO18* and *MdMLO19)*. Insertion T-1201 in *MdMLO19* caused the formation of an early stop codon, resulting in a truncated protein lacking 185 amino-acids and the calmodulin-binding domain. The presence of the insertion was evaluated in a collection of 159 apple genotypes: it was homozygous in 53 genotypes, 45 of which were resistant or very resistant to PM, four partially susceptible and four not assessed. These results strongly suggest that this insertion is causative for the observed PM resistance. The absence of a clear fitness cost associated to the loss-of-function of *MdMLO19*, might have contributed to the high frequency of the mutation in breeding germplasm and cultivars. Among the genotypes containing the homozygous insertion, 'McIntosh' and 'Fuji' are commonly used in apple breeding. After barley and tomato, apple is the third species with a reported natural non-functional *mlo* allele in its germplasm, with the important difference that the allele is present in a relatively large number of apple genotypes, most of which not related to each other.

Chapter 5 reports the knock-down through RNA interference of four grapevine MLO genes, all members of clade V. *VvMLO7*, *11* and *13* are up-regulated in early stages of infection, whereas *VvMLO6* is not responsive to the pathogen. Knock-down of *VvMLO6*, *11* and *13*, alone or combined, did not decrease PM severity, whereas the knock-down of *VvMLO7,* alone or in combination with *VvMLO6* and *VvMLO11,* caused a reduction of severity of 77%. Cell wall appositions (papillae), a response to PM attack, were present in both resistant and susceptible lines, but were larger in resistant lines. Thirteen genes involved in defense were less up-regulated in resistant plants, highlighting the reduction of PM disease severity.

In **Chapter 6** we discuss the results presented in this thesis. The pivotal role of *MLO* genes in the interaction of PM pathogens with apple and grapevine is described and further experiments aimed at addressing open questions are proposed. The results described in this thesis open interesting avenues in *MLO* genes research, particularly the finding that a natural *mlo* mutation in apple appeared to be more common than expected. This mutation is directly applicable in marker assisted breeding for durable PM resistance in apple.

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Four years can be a very long time and yet so short. The day I arrived in the Netherlands there were a lot of “firsts” for me: first life experience in a foreign country, first real job, first day as a real scientist (or at least, I thought so!). It might sound like a cheesy movie line, but it really felt like “the first day of the rest of my life”. And now here I am contemplating the end of another chapter of my life. I am extremely happy about the last four years: they have been more than fulfilling, both professionally and personally.

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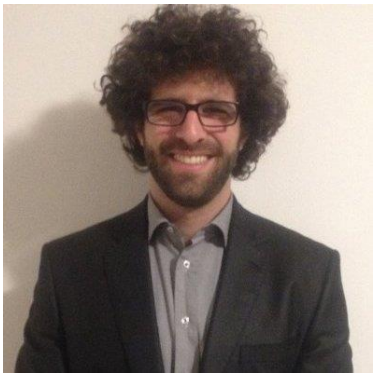
Stefano

About the author



Stefano Pessina was born on December 24th, 1986 in Monza (Italy). He started his bachelor studies in plant biotechnology at the University of Milano in 2005. After graduating in 2008, he continued his studies with a master in plant, food and environmental biotechnology in the same University, where he graduated in 2011. His master thesis research was carried out at the laboratory of molecular agri-food sciences at University of Milano, under the supervision of Dr. Alessio Scarafoni and Prof. Dr. Marcello Duranti. The research carried out during the thesis aimed at characterizing the role of a protein abundant in lupin (*Lupinus albus)* seeds, through site-specific mutagenesis and heterologous expression in yeast. Shortly after graduating, he obtained a scholarship from Fondazione Edmund Mach (Italy) and moved to Wageningen to start his PhD project. In 2012 he moved back to Italy to continue his work at Fondazione Edmund Mach. His PhD project aimed at characterizing the role of *MLO* genes in susceptibility to powdery mildew in apple and grapevine. This project was supervised by Prof. Dr. Richard G. F. Visser, Dr. Henk J. Schouten, Dr. Mickael Malnoy and Dr. Yuling Bai and the results of his studies are described in this thesis.

List of publications

Pessina S, Pavan S, Catalano D, Gallotta A, Visser RGF, Bai Y, Malnoy M, Schouten HJ: **Characterization of the MLO gene family in Rosaceae and gene expression analysis in *Malus domestica.*** *BMC genomics* 2014, 15:618

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Stefano Pessina
Date: 22 January 2016
Group: Laboratory of Plant Breeding
University: Wageningen University & Research Centre

1) Start-up phase	date
► **First presentation of your project**	
Silencing Mlo--like susceptibility genes to achieve broad-spectrum resistance to powdery mildew in apple	Nov 17, 2011
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*1.5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD student day, Leiden University	Nov 29, 2013
► **EPS theme symposia**	
EPS theme 4 symposium 'Genome Biology', Wageningen University	Dec 09, 2011
EPS Theme 1 Symposium 'Developmental Biology of Plants', Wageningen University	Jan 19, 2012
EPS Theme 2 Symposium 'Interactions between Plants and Biotic Agents', Wageningen University	Feb 10, 2012
EPS Theme 3 Symposium 'Metabolism and Adaptation', Utrecht University	Apr 26, 2012
► **NWO Lunteren days and other National Platforms**	
► **Seminars (series), workshops and symposia**	
Plant Sciences Seminar 'Does gender matter (in management)?'	Nov 08, 2011
Mini-Symposium 'Plant Breeding in the Genomics Era'	Nov 25, 2011
Extreme Science Make-over?'	Dec 13, 2011
Impacts of global warming on plant biodiversity and ecosystem function: a 200 million year old case study from East Greenland'	Jan 20, 2012
Debate: How realistic is our "Two times more with two times less"-ambition?	Jan 31, 2012
SPICY symposium: improving yield prediction	Feb 07, 2012
Metabolic engineering of high-value industrial and nutritional isoprenoids in plants	Feb 16, 2012
Lessons from photosynthetic analyses in three widely used Arabidopsis ecotypes	Feb 21, 2012
Participatory plant breeding - Salvatore Ceccarelli (ICARDA)	May 29, 2012
Single-Molecule, Real-Time (SMRT™) DNA Sequencing: Technology Overview and Recent Applications - Ralph Vogelsand (Pacific Biosciences)	Mar 26, 2013
FIRST training - series of 6 seminars by Vittorio Sgaramella: The Genome as a read-write system: the pursuit of a natural form of genetic engineering	Apr 18-Oct 24, 2013
FIRST training - series of 3 seminars by various: Soft Skills	May 22-Jun 05, 2013
Biodays 2013	Jun 03-04, 2013
Technology drives insight: Multiscale analysis of signaling networks in A. thaliana - Klaus Palme	Oct 07, 2013
FIRST training - series of 8 seminars by various: Knowledge and Innovation Soft Skills Seminars	Oct 16, 2013- Feb 19, 2014
Investigating the role of plants in the diet on the prevention and amelioration of chronic disease - Cathie Martin (John Innes Centre, Norwich UK)	Nov 07, 2013
Calcium regulates interactions between the bacterial plan pathogen Xylella fastidiosa and its plant host - Leonardo De La Fuente (Auburn University, USA)	Oct 03, 2014
The scientific and economic consequences of the Pseudomonas syringae pv actinidiae outbreak in New Zealand - Joel L. Vanneste (Plant & Food Research, New Zealand)	Oct 17, 2014
International workshop - Molecular Basis of Fire Blight	Nov 15, 2014
Good Pipetting Practises (organised by Mettler-Toledo)	Mar 03, 2015
► **Seminar plus**	
► **International symposia and congresses**	
Rosaceus Genome Conference 6 (Italy)	Oct 01-03, 2012
Next Generation Breeding conference (Netherland)	Nov 12-14, 2012
Plant diseases and resistance mechanisms (Austria)	Feb 20-22, 2013
XVI International Congress on Molecular Plant-Microbe Interactions (Greece)	Jul 06-10, 2014
Eucarpia Fruit 2015 (Italy)	Jun 14-18, 2015
► **Presentations**	
Poster: Plant diseases and resistance mechanisms	Feb 20-22, 2013
1 oral presentation	Mar 30, 2012
Talk: Eucarpia 2015 (Bologna)	Jun 15, 2015
► **IAB interview**	
► **Excursions**	
Subtotal Scientific Exposure	*13.8 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
GMPF international Summer school - introduction to plant metabolomics (FEM, Italy)	Jul 04-08, 2011
GMPF international Summer school - introduction to bioinformatics (FEM, Italy)	Jun 28-Jul 01, 2011
SPICY Workhosp: bioinformatics and statistical genetics and genomics	Mar 08, 2012
SPICY Workhosp: Crop growth modelling	Mar 09, 2012
GMPF international Summer school - Gene Expression and Pathway Reconstruction (FEM, Italy)	Jun 26-29, 2012
GMPF international Summer school - population and quantitative genetics (FEM, Italy)	Jul 02-07, 2012
GMPF international Winter school - symbiomes, system metagenomics of host microbe interactions	Mar 11-13, 2013
Italian Society for Plant Pathology - 5th summer school in Physiological Plant Pathology (Pieve Tesino, Italy)	Jun 17-21, 2013
FEM-FIRST international summer school - new frontiers in photosynthesis	Jul 29-30, 2013
FEM-FIRST course - Evolution, a view from the 21st century, held by J. Shapiro (FEM, Italy)	Nov 12-15, 2013
EPS course - transcription factors and transcriptional regulation	Dec 17-19, 2013
EPS+VLAG course - Microscopy and Spectroscopy in Food and Plant Sciences	May 06-08, 2014
FEM>FIRST international fall school in applied genomics	Nov 03-06, 2014
► **Journal club**	
Participation in a literature discussion group	2011, 2012
► **Individual research training**	
Subtotal In-Depth Studies	*13.6 credits**

4) Personal development	*date*
► **Skill training courses**	
Competence Assesment	Jan 24 and Feb 29, 2012
Wageningen Graduate Schools Courses - Techniques for Writing and Presenting Scientific Papers	Aug 27-30, 2013
FEM>FIRST - Linux Basic, a practical course on linux command line - 7 lessons of 2 hours (Italy)	Nov 18, 2014-Jan 27, 2015
Wageningen Graduate Schools Courses - Writing Grant Proposals	Jan 13-16, 2015
Training course - How to Write a Competitive Proposal for Horizon 2020 (Italy)	Jan 23, 2015
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*4.3 credits**

TOTAL NUMBER OF CREDIT POINTS*	**33,2**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS

** A credit represents a normative study load of 28 hours of study.*