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"Improving fire blight resistance in susceptible apple cultivars by different biotechnological approaches"

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SUMMARY

Fire blight, caused by the bacterium *Erwinia amylovora* (*E. amylovora*), is one of the most economically important apple (*Malus x domestica*) pathogens worldwide. Various chemical and biological approaches can be applied to deal with the disease, but none of these is decisive. Such strategies are also prohibited in many countries due to their potential impact on human health and environment. To date, the most efficient strategy for controlling *E. amylovora* is thus to breed resistant/tolerant apple cultivars by manipulating one or multiple plant genes, which are associated with resistance or susceptibility to the disease. Within this context, classical breeding or genetic engineering can be applied. While conventional breeding is still considered a time-consuming and laborious process, genetic engineering methodologies represent rapid, precise and powerful alternatives to insert the desired trait into the crop of interest.

In this thesis, we exploit different biotechnological approaches on the one hand to improve fire blight resistance trait by knocking-out a known susceptibility gene and on the other hand to investigate potential disease-related key genes.

At first, we develop a CRISPR/Cas9-FLP/FRT-based gene editing system, mediated by *Agrobacterium tumefaciens*, to knock-out the fire blight susceptibility gene *MdDIPM4* and generate apple ('Gala' and 'Golden Delicious') cultivars with reduced susceptibility to the disease and a minimal trace of exogenous DNA. Several transgenic lines were screened by sequencing to identify mutations in *MdDIPM4*. An editing efficiency of 75% was observed. Candidate lines showing loss-of-function mutation were inoculated with *E. amylovora* and a significant reduction (of about 40%) in disease symptoms was observed compared to *wild-type* plants. No CRISPR/Cas9 off-targeting activity was detected in five potential off-target regions. Thus, with the aim of removing the 'entire' T-DNA in those lines with reduced susceptibility to the pathogen, the FLP/FRT system was induced and the excision of the T-DNA was validated. This work demonstrates for the first time the development and application of a CRISPR/Cas9-FLP/FRT-based editing system for the production of 'clean' fire blight resistant apple cultivars.

Secondly, we investigate the apple miRNA *MdmiR285N* which is predicted to play a key role in the post-transcriptional regulation of 35 RNA transcripts coding for different disease resistance proteins. A complex network of potential transcriptional regulatory elements involved in plant growth and development, and in response to different hormones and stress conditions has been identified in *MdmiR285N* promoter in both apple and the model plant species *Arabidopsis thaliana*. Moreover, spatio-temporal expression of *MdmiR285N* has been assessed in plants at physiological growth conditions and in response to bacterial pathogens. Our results suggest that MdmiR285N is a multifunctional microRNA which may control different processes, such as biotic stress response, plant growth and development.

In parallel, a methodological work has been carried out for a precise and rapid characterization of the transgenic apple lines produced. A quantitative, rapid and cost-effective method has been developed, based on real-time PCR to quantify the copy number of *nptII* marker gene in apple lines and to evaluate its elimination after the activation of the recombinase system. This method may be valuable for those institutions committed to tracing 'gmo' apple products.

CHAPTER 1

General introduction

Apple (*Malus x domestica*)

The domesticated apple (*Malus x domestica, M. x domestica*) belongs to the genus *Malus* and to the family *Rosaceae*, the latter including over 100 genera and 3000 species distributed worldwide, most commonly in temperate regions of the northern and southern hemispheres (Velasco et al. 2010). Among the species of agronomic and economic impact, *M. x domestica* is the most cultivated. *M. x domestica* originated approximately 4000 years ago in central Asia, where the original ancestor *Malus sieversii* is still found growing wild. There were however additional genomic contributions from *Malus orientalis* and *Malus sylvestris* as the domesticated species spread westward (Duan et al., 2017).

Domesticated apple is a self-incompatible tree, and apple cultivars are propagated by grafting scions onto dedicated rootstocks. However, throughout its history of cultivation more than ten thousand cultivars of *M. x domestica* have been developed, and new are currently still produced by modern breeding techniques (Qian et al., 2010; Velasco et al. 2010). Most domesticated apple cultivars are diploid (n=17) (e.g. 'Delicious', 'Fuji', 'Golden Delicious', 'Gala', 'Granny Smith', 'Jonathan', 'McIntosh'), although triploid (e.g. Jonagold) and tetraploid (e.g. 'Antonovka Ploskaya', 'McIntosh Tetraploidnyi', 'Papirovka Tetraploidnaya', 'Wealthy Tetraploidnyi') cultivars are also documented (Hampson and Kemp, 2003; Sedov and Makarkina, 2008; Westwood, 1993). The estimated genome size for domesticated apple is roughly 750 Mb, based on the genome sequence for the 'Golden Delicious' cultivar (Velasco et al. 2010).

Economic evidences of apple production

Apple is indisputably the most cultivated among the several edible fruits coming from the family *Rosaceae*, e.g. pears, apricots, plums, cherries, peaches and strawberries. It is a good source of fiber and energy, and can be consumed in a variety of ways, such as fresh fruit, juice, cider, cooked into pies or sauces and other baked dishes. It is also rich in flavonoids and many other phenolic

compounds, which may play central roles in reducing risk of chronic disease in humans (Boyer and Liu, 2004).

In 2017 apple represented the 17th most valuable cultivated crop and the 3rd most valuable fruit plant after watermelon and banana, globally (FAOSTAT, 2017). The total worldwide apple production was estimated to have increased by almost the 50% between 2000 and 2016 reaching approximately 83 million tonnes in 2017 (up by 2 million tonnes (2.5%) more than the year before) and a global value of 45 billion US dollars (FAOSTAT, 2017). However, there was a relatively stable trend over the last six years with only mild fluctuations. In 2017, most important apple producers were China, United States, Turkey and Poland with a global production of 41, 5, 3 and 2.4 million tonnes, respectively (FAOSTAT, 2017). China and United States were also the main global suppliers of apples followed by Italy, Poland and Chile (FAOSTAT, 2017).

Apple production faces continual new challenges, such as increased marketability (in terms of improved taste, texture, storage, and resistance to browning) or increased resistance to several environmental stress conditions. Besides non-infectious factors (e.g. extreme temperatures, irradiation, soil conditions and chemicals), many infectious agents can cause disease in apple tree. Among them, the bacterium *Erwinia amylovora* (*E. amylovora*, Burrill; Winslow et al., 1920), the causative agent of fire blight disease, is probably the most destructive pathogen affecting apple and its production (Figure 1).

Fire blight disease in apple

The pathogen

E. amylovora is the type species for the genus *Erwinia*, a genus of the Enterobacteriaceae family which includes the Gram-negative, motile, aerobic to facultative anaerobic, non-sporulating bacteria associated with plants (Brenner, 1984). The estimated genome size for *E. amylovora* is approximately 3.8 Mb, based on the genome sequence for the 'ATCC 49946' strain (Sebaihia et al., 2010). The *E. amylovora* cell is a Gram-negative rod with 2-7 peritrichous flagella and it is surrounded by an exopolysaccharide capsule (Figure 1a).

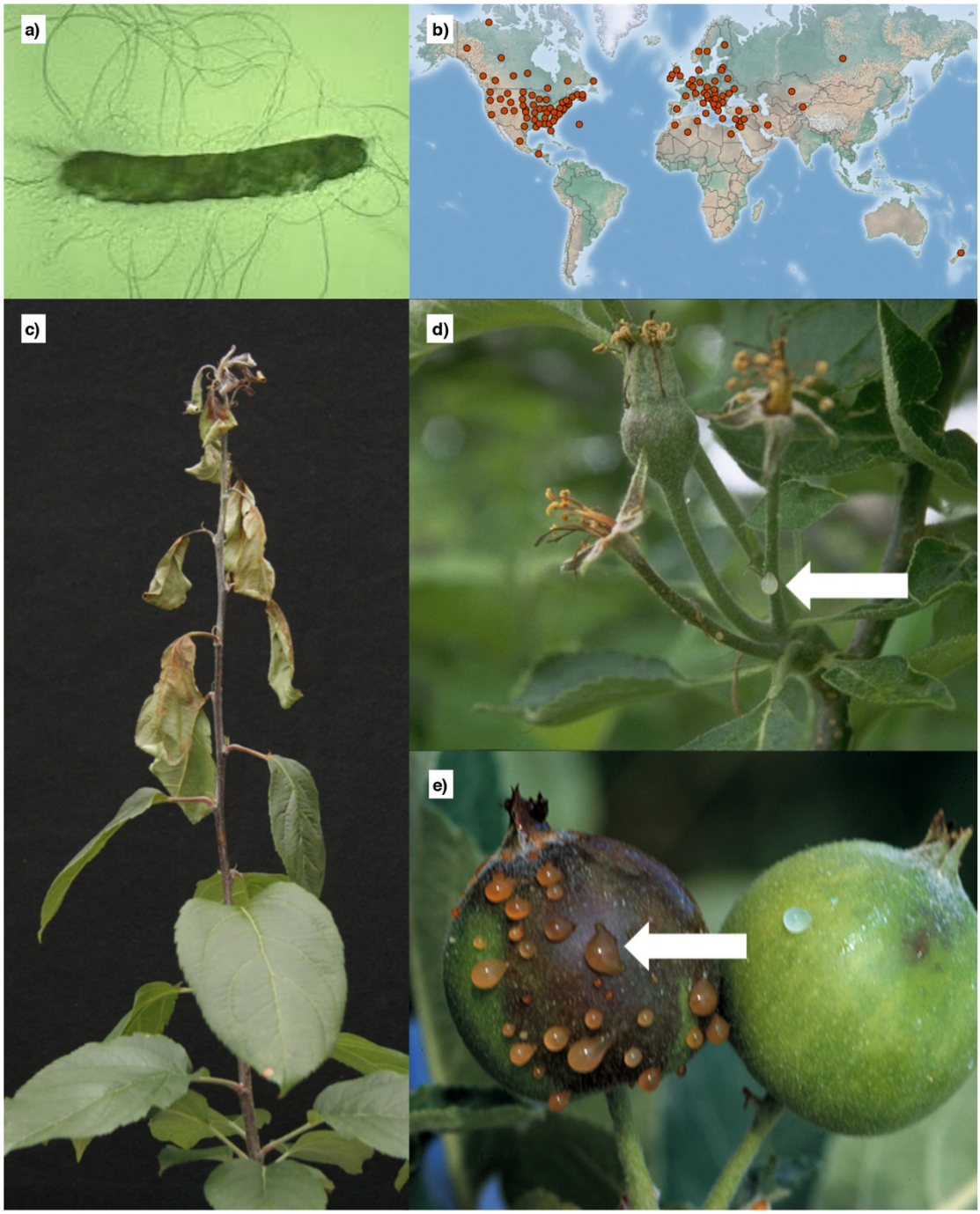

Figure 1. Fire blight disease in apple. (a) *Erwinia amylovora* cell (b) Worldwide fire blight epidemics (c) Shoot blight (d) Blossom blight (e) Fruit blight. Arrows point to bacterial ooze. (modified versions from CABI, 2018; Johnson, 2000; Norelli et al., 2003)

The optimal growing temperature is 25-27°C, while, for natural infection, the optimal temperature is between 18°C and 30°C (Billing, 1992). *E. amylovora*

forms colonies of characteristic color and colony formation on most culture media (Bereswill et al., 1998). In infected plants, the bacteria are however not visible to the naked eye, but when symptoms are present they would be visible to the naked eye.

E. amylovora is highly virulent and capable of rapid systemic movement and dissemination within apple host. The internal movement of the pathogen through the vascular system of the plant and the ability of the pathogen to infect flowers, actively growing shoots, and rootstocks makes the management of fire blight extremely difficult in the orchard (Dellagi et al., 1998; Koczan et al., 2011; Malnoy et al., 2012; Norelli et al., 2003; Thomson, 1986; Vanneste and Eden-Green, 2000; Winslow et al., 1920; Zeng and Sundin, 2014).

The disease

Fire blight is among the most serious bacterial disease affecting *M. x domestica* cultivars worldwide. Epidemics are sporadic and are dependent on a number of factors, including favorable environmental conditions, sufficient inoculum level present in the orchard and host susceptibility. Wherever they have been reported (Figure 1b), the economic damages following fire blight outbreaks are estimated in millions of US dollars (Hasler et al., 2002; Norelli et al., 2003). The disease is easily dispersed by birds, insects, rain or wind, and depending on the affected plant part the disease produces blossom blight, shoot or twig blight, leaf blight, fruit blight, limb or trunk blight, or collar or rootstock blight (Norelli et al., 2003; Thomson, 2000; Vanneste and Eden-Green, 2000). The disease begins in spring with the production of the primary inoculum from bacteria overwintering in cankers causing blossom infection (Figure 1c). Infected blossoms become water-soaked, then wilt, shrivel and turn orange or brown to black. The disease continues into summer with shoot and fruit infection (Figure 1d, 1e). Infected leaves wilt, shrivel, then turn brown. Similarly, upon infection fruits appear oily or water-soaked, become brown to black, and often ooze droplets of bacterial exudate. Finally, fire blight ends in winter with the

development of cankers throughout the dormant period of the host (Norelli et al., 2003; Thomson, 2000; Vanneste and Eden-Green, 2000).

Prevention and control of fire blight

The apple world market is dominated by just a few cultivars (e.g. 'Braeburn', 'Golden Delicious', 'Gala', 'Red Delicious', 'Jonagold', 'Pink Lady' and 'Fuji') susceptible to various diseases. In many production areas of the world major diseases such as apple scab, powdery mildew and fire blight are considerable threats to production and require a greater number of chemical treatments. However, while some fungicides are available to control apple scab and powdery mildew, no effective strategies are currently available for fire blight. Below, some strategies to prevent/combat fire blight disease are reported.

- Cultural control

  Cultural methods include the sanitation of trees, obtained by a prompt pruning out of symptoms as soon as they are detected in an orchard or a plantation (Chen et al., 2018; Steiner, 2000). The suppression of primary and secondary blossoms by severe trimming has also been recommended (Lecomte and Paulin, 1992; Meijneke, 1984). Apple losses can also be prevented by selecting trees propagated on resistant rootstocks for new orchards (Cline et al., 2001; Norelli et al., 2003).

- Chemical control

  Bordeaux mixture and fixed coppers were the first compounds used to control fire blight (Psallidas and Tsiantos, 2000). Antibiotics (streptomycin, oxytetracycline, oxolinic acid and gentamicin) are also used, especially to prevent flower and shoot infections. The most effective antibiotic is streptomycin. To date, streptomycin is however not registered for commercial use in many countries and streptomycin-resistant *E. amylovora* strains have arisen where streptomycin was used frequently (Jones and Schnabel 2000). An effective strategy is the

application of the prohexadione-calcium, a compound which reduces apple shoot elongation resulting in less susceptibility to fire blight (Sobiczewski et al., 2001). Finally, the acibenzolar-S-methyl can stimulate the tree's natural defense mechanisms and provide a significant level of fire blight control (Brisset et al., 2000; Maxson-Stein et al., 2002).

- Biological control

  Experiments with antagonistic bacteria have been conducted with strains of *Pseudomonas agglomerans*, *Pseudomonas fluorescens*, *Lactobacillus plantarum, Pantoea vagans and Bacillus subtilis* (Aldwinckle et al., 2002; Broggini et al., 2005; Johnson and Stockwell, 1998, 2000; Mercier and Lindow, 2001; Roselló et al., 2013; Smits et al., 2010; Vanneste, 1996; Vanneste et al., 2002b). However, the biological control of fire blight is not widely practiced at present, as consistency in the level of control has not been easy to obtain.

- Host-plant resistance control

  Several studies on fire blight susceptibility of seedlings, cultivars and rootstocks have been carried out to identify resistant cultivars or sources of fire blight resistance, and these sources of resistance are being used by breeding programs in several countries for apple (Khan and Chao, 2017; Lespinasse and Aldwinckle, 2000; Malnoy et al., 2012). In addition to the use of traditional breeding methods to produce new resistant cultivars, genetic engineering methods to enhance the resistance of existing cultivars have also been evaluated and applied (Broggini et al., 2014; Kost et al., 2015; Norelli and Aldwinckle, 2000). The use of resistant cultivars and rootstocks in apple production is considered a promising method to manage the disease. However, dominantly inherited resistance has been shown to be potentially overcome within years (Mundt, 2014). The need of breeding continuously new resistant

apple cultivars is thus an essential aspect for a long-lasting management of the disease. Within this context, the identification of susceptibility (S) genes, required by the pathogen to infect the host-tissue, has been identified as a successful alternative strategy to breed resistant apples (Borejsza-Wysocka et al., 2004; Borejsza-Wysocka et al., 2006; Campa et al., 2018).

Erwinia amylovora: the major virulence determinants

During the last three decades, several genetic studies were performed to identify and characterize genes involved in the *E. amylovora* virulence (Oh and Beer, 2005), including the Harpin (Hrp) Type III Secretion System (T3SS), the exopolysaccharide (EPS) amylovoran, biofilm formation and motility. Among them, the T3SS has been described as the major virulence determinant required for *E. amylovora* to infect and cause disease on host plants (Vrancken et al., 2013). The T3SS is a specialized pilus-like structure extruded on the bacterial surface able to release proteins named harpins that are effectors delivered into the apoplast or directly into the plant cell. To date, twelve secreted effectors proteins have been reported (e.g. Eop1, Eop3, Eop4, DspA/E, Eop2, HrpK, HrpN, HrpW and HrpJ) (Nissinen et al., 2007). The first T3SS effectors identified were harpins (Hrp) (HrpN and HrpW) and DspA/E, and their characterization is still an important challenge to understand.

- DspA/E

  DspA/E belongs to the AvrE-family of T3 effectors. Effectors of this family are all encoded by genes adjacent to the *Harpin-T3SS* gene cluster. These effectors are important to promote bacterial growth after infection and, most importantly, in *E. amylovora* DspA/E is central to the disease process, as *DspA/E* mutants do not grow on host plants and can not induce the disease (Barny, 1995; Degrave et al., 2013; Gaudriault et al., 1997; Siamer et al., 2014). DspA/E is required to block callose

deposition, a plant basal defense response which strengthens the plant cell wall at the site of infection (DebRoy et al., 2004), and to repress Pathogenesis-Related 1 (PR1) expression (Boureau et al., 2006). DspA/E ectopic expression induces necrosis in apple and tobacco (Boureau et al., 2006; Oh et al., 2007). In apple, yeast two hybrid assay performed with the N-terminal half of DspA/E as bait identified four serine/threonine receptor kinases of apple that were designated 'DspE-Interacting Proteins from *Malus* (DIPM). When the C-terminal half was used as bait, the only interactor identified was a cytoplasmatic precursor of ferredoxine (Oh and Beer, 2005). It is however still unclear how the identified interactors from *Malus* are involved in the functions of DspA/E in plants.

- Harpins

  Harpin proteins, a subset of T3 effectors found in many phytopathogenic bacteria, are able to induce both hypersensitive reaction in non-host plants such as tobacco (Gaudriault et al., 1998; Kim and Beer, 1998), and symptoms development in susceptible plants (Beer et al., 1991). In the pool of Hrp proteins, HrpN and HrpW are the most abundant (Nissinen et al., 2007). While *HrpW* mutants are pathogenic like the wild-type strain and elicit hypersensitive response more strongly than the wild-type strain, *HrpN* mutants are strongly altered in virulence on host plants and elicit weak hypersensitive response on non-host plants (Dong et al., 1999; Gaudriault et al., 1998; Kim and Beer, 1998; Wei et al., 1992). This observation suggests that HrpN is mandatory for *E. amylovora* pathogenicity. Briefly, HrpN has been shown to trigger an oxidative burst (Baker et al., 1993), mitogen-activated protein kinase activation (Adam et al., 1997), cytosolic [Ca^{2+}] elevation (Cessna et al., 2001; Pike et al, 1998), defense gene expression (Degrave et al., 2008; Dong et al., 2004; Liu et al., 2010; Peng et al., 2003), ion fluxes modulation (El-Maarouf et., 2001; Popham et al., 1993), and drought

tolerance through an abscisic acid dependent signaling (Dong et al., 2005). HrpN was also reported to enhance plant growth (Jang et al., 2006; Oh and Beer, 2006). This effect is likely to be mediated by HIPM (HrpN-Interacting Protein of *Malus*), a plasma membrane localized protein, which interacts with HrpN in yeast and *in vitro* and functions as a negative regulator of plant growth (Oh and Beer, 2006).

Fire blight resistance and susceptibility sources in apple

Resistance genetic sources

Over the past two decades, different fire blight resistant sources have been found, particularly in wild apples. Major QTLs conferring a good level of host resistance were identified, described and mapped in wild apples (Figure 2) such as *Malus x robusta* 5 (MR-5) (chromosome 3), *Malus fusca* (chromosome 10), *Malus floribunda* 821 (chromosome 12), *Malus x arnoldiana* (chromosome 12) and the ornamental cultivar 'Evereste' (chromosome 12) (Durel et al., 2009; Emeriewen et al., 2014; Emeriewen et al., 2017; Peil et al., 2007) (Figure 2). The strong QTL of MR-5 (FB_MR5) explained 67-83% of the phenotypic variation. In the progeny, about 20-25% of the seedlings remained resistant to the pathogen (Peil et al., 2008). Within the QTL region on chromosome 3 of MR-5, a candidate resistance gene was identified by a map-based cloning approach (Fahrentrapp et al., 2013). This candidate, which belongs to the CC-NBS-LRR resistance gene family, was transferred into the genome of the apple cultivar 'Gala' resulting in strong phenotypic resistance of the transformed plant lines against *E. amylovora* (Broggini et al., 2014). Another QTL explaining 50-70% of phenotypic variation was identified in 'Evereste' (FB_E) (Durel et al., 2009) and an additional one in *Malus fusca* (FB_Mfu10), explaining about 66% of the phenotypic variation (Emeriewen et al., 2014). The QTLs FB_MR5, Fb_E and FB_Mfu10 confer a stronger resistance in contrast to the QTL in chromosome 7 from 'Fiesta' (FB_F7) (Calenge et al., 2005; Khan et al., 2006). For all these QTLs, markers for assisted selection were developed (Emeriewen

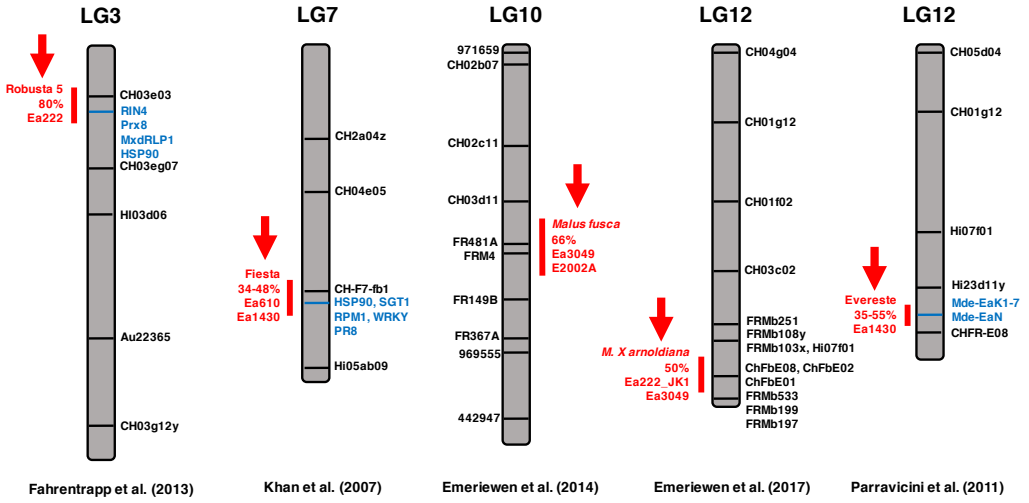

Figure 2. Schematic illustration of the major QTLs conferring good level of fire blight resistance in apple.

et al., 2014; Emeriewen et al., 2017; Fahrentrapp et al., 2013; Khan et al., 2007; Parravicini et al., 2011).

However, as these strong resistance QTL's were found in crab apples with low fruit quality, several backcross generations are required before a new commercial cultivar carrying this resistance can be obtained. Besides wild apples, a range of traditional and modern varieties and selections showed low fire blight susceptibility (Kellerhals et al., 2012; Szobiczewski et al., 2011). These varieties are interesting in terms of breeding and marketability. Nonetheless, resistance loci have not been mapped and no related molecular markers have been developed for those resistances (Kellerhals et al., 2017).

Besides minor QTLs of 'Fiesta' (FB_F7) (Calenge et al., 2005) and 'Florina' (chromosomes 10 and 5) (Le Roux et al., 2010), a few novel fire blight minor QTLs were recently identified in a 'Royal Gala' *x Malus sieversii* population (Desnoues et al., 2018).

Susceptibility genetic sources

To date, many efforts have been made to better understand the main molecular mechanisms underlying both plant host resistance and *E. amylovora*

pathogenesis. In this context, the centrality of the T3SS and the effector proteins that it delivers to the host cells represent attractive targets for further investigation and potential manipulation. Most effector proteins act inside the host cell and some have been shown to interact with factors encoded by the apple genome. These apple genes are of primary interest, as they may represent susceptibility (S) genes, i.e., plant genes that facilitate the compatible interaction between plants and pathogens and, therefore, the progression of the disease (van Schie and Takken 2014). To date, two apple gene families were shown to have role in plant susceptibility to fire blight.

Previous studies with HrpN identified an apple transmembrane receptor protein via a yeast two-hybrid analysis that interacts with HrpN and termed HIPM (for HrpN-interacting protein of *Malus*) (Oh and Beer 2007). The authors hypothesized that HIPM was as a pivotal receptor for the important *E. amylovora* effector HrpN, which is necessary for infection. Recently, Campa and colleagues (2018) investigated silencing of HIPM in apple (cv. Galaxy) which resulted in significantly reduced (ca. 40%) susceptibility of plants to *E. amylovora*. Moreover, the authors identified an oxygen-evolving enhancer-like protein (OEE) from apple (MdOEE) that interacts with HIPM, thus giving new inputs in the understanding of the plant response mechanism to *E. amylovora* and the progression of the fire blight disease.

Next to the *HrpN* cluster of the *E. amylovora* pathogenicity island is the Disease specific (*Dsp*) region which encodes the pathogenicity effector protein DspA/E, essential for the development of fire blight. This effector interacts specifically and physically with four Disease Interacting Proteins from *Malus* (DIPM1-4) (Meng *et al*., 2006). The interaction between DIPMs and DspA/E is thought to be involved in fire blight spread by acting as a susceptibility determinant. Borejsza-Wysocka and colleagues (2004, 2006) verified and confirmed this hypothesis by preventing DIPMs-DspA/E interactions in transgenic apple lines with silenced *DIPM* genes. The produced transgenic lines showed increased (ca. 50%) resistance after artificial inoculations with the pathogen.

Overall, through the knock-down of their expression, HIPM and DIPM genes have been highlighted to play crucial role in the susceptibility of apple to *E. amylovora* infection. From an applied point of view, this is a major step forward, since new breeding strategies directed to their suppression, i.e., genome editing techniques like CRISPR/Cas9, could provide a durable solution to fire blight.

Genome editing tools for crop improvement

The history of plant genetic improvement began with the selection of especially desirable agricultural traits. This was obtained by cross, hybrid and mutation breeding, and later genetic engineering and marker-assisted breeding. These developments were necessary to provide new solutions for the steady increase of requirements from society. Within this context, the novel gene editing technologies may expand these possibilities.

Genome editing is a pool of advanced molecular biology techniques that allow precise, efficient, and targeted modifications of a gene of interest (Chen and Gao, 2013, Gao, 2015) (Figure 3). Over the past decades, genome editing techniques such as Zinc-Finger Nucleases (ZFNs) (Kim et al., 1996) and Transcription Activator-Like Effector Nucleases (TALENs) (Christian et al., 2010) has been widely used for crop improvement (Martínez-Fortún et al., 2017; Ran et al., 2017; Zhang et al., 2018).

ZFNs are fusions of zinc-finger-based DNA-recognition modules and the DNA-cleavage domain of the FokI restriction enzyme (Figure 3a). Each individual zinc finger typically recognizes and binds to a nucleotide triplet, and fingers are often assembled into groups to bind to specific DNA sequences (Urnov et al., 2010). Like ZFNs, TALENs are fusions of TALE repeats and the FokI restriction enzyme (Boch et al., 2009) (Figure 3b). However, each TALE repeat individually targets a single nucleotide, allowing for more flexible target design and increasing the number of potential target sites compared with ZFNs.

Recently, it has been developed a new gene editing technology, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR associated

protein 9 (Cas9) (CRISPR/Cas9) system (Doudna et al., 2014) (Figure 3d). Compared with ZFNs and TALENs, CRISPR/Cas9 is more efficient, rapid, precise and low-cost, and it can also edit multiple target genes simultaneously (Zhao et al., 2015). CRISPR/Cas9 cleaves foreign DNA through two components, Cas9 and sgRNA. Cas9 is a DNA endonuclease that can be derived from different bacteria, such as *Brevibacillus laterosporus*, *Staphylococcus aureus*, *Streptococcus thermophilus*, and *Streptococcus pyogenes* which is the most widely used. Cas9 contains two domains, i.e. HNH domain and RucV-like domain. The HNH domain cuts the complementary strand of crRNA, while the RucV-like domain cleaves the opposite strand of DNA. The sgRNA is a synthetic RNA with a length of about 100 nucleotides (nts). Its 5′-end has a 20-nts sequence that acts as a guide sequence to identify the target sequence next to a Protospacer Adjacent Motif (PAM) sequence, which is often the consensus 5′-NGG-3′. The loop structure at the 3′-end of the sgRNA can anchor the target sequence by the guide sequence and form a complex with Cas9, which then cleaves the DNA (Doudna et al., 2014).

Overall, all of these technologies can thus be induced to recognize specific DNA sequences and to generate double-stranded breaks (DSBs) (Figure 3c). After DSBs, the plant's endogenous repair systems fix the DSBs either by Non-Homologous End Joining (NHEJ), which can lead to the insertion or deletion of nucleotides thereby causing gene knock-outs, or by Homologous Recombination (HR), which can cause gene replacements and insertions (Figure 3c) (Symington and Gautier, 2011). To date, many knock-out mutants, gene replacement and insertion mutants have been produced through the use of gene editing technologies, especially CRISPR/Cas9, in large variety of plants (Martínez-Fortún et al., 2017; Ran et al., 2017; Zhang et al., 2018).

Although CRISPR/Cas9 has been widely applied to gene functional studies and molecular breeding in several (agronomically important or model) plant species, very little is known on its potentiality in apple. Two previous studies successfully

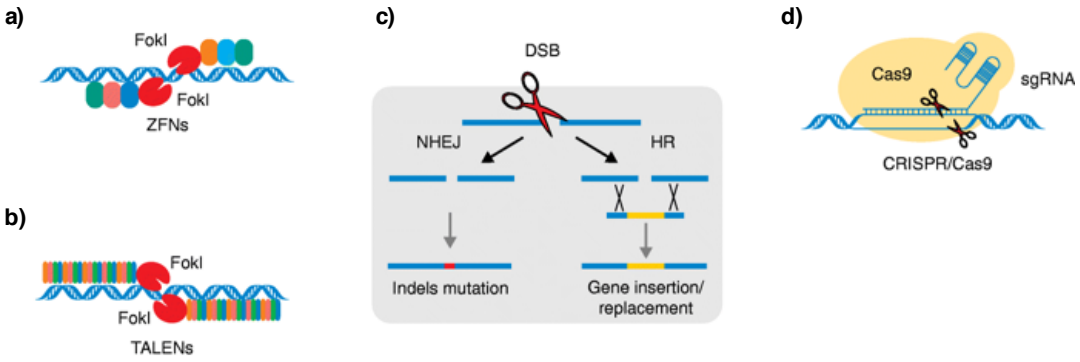

Figure 3. Genome editing tools and DNA repair mechanisms. (a) ZFNs. (b) TALENs. (c) endogenous repair systems for DSBs (NHEJ or HR). (d) CRISPR/Cas9 system. (modified version from Zhang et al., 2018)

conducted proofs-of-concept to investigate the feasibility of knocking-out by CRISPR/Cas9 delivered *via Agrabacterium tumefaciens* (*A. tumefaciens*) *PDS* and *TFL1* genes (Charrier et al., 2019; Nishitani et al., 2016). Differently, Malnoy and colleagues (2016) tested the editing produced in *DIPM* genes by Cas9-sgRNA delivered as ribonucleoproteins in protoplasts of the apple cultivar 'Golden Delicious'. This strategy, which represents one of the most recent applications of CRISPR/Cas9, is 'clean' if compared with the classical *A. tumefaciens*-mediated delivery. Following the editing at the targeted genomic site, the Cas9-sgRNA ribonucleoprotein is processed resulting in edited protoplasts free from foreign editing machinery, which can be later regenerated into a new plant (Zhang et al. 2018). However, in apple applications of CRISPR/Cas9 system can not rely on this methodology as efficient protocols for protoplast regeneration are still not available in literature. Currently, conventional transformation methodologies *via A. tumefaciens* are thus pivotal for this plant species, although these procedures lead to the generation of edited plants containing exogenous DNA.

Strategies for the removal of exogenous DNA sequences from the genome of transgenic plants

Genetic engineering techniques mediated for instance by *A. tumefaciens* involve the addition of exogenous marker genes. These selectable marker genes are required for obtaining transgenic plants, but undesirable to be retained in commercial transgenic plants because of their possible toxicity or allergenicity to humans and their unpredictable hazards to the environment (Rosellini, 2012). Based on these information, and given the need to obtain plants without superfluous exogenous DNA elements, over the years researchers have looked for solutions to eliminate marker genes (or T-DNA) once the plant transformation has been verified. Several approaches have been developed, such as T-DNA mediated segregation between selectable marker gene and gene of interest (Mcormac et al., 2001; Xing et al., 2000), homologous recombination between direct repeats (Linchenstein et al., 1994; Zubcko et al., 2000) and a site-specific recombination mediated approach (Sugita et al., 2000; Zuo et al., 2001). In comparison with the former two approaches, the latter is easier to manipulate and more efficient.

Site-specific recombination approaches most rely on the application of three different recombination systems:

- Cre/lox, isolated from bacteriophage P1 (Dale and Ow, 1991);
- FLP-FRT, isolated from *Saccaromyces cerevisae* (Golic and Lindquist, 1989);
- R/Rs, isolated from *Zygosaccharomyces rouxii* (Onouchi et al., 1991).

A common feature of these systems is that each system consists of a recombinase Cre, FLP, or R and two identical or similar palindromic recognition sites, lox, FRT, or RS (Figure 4). Each recognition site contains a short

Figure 4. Simplified illustration of Cre/lox, FLP/FRT and R/Rs mechanism of action.

asymmetric spacer sequence where DNA strand exchange takes place, flanked by inverted repeat sequences where the corresponding recombinase specifically binds. If two recognition sites are located in *cis* on a DNA molecule, the DNA segment can be excised if flanked by two directionally oriented sites or inverted if flanked by two oppositely oriented sites. If two recognition sites are located in *trans* on two different DNA molecules, a reciprocal translocation can happen between the two DNA molecules or the two molecules can integrate if at least one of them is a circular DNA (Groth and Calos, 2003; Ow, 2002).

In apple, grape, potato and many other crops, these systems have been successfully applied for the removal of exogenous DNA elements, as for instance the most common antibiotic resistance gene *nptII*, after the transformation event (Cuellar et al., 2006; Dalla Costa et al., 2016; Herzog et al., 2012; Kost et al., 2015).

MicroRNAs and their potential role in genetic engineering of apple

MicroRNAs (miRNAs) are non-coding RNAs of approximately 20-24 nts in length that act as central regulators of gene expression by targeting messenger RNAs for cleavage or translational repression. In plants, miRNAs are associated with numerous regulatory pathways in growth and development, and defensive responses upon plant-pathogen interactions (Djami-Tchatchou et al., 2017).

In apple, approximately three hundred miRNAs have been identified and characterized (www.mirbase.com). A previous study showed that many miRNAs are expressed in the phloem tissue and phloem sap (Varkonyi-Gasicet et al., 2010). This study demonstrated that some miRNAs in the phloem sap can play

a long distance signaling role for the regulation of plant phosphate homeostasis with a shoot to root transport activity (Pant et al., 2008; Varkonyi-Gasicet et al., 2010). Other studies identified and characterized apple miRNAs potentially implicated in diverse aspects of plant growth, development, flower induction and fruit production (Fan et al., 2018; Gleave et al., 2008; Qu et al., 2016; Song et al., 2017; Xia et al., 2012; Xing et al., 2016; Yao et al., 2015). Ma et al. (2014) and Zhang et al. (2018) showed that a single apple miRNA regulates the expression of a NBS–LRR protein during pathogen infection leading to resistance against the plant pathogenic bacterium *Alternaria alternata* f.sp. mali. Similarly, other works focused on the identification and characterization of miRNAs putatively involved in apple response to ring rot disease (Yu et al., 2014), *Glomerella* leaf spot (Zhang et al., 2019) and *Valsa* canker (Feng et al., 2017). Recently, different miRNAs were also reported to be potentially involved in resistance to fire blight by targeting stress response proteins (Kaja et al., 2015).

Based on these information, the manipulation of miRNA expression levels may represent a promising strategy for improving the responses of apple to environmental stresses, attacks by pathogens as well as plant growth and development. More efforts are however required to better elucidate apple miRNAs and their role with the aim of setting up potential and suitable miRNA-based strategies for genetic engineering of this important plant species.

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AIM OF THE THESIS

Fire blight, caused by the bacterium *E. amylovora*, is among the most economically important and contagious diseases affecting apple. The goal of this thesis is to improve fire blight resistance trait in susceptible commercial apple cultivars through biotechnology, by exploiting different strategies as below described.

In Chapter 2, we develop a CRISPR/Cas9-FLP/FRT-based gene editing system for the production of apple cultivars with reduced susceptibility to the disease and a minimal trace of exogenous DNA. The target of the editing is a member of the DIPM gene family which is involved in plant susceptibility to fire blight, according to the available literature. Within the context of a sustainable agriculture, this work provides a cutting-edge strategy for the production of improved apple cultivars.

In Chapter 3, we investigate the apple miRNA *MdmiR285N* that is predicted to play a key role in the regulation of plant immunity. By studying its physiological and pathogen-induced transcriptional regulation in *M. x domestica* and in the heterologous plant species *A. thaliana*, this work represents an initial part of a long term-study aiming at setting up a miRNA-based strategy for the genetic improvement of fire blight resistance.

The Chapter 4 is methodological. We set up a quantitative real-time PCR method for the estimation of the copy number of T-DNA integration in transgenic apple lines. This method helps to characterize new breeding technologies apple products (chapter 2) or, more in general, transgenic apple plants used in basic gene functional studies (chapter 3).

CHAPTER 2

Reduced fire blight susceptibility in apple cultivars using a high-efficiency CRISPR/Cas9-FLP/FRT-based gene editing system

CHAPTER 2

Reduced fire blight susceptibility in apple cultivars using a high-efficiency CRISPR/Cas9-FLP/FRT-based gene editing system

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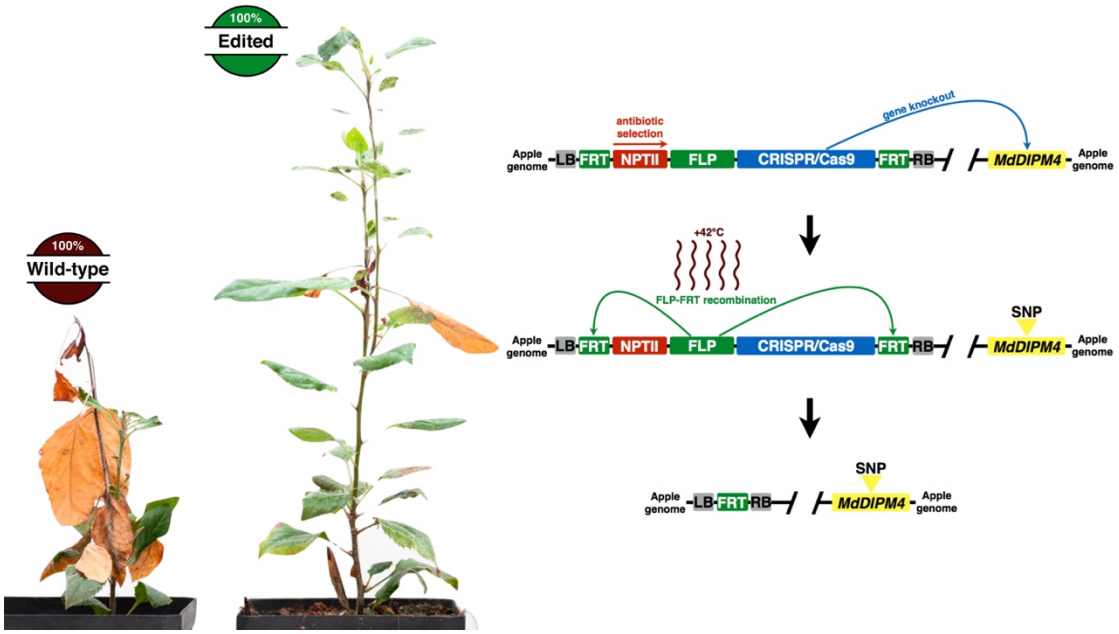
GRAPHICAL SUMMARY



Application of a CRISPR/Cas9-FLP/FRT-based editing system for the production of apple plants with reduced fire blight susceptibility and a minimal trace of exogenous DNA. The strategy exploits the use of a transformation vector containing a kanamycin resistance system for plant selection after transformation, a CRISPR/Cas9 editing system to specifically knock-out the fire blight-susceptibility apple gene *MdDIPM4*, and a heat-shock inducible FLP/FRT recombination system for a later excision of the integrated T-DNA.

This illustration was submitted as potential cover of the volume of Plant Biotechnology Journal in which the presented work will be inserted.

SUMMARY

The bacterium *Erwinia amylovora,* the causal agent of fire blight disease in apple, triggers its infection through the DspA/E effector which interacts with the apple susceptibility protein MdDIPM4. In this work, an *MdDIPM4* knock-out has been produced in two *Malus x domestica* susceptible cultivars using the CRISPR/Cas9 system delivered *via Agrobacterium tumefaciens*. Fifty-seven transgenic lines were screened to identify CRISPR/Cas9-induced mutations. An editing efficiency of 75% was obtained. Seven edited lines with a loss-of-function mutation were inoculated with the pathogen. Highly significant reduction of susceptibility was observed compared to control plants. Sequencing of 5 potential off-target sites revealed no mutation event. Moreover, our construct contained a heat shock-inducible FLP/FRT recombination system designed specifically to remove the T-DNA harbouring expression cassettes for CRISPR/Cas9, the marker gene and the FLP itself. Six plant lines with reduced susceptibility to the pathogen were heat-treated and screened by real-time PCR to quantify the exogenous DNA elimination. The T-DNA removal was further validated by sequencing in one plant line. To our knowledge, this work demonstrates for the first time the development and application of a CRISPR/Cas9-FLP/FRT gene editing system for the production of edited apple plants carrying a minimal trace of exogenous DNA.

KEYWORDS

Malus x domestica, fire blight, DIPM, gene editing, FLP/FRT recombination

INTRODUCTION

Apple (*Malus x domestica*) is one of the most cultivated fruit crops throughout the temperate regions of the world. Its production faces continual new challenges such as a constant change in consumer demand, based on a variation of tastes and flavors, and, from an agronomic point of view, climate change and harmful biotic agents (insects or bacterial, fungal and viral pathogens). Among them, one of the major economic threats to apple

production worldwide is the necrogenic and highly infectious Gram-negative bacterium *Erwinia amylovora* (Burrill) (Winslow et al., 1920), the causative agent of fire blight disease.

E. amylovora uses a complex regulatory network of many virulence determinants to establish infection. However, recent studies have been particularly focused on DspA/E, a 198 kDa effector protein homologous to the type III effector AvrE of *Pseudomonas syringae* pv. *tomato* (Gaudriault et al., 1997). It has been shown that the capacity of the bacterium to induce disease mainly depends on this single delivered effector (Siamer et al., 2014). Degrave et al. (2013) showed that DspA/E was required for transient bacterial growth in nonhost *Arabidopsis thaliana* leaves, while an *E. amylovora dspA/E* mutant was unable to grow. In addition, after its secretion into the cytoplasm of host plant cells, DspA/E was shown to interact with the intracellular domains of host plant receptor kinases (Boureau et al., 2006, Oh et al., 2007). In apple, using a yeast two-hybrid assay and an *in vitro* protein pull-down assay, Meng et al. (2006) demonstrated that DspA/E physically and specifically interacts with the kinase domain of four leucine-rich repeat (LRR) receptor-like serine/threonine kinases (RLK), called DspA/E-Interacting Proteins of *M. x domestica* (DIPM1 to DIPM4). The structures of DIPMs indicate that they might function in signal transduction, perhaps by sensing extracellular signals with LRRs and interacting with effectors through the RLKs. Thus, Meng and colleagues suggested that the interaction of DspA/E with the DIPMs may suppress a defense response by interrupting DIPMs signal transduction and that DIPM proteins may act as susceptibility factors during the *E. amylovora*–apple interaction.

Among the most advanced technologies of genetic engineering (New Breeding Technologies, NBT), gene editing *via* the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 (CRISPR/Cas9) has emerged as an effective tool for gene functional analysis in plants. It can directly introduce mutations into the plant genome by operating through guide RNA (designed to target a specific genomic sequence) and the Cas9 protein (which cleaves the specific site within the target gene) activating the error-

prone non-homologous end-joining (NHEJ) pathway for DNA repair (Barabaschi et al., 2016). CRISPR/Cas9 system, which involves simple designing and cloning methods, has emerged as a powerful strategy to precisely and quickly insert the desired traits into a plant genome, with the aim of facing biotic and abiotic stresses as well as improving other important agronomic traits (Jaganathan et al., 2018). To date, there have been many studies reporting the use of CRISPR/Cas9 in plants of agricultural interest, such as tomato (Pan et al., 2016, Ueta et al., 2017), potato (Andersson et al., 2017), wheat (Gil-Humanes et al., 2017, Liang et al., 2017), orange (Jia and Wang, 2014), grape (Malnoy et al., 2016, Nakajima et al., 2017, Ren et al., 2016, Wang et al., 2018), pear (Charrier et al., 2019), and apple (Charrier et al., 2019, Malnoy et al., 2016, Nishitani et al., 2016). In plants, one of the most recent applications of CRISPR/Cas9 relies on the delivery of the Cas9-guide RNA ribonucleoprotein complex directly into plant protoplasts. Following the generation of mutations at the targeted genomic site, the Cas9-guide RNA complex is processed and degraded resulting in edited protoplasts free from exogenous editing machinery, some of which can be regenerated into a new plant. In *Arabidopsis*, lettuce, petunia, rice, tobacco and wheat this methodology was successfully reported (Subburaj et al., 2016, Woo et al., 2015, Zhang et al. 2018). Nevertheless, the strategy can neither be widely applied nor represent an alternative to the conventional *Agrobacterium tumefaciens* (*A. tumefaciens*)-mediated transformation as, to date, efficient protocols for protoplast regeneration are still not available for many plant species. In fact, especially in apple, applications of the CRISPR/Cas9 system still relies on conventional transformation methodologies *via A. tumefaciens*, which however lead to the production of edited plants containing exogenous DNA.

Besides CRISPR/Cas9, multiple recombination systems such as Cre/loxP, R/Rs or FLP/FRT have been developed to remove unwanted foreign DNA elements from transformed crops with the aim of alleviating consumer and regulatory concerns. This approaches for transgene elimination are based on transformation vectors containing the recombinase gene and transgenes

between two directly repeated recombinase recognition sites. For many crops, these recombination systems have been successfully used to excise selectable marker genes from the genome of transgenic plants. For instance, in transgenic apple (Herzog et al., 2012, Righetti et al., 2014, Würdig et al., 2013) and pear (Righetti et al., 2014), proofs-of-concept were conducted to investigate the feasibility of eliminating the *nptII* marker gene using the FLP/FRT and R/Rs recombination systems. Similarly, Dalla Costa and colleagues (2016) demonstrated the removal of *nptII* gene in grape. Moreover, from an applicative point of view, these recombination systems were also applied in transformation vectors to produce cisgenic plants carrying the genetic trait of interest and free from undesirable T-DNA sequences. In the work of Kost and colleagues (2015), to improve fire blight resistance in a susceptible apple cultivar, one plant was generated using the *FB_MR5* gene in a cisgenic approach based on the FLP/FRT recombinase system. In addition, scab resistance was improved in different apple cultivars by a similar cisgenic approach to introduce the *Rvi6* gene into the apple genome (Würdig et al., 2015).

In this work, we have used a CRISPR/Cas9 gene editing approach to knock out the *MdDIPM4* susceptibility gene to reduce fire blight susceptibility in *M. x domestica* cultivars, 'Gala' and 'Golden Delicious'. Moreover, a strategy based on the heat-shock inducible FLP/FRT recombination system was applied to remove the T-DNA region containing expression cassettes of the editing machinery and selectable marker in those edited lines with reduced susceptibility to the disease.

RESULTS

Generation of edited transgenic apple lines

A total of 2000 'Gala' and 1370 'Golden Delicious' leaf explants were infected with *A. tumefaciens* containing the binary vector carrying the CRISPR/Cas9 machinery (Figure 1), specifically targeting a *MdDIPM4* region with no homology to other members of the DIPM gene family (Figure S1). The target

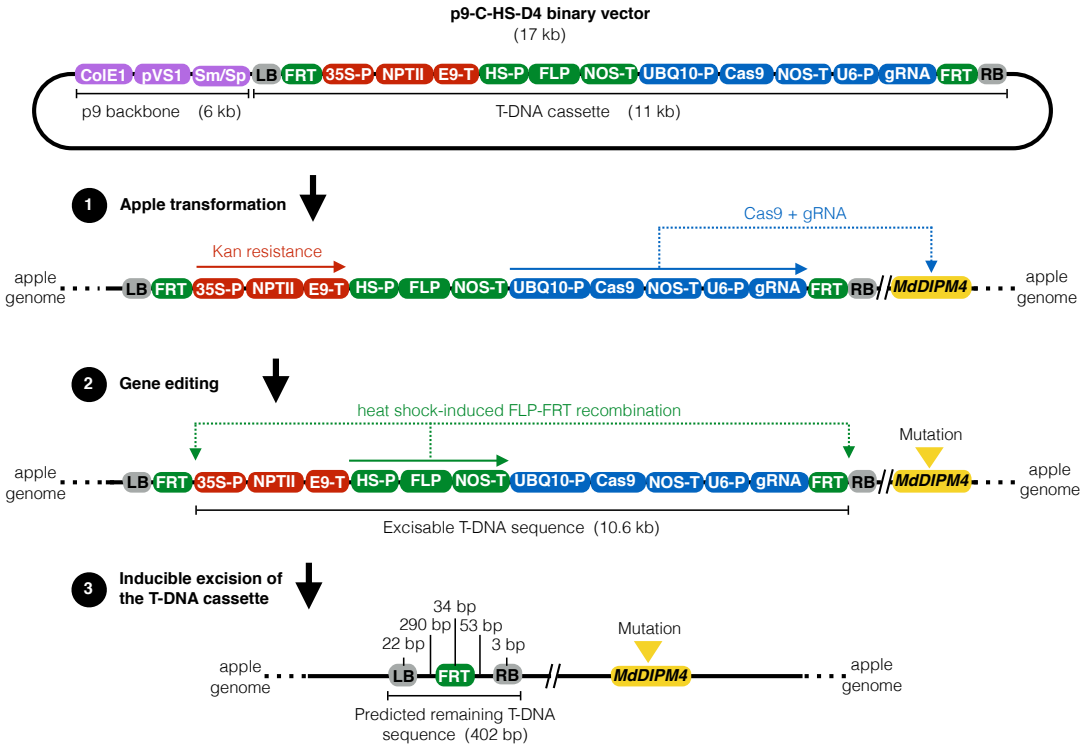


Figure 1. Schematic representation of structure and mechanism of action of the p9-C-HS-D4 binary vector based on the CRISPR/Cas9-FLP/FRT gene editing system. The binary vector is produced by cloning a 10.9 kb T-DNA cassette into a 6 kb p9 vector backbone (violet boxes). The T-DNA cassette contains a kanamycin resistance system (red boxes) for plant selection after the apple transformation (step 1), a gene editing system (blue boxes) to target genomic *MdDIPM4* (yellow box) (step 2) and a heat-shock inducible FLP/FRT recombination system (green boxes) for the excision of the exogenous DNA (step 3). *Colicin E1* and *pVS1* origins of replication (ColE1 and pVS1); Streptomycin/Spectinomycin resistance genes (Sm/Sp); Left and Right Borders (LB and RB); Flippase Recognition Target site (FRT); *Cauliflower Mosaic Virus 35S* Promoter (35S-P); *Neomycin phosphotransferase II* (NptII); E9 Terminator (E9-T); Heat Shock-inducible Promoter (HSP); *Flippase* gene (FLP); *Nopaline Synthase* Terminator (NOS-T); *Arabidopsis thaliana Ubiquitin-10* Promoter (UBQ10-P); *Crispr associated protein 9* (Cas9); *Arabidopsis thaliana U6* Promoter (U6-P); guide RNA for *MdDIPM4* target (gRNA); kanamycin (Kan).

site is identical between the two cultivars without allelic variations. Respectively 40 and 46 regenerants of 'Gala' and 'Golden Delicious' were collected approximately 6-7 months after culture in selective medium (Table 1). Regenerants were tested by PCR to screen for the integration of T-DNA (primer listed in Table S1). A total of 31 'Gala' and 35 'Golden Delicious' apple lines had the *Cas9* gene integrated in the genome and no *A. tumefaciens* contamination,

resulting in transformation efficiencies of 1.55% and 2.55%, respectively (Table 1).

Characterization of *MdDIPM4* mutants and selection of candidate apple lines

The *MdDIPM4* target region was screened in 57 transgenic lines (respectively 27 and 30 for 'Gala' and 'Golden Delicious' backgrounds, hereafter 'G' and 'GD') using high-throughput sequencing (HTS) on the Illumina MiSeq Platform (Figure 2a). On average, 3000 raw sequence reads were obtained for each of the analyzed plants. Editing results were very similar between the two cultivars, showing a percentage of non-edited plants of 22.2% for 'G' and 26.7% for 'GD', corresponding to editing efficiencies of 77.8% and 73.3%, respectively. Among the edited plants, some were completely edited showing a single type (homozygous) mutation (7.4% 'G'- 20% 'GD') or multiple mutation (heterozygous) profiles (63% 'G'- 50% 'GD'), while others had a partially edited genotype as a *wild-type* background was maintained (7.4% 'G' and 3.3% 'GD'). Several types of mutation were identified: a small insertion (+1 nt), a small replacement (R1 nt), small deletions (-1, -2, -3, -4, -5, -6, -7, - 8, -9, -10 nts), and large deletions (-22, -25, -27 nts) (Figure 2a). The most frequent type of mutation was nucleotide deletion, with a 91.7% in 'GD' and a 92.5% in 'G', followed by nucleotide insertion (5% 'G' and 8.3% 'GD') and nucleotide replacement (2.5% only for Gala) (Figure 2b). In both apple cultivars, -1 nt (21.6% 'GD' - 30% 'G') and -2 nts (16.2% 'GD'- 25% 'G') were the most abundant, followed by -3 nts (10%) and -4 nts (15%) for 'G' and -4 nts (10.8%) and -5 nts (13.5%) for 'GD' (Figure 2c).

These data were partially consistent with a previous analysis based on Sanger sequencing (Sanger experimental procedure in Supporting Information) of the target site (Figure S2) according to which three plant lines (V1-4, V1-7, V4-3) did not contain mutations and the others showed a short insertion (+1 nt) and short deletions (-1, -2, -3, -4, -5, -6, -10 nts). The discrepancy in the results

Table 1. Efficiency of *Agrobacterium tumefaciens*-mediated transformations in 'Gala' and 'Golden Delicious' apple cultivars.

Cultivars	Transformation name	No. of leaf explants infected	No. of regenerants collected	PCR screening			Transformation efficiency†
				No. of regenerants tested	No. of positive regenerants		
					Cas9	*VirG*	
Gala	V1	920	29	27	24	0	1.55%
	V3	1080	11	8	8	1	
Golden Delicious	V2	330	6	3	3	0	2.55%
	V4	1040	38	32	32	0	

†The transformation efficiency was calculated by dividing the number of regenerants positive for *Cas9* and negative for *VirG* by the number of leaf explants infected (considering V1 + V3 and V2 + V4).

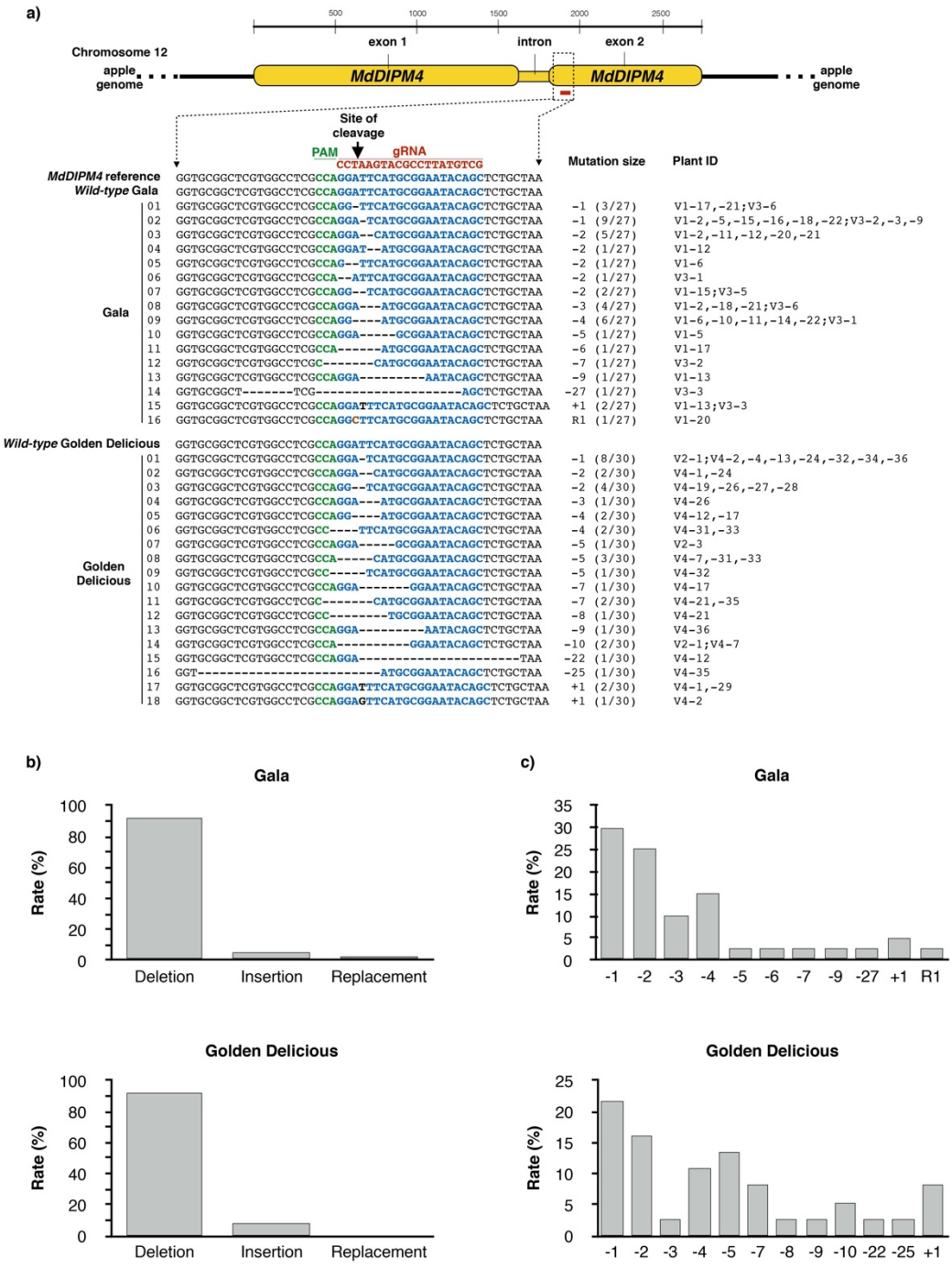


Figure 2. CRISPR/Cas9-editing in *Malus x domestica* cultivars 'Gala' and 'Golden Delicious' detected by high-throughput sequencing. (a) Representation of NHEJ mutation events generated by the CRISPR/Cas9 system in *MdDIPM4* gene. NHEJ mutations were detected on the predicted site of cleavage using as reference the *MdDIPM4* genomic sequence of the apple genome assembly GDDH13 v1.1 (Daccord et al., 2017) in addition to *wild-type* plants. The *MdDIPM4* target sequence is colored in blue. Within the sequences alignment, deletions are represented by traits. Insertions and replacement are shown

respectively with black and orange bold letters. Mutations and plant ID are shown on the right. Numbers on the left refer to those reported in Figure 3. Guide RNA (gRNA); Protospacer Adjacent Motif (PAM). (b) Rate of mutation types. (c) Rate of mutation sizes. Percentages in (2B) and (2C) were calculated by dividing the number of total events (respectively of each mutation type and size) by the sum of total mutation events.

produced by the two sequencing methods concerned the profile of two plant lines (V1-6 and V4-7).

The deduced amino acid sequences of the MdDIPM4 protein for all the analyzed plants are shown in Figure 3a. A total of 16 and 18 MdDIPM4 edited sequences were transduced for 'G' and 'GD' respectively. In 'G', 11 sequences showed frame-shifting and early termination mutations with the introduction of stop codons responsible for the premature termination of protein transduction. The remaining 5 sequences showed ORF-preserving mutations that caused the loss of 1 or few amino acids without affecting the protein translation. In 'GD', 16 sequences had early termination mutations and only 2 ORF-preserving mutations (Figure 3a). The summary of the editing rate of *MdDIPM4* for all the transgenic apple plants confirmed with T-DNA insertion for both cultivars is shown in Figure 3b.

All the transgenic plants evaluated for the editing in the target site, were subsequently characterized for T-DNA integration copy number (CN) by quantifying the *nptII* selection marker gene (Table S2). *NptII* CN ranged from a minimum of 0.2 to a maximum of 4.9. According to mutation profiles and T-DNA integration CN, lines V1-4, V1-6, V1-10, V1-14 for 'G' and V2-3, V4-5, V4-7, V4-28, V4-34 for 'GD' (Figure 3b) were used for the subsequent assays of fire blight resistance and removal of the T-DNA following the workflow shown in Figure S3 (workflow experimental procedure in Supporting Information). Those lines were re-screened by HTS in order to confirm their editing profile after 6 months of micropropagation (corresponding to 7 months after the regeneration of transgenic plants). The analysis of the reads obtained (ca. 10000 raw reads/plant) confirmed the previous data (data not shown). Moreover, with regard to putative pleiotropic effects caused by the knock-out of *MdDIPM4*,

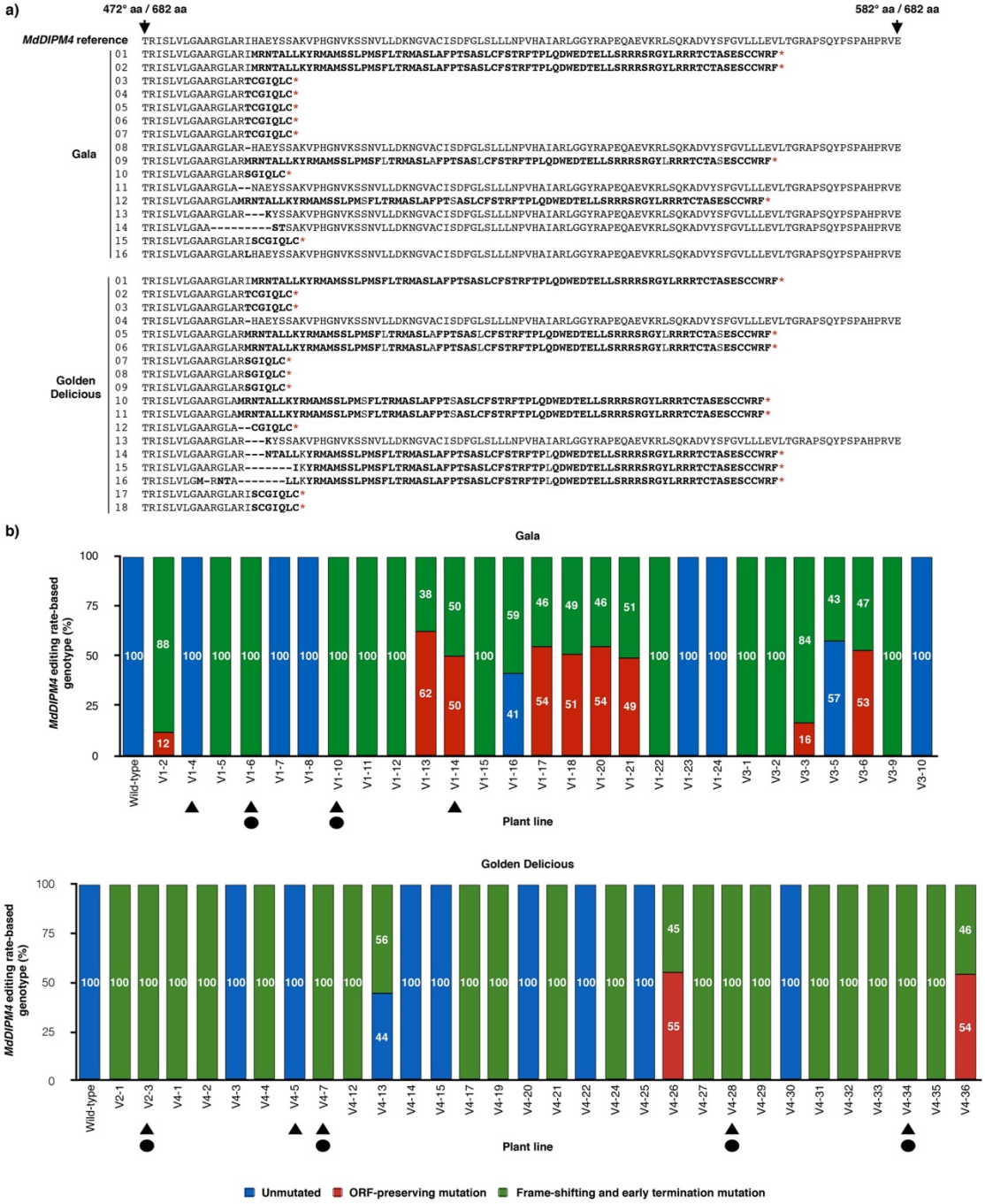


Figure 3. MdDIPM4 protein and *MdDIPM4* editing rate-based genotypes of CRISPR/Cas9-edited apple plants. (a) A fragment of the translated MdDIPM4, from 472 to 582 out of 682 amino acids (aa), containing the CRISPR/Cas9 site of cleavage is shown. Amino acids in bold indicate transcoding sequences compared to the MdDIPM4 reference. Deleted amino acids and stop codons are represented respectively by traits and red asterisks. Numbers on the left refer to those reported in Figure 2. (b) The summary of *MdDIPM4* editing rate-based genotypes of transgenic apple plants compared to *wild-type* plants is reported. Triangles and circles indicate candidate apple lines selected respectively for the analysis of plant resistance to *Erwinia amylovora* and heat-shock inductions.

selected plant lines were observed during several rounds of micropropagation and acclimation to soil and no visible phenotypic differences were found between *wild-type* and transgenic plants.

Fire blight resistance test in *MdDIPM4* knock-out mutants

Selected apple lines were infected with *E. amylovora* strain Ea273 in three independent experiments. Results of necrotic symptoms are shown in Figure 4. For the cultivar 'G', *wild-type* plants (control) were susceptible showing a necrosis percentage of 81% ± 10% (Figure 4a). Similarly, one transgenic but non-edited line (V1-4) had a necrosis phenotype of 76% ± 16%. On the contrary, all other edited lines showed a highly significant (p-value < 0.001) reduction of symptoms compared to control. The percentage of necrotic symptoms varied from 61% ± 10% for the chimeric line V1-14 to 44% ± 18% for the line V1-6 and 35% ± 11% for the line V1-10. For the cultivar 'GD', control plants and the transgenic but non-edited line V4-5 had similar necrosis percentages of 73% ± 9% and 79% ± 13%, respectively. Conversely, all other edited lines showed a highly significant (p-value < 0.001) reduction of susceptibility compared to control. Lines V2-3, V4-28 and V4-34 had a necrosis percentage of respectively 35% ± 12%, 40% ± 15% and 42% ± 11%, while for the line V4-7 the disease symptoms were even lower showing a necrosis percentage of 25% ± 11% (Figure 4a). In summary, for 'G' and 'GD' completely edited lines symptoms were reduced on average of 50%. The 'G' line V1-14 showed a lower reduction (25%) due to its chimeric profile. For this line, the presence of T-DNA chimeric tissues was based on the *nptII* CN lower than 1 (i.e.=0.6). In this case, the partial editing rate in the target site (half edited/half *wild-type* - Figure 3b) is due to chimerism and not to a heterozygous state. Moreover, in both cultivars, at the time of data collection (21 days after infection) an interruption of necrosis was detected in the edited plants and new shoots developed without symptoms, while control plants showed a continuous progression of the disease (Figure 4b).

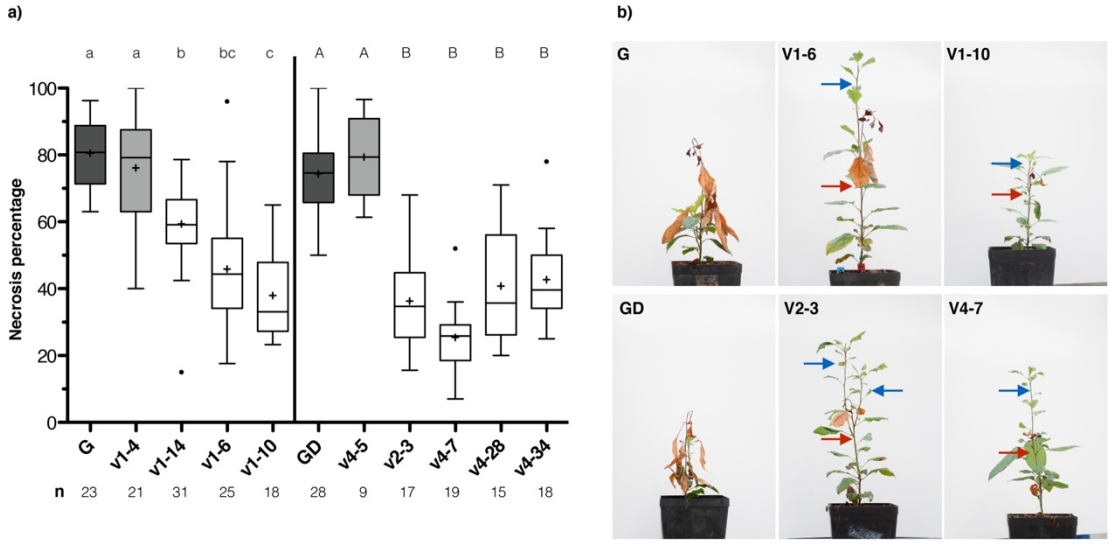


Figure 4. Fire blight severity in *MdDIPM4*-edited plants of 'Gala' and 'Golden Delicious' cultivars. (a) Boxplot summarizing the percentage of necrosis (calculated as length of the necrosis/total length of the shoot × 100) of candidate *MdDIPM4* CRISPR/Cas9-edited plants inoculated by the method of scissor with *Erwinia amylovora* strain Ea273. The number of inoculated biological replicates for each line is indicated (n). Boxes comprise values between 25% and 75% of the group. Horizontal central lines represent medians. Mean is shown as +. Whiskers (Tukey) determine values within ± 1.5 interquartile ranges from the median. Circles indicate outliers. Lettering indicate statistically significant differences between plant lines (for 'Gala' lower case, for 'Golden Delicious' upper case) according to Kruskal-Wallis test followed by multiple comparison of mean rank (α=0.05). (b) Pictures, taken one month after inoculation, showing the fire blight-induced necrotic phenotype in *wild-type* and some transgenic lines. Red and blue arrows indicate the interruption of necrosis and new regenerated shoots, respectively. Gala (G); Golden Delicious (GD).

Screening of exogenous DNA elimination in heat shock-treated edited apple plants

Apple lines V1-6, V1-10, V2-3, V4-7, V4-28, and V4-34, which showed a complete *MdDIPM4* knock-out and a reduced susceptibility to fire blight disease, were subjected to heat-shock treatments in order to activate the FLP/FRT recombination system for the removal of T-DNA flanked by the two FRT sites (10.6 kb) (Figure 1). To check the level of exogenous DNA elimination, the marker gene *nptII*, a crucial element of the T-DNA cassette, was quantified in shoots (10 for each line) regenerated from central nodes of heat-induced plantlets (Figure 5). On average, for each edited line, 1-2 shoots showed a high

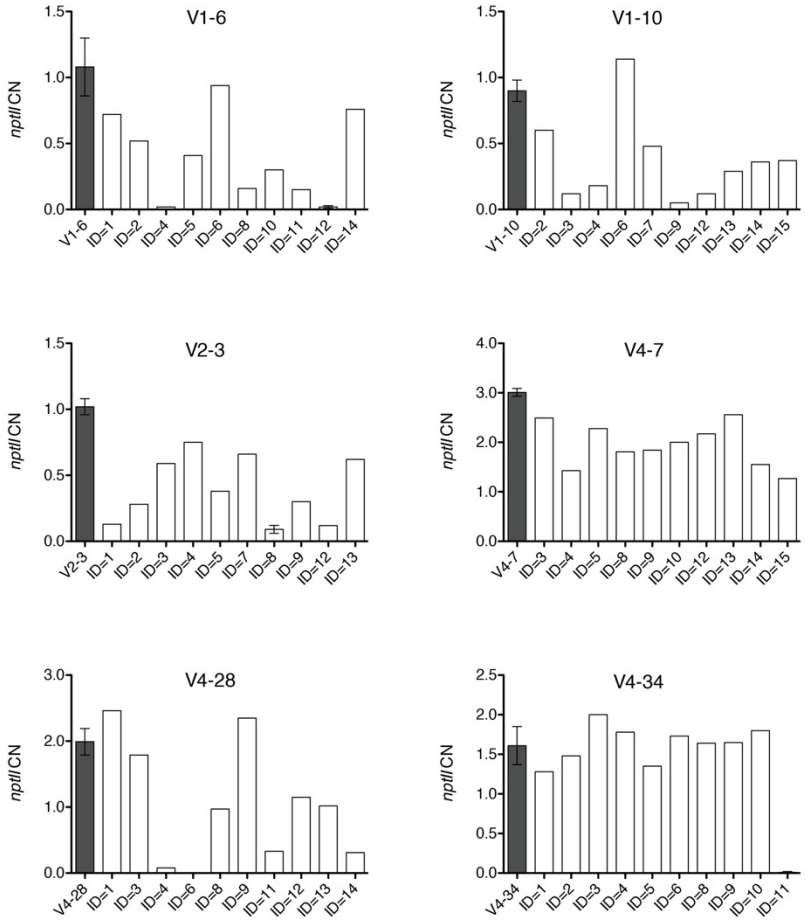


Figure 5. Summary of the *nptII* removal in heat shock-treated *MdDIPM4*-edited apple plants with reduced susceptibility to fire blight. *In vitro* biological replicates (n= from 6 to 10) of six candidate lines were heat-treated and the 2 central nodes of each plant were subsequently propagated. After 1 month, 10 new shoots regenerated from induced nodes were analyzed by Taqman real-time PCR to quantify *nptII* copy number (CN). *NptII* CN of untreated plants (dark grey bars) and plants V1-6.12, V2-3.8 and V4-34.11 is the mean ± SD of three biological replicates while the other plants were analyzed in a single biological replicate. Primers sequences are listed in Table S1.

percentage of T-DNA excision (91-100%) except for lines V4-7. Among 60 plants, 7 showed more than 90% of T-DNA removal, 21 exhibited between 50-90%, 21 between 10-50%, while in 11 plants a removal of less than 10% was observed. Some plants showing *nptII* CN equal or near to zero (V1-6.12, V2-3.8, V4-34.11) were propagated for 2 months and subsequently re-tested. The confirmation of the CN values demonstrated that in those plants the removal of the T-DNA was stable (Figure 5).

Validation of the T-DNA excision in edited apple line V4-28

The apple line V4-28 (CN=1.99 ± 0.2 - Figure 5) and related heat-induced clones V4-28.4 and V4-28.6, which showed a T-DNA elimination respectively of 96% (CN=0.08) and 100% (CN=0.00), were selected to further demonstrate the T-DNA excision by genomic sequencing. At first, the unheated clone V4-28 was used to identify the predicted two T-DNA insertion sites (Figure 6a) by a procedure combining an enrichment PCR with high-throughput sequencing as detailed in the experimental procedures section. The putative genomic DNA sequences derived from sequencing were blasted against the apple genome GDDH13 v1.1 (Daccord et al., 2017) and two sites with perfect sequence identities were found on chromosomes 13 (first T-DNA insertion site, S1) and 6 (second T-DNA insertion site, S2) (Figure 6a). No gene was annotated at these two genomic sites. In addition, in S1 the T-DNA left border (22 bps) and 57 bps of vector backbone were absent and similarly a 128 bps sequence of the T-DNA left-border region has been lost in S2 (Figure 6a). This truncation was probably due to the T-DNA translocation mechanism during the *Agrobacterium*-mediated plant transformation. The two identified T-DNA insertion sites were confirmed by PCR with a specific forward primer annealing on the upstream genomic region (C13_F or C06_F) and with a reverse primer annealing on the promoter 35S (35S-P_R) (Figure 6b). Predictably, the amplification occurred only for the unheated plant V4-28 and not for plants V4-28.4 and V4-28.6 which lost the 35S promoter after the heat-shock induction (Figure 6c). The removal of exogenous DNA and resulting residual T-DNA sequence were checked in apple clones V4-28.4 and V4-28.6 at the integration site 1 by means of a PCR amplifying the Chr13 region containing the T-DNA insertion site (Figure 6d). As expected, for the plant V4-28, characterized by the whole T-DNA cassette, no amplification was obtained (the amplification fragment would have been >10 kb, a non-amplifiable size with the conditions used in PCR). On the contrary, in plants V4-28.4 and V4-28.6 the amplification occurred (Figure 6e). In all plants tested, a band (206 bps - Table S3) was visible due to the amplification of the corresponding DNA fragment on the *wild-type* copy of homologous

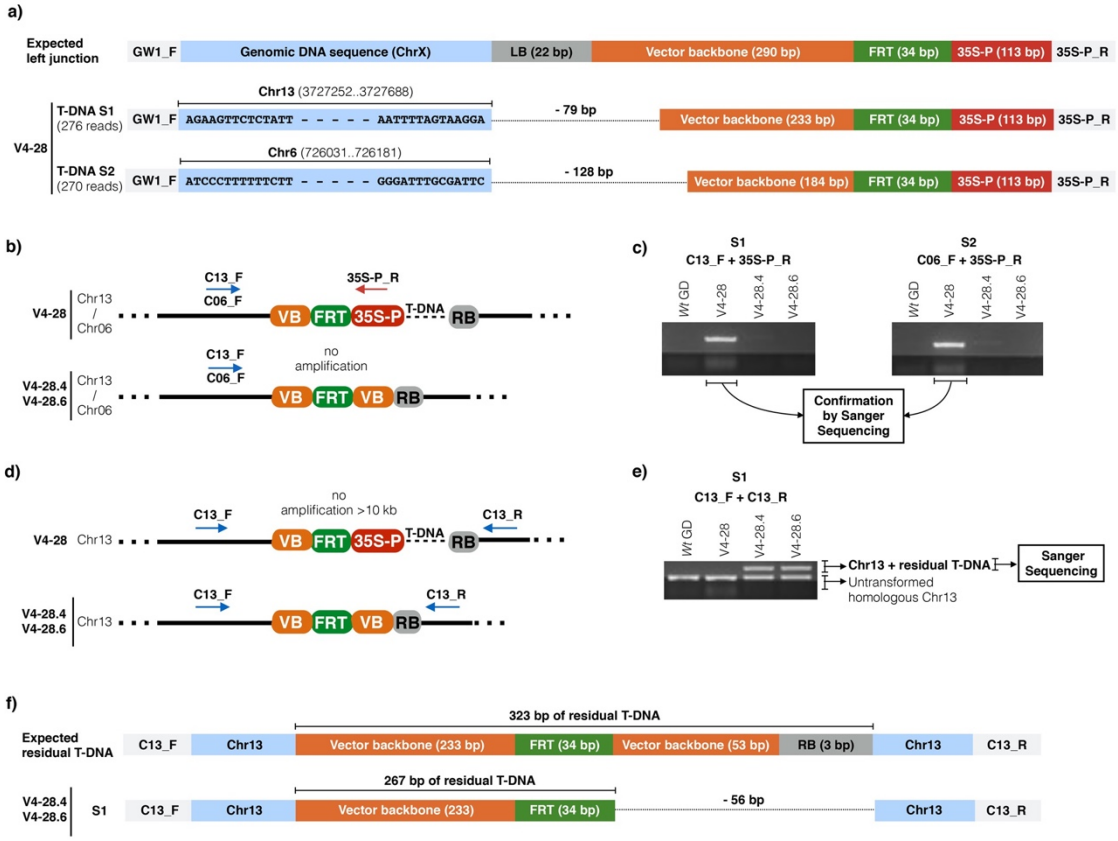


Figure 6. Identification of the T-DNA insertion site and validation of the T-DNA removal in the CRISPR/Cas9-edited apple line V4-28. a) Visualization of the two T-DNA insertion sites (S1-2) identified by high-throughput sequencing on chromosomes 13 and 6, respectively. In both cases, compared to the expected sequence, the left end of T-DNA was digested of 79 bps and 128 bps, respectively. b) Schematic representation of the amplification expected with the combinations of primers C13_F + 35S-P_R and C06_F + 35S-P_R, in not-treated V4-28 plant and in heat-shock induced shoots (V4-28.4 and V4-28.6) c) Results of the PCR described in 6B d) Schematic representation of the amplification expected with primers C13, during the validation of the T-DNA removal in S1. e) Results of PCR described in 6D. f) Visualization of the T-DNA removal and corresponding residual T-DNA in S1. GenomeWalker (GW); Left Border (LB); Flippase Recognition Target site (FRT); *Cauliflower Mosaic Virus 35S* Promoter (35S-P); Site (S); Chromosome (Chr). Vector Backbone (VB); Right Border (RB).

chromosome 13. DNA bands corresponding to the fragment of interest (Chr13 + residual T-DNA) were gel purified and Sanger sequenced (Figure 6e). The previously identified digestion pattern at the left end of the T-DNA cassette (79 bps) was validated and, similarly, a 56 bps sequence truncation was identified at the right end (Figure 6f).

Detection of the CRISPR/Cas9-editing activity in off-target genomic sites

In addition to the heat-shock treatments, apple lines V1-6, V1-10, V2-3, V4-7, V4-28, and V4-34 were further investigated to screen the editing activity of the CRISPR/Cas9 machinery on potential off-target (OT) genomic sites (Figure 7). The prediction of the OT regions revealed that our guide RNA was highly specific as no target was found with 1, 2 and 3 mismatches. However, 7 OT sites showing 4 mismatches with the guide RNA were predicted. Among them, 5 OTs (Figure 7) characterized by different CFD (Cutting Frequency Determination) scores were selected and screened by high-throughput sequencing. The obtained raw reads (ca. 6000/OT/plant) were processed and visualized to detect the OT editing. No mutation was identified in any of the 30 (6 lines x 5 putative OT sites) tested samples (Figure 7).

DISCUSSION

Most of today's elite cultivars such as 'Braeburn', 'Cripps Pink', 'Gala', 'Golden Delicious', 'Fuji' and 'Jonagold' are susceptible to fire blight (Norelli et al., 2003). To manage this disease, the use of resistant cultivars commercially available (e.g. 'Rewena' and 'Enterprise') (Kellerhals et al., 2014; Richter and Fisher, 1999) is currently not widespread, as the quality of their fruits does not fulfill consumers demand. As alternative, in the last decade genetic engineering has been used as a strategy to combat fire blight in apple by introducing resistance gene from crab apple genotypes into cultivars of interest. Broggini et al. (2014) transferred the *FB_MR5* gene, deriving from the wild apple accession *Malus* x *robusta* 5, into the susceptible genotype 'Gala' *via A. tumefaciens* and observed a reduction of disease symptoms (on average 80%) in transgenic lines after inoculation with two different *E. amylovora* strains. Using the same gene, Kost and colleagues (2015) produced the first cisgenic 'Gala' apple plant showing a reduction of fire blight susceptibility between 50%-80%. However, it is known that the pathogen can overcome this resistance by a non-synonymous single nucleotide mutation in the *AvrRpt2EA* effector gene responsible for an

Off-Target Gene	No. of Mismatches	Similarity guide RNA-off-target	CFD score	Editing detected
OT1: MD13G1085100 (Chr13)	4	gRNA: GCTG T A TT CCGCATGAAT C C OT1: GCTG G A AA CCGCATGAAT G CAGG	0.05	0 %
OT2: MD08G1095900 (Chr08)	4	gRNA: G CTGTA T TC C G C ATGAATCC OT2: A CTGTA G TC A G ATGAATCCCGG	0.20	0 %
OT3: MD01G1037700 (Chr01)	4	gRNA: GCTGT A TTC CG CATGAA T CC OT3: GCTGT T TTC TA CATGAA G CCCGG	0.22	0 %
OT4: MD11G1083100 (Chr11)	4	gRNA: G CTGT A TTCC G CATGAAT C C OT4: T CTGT T TTCC C CATGAAT A CAGG	0.13	0 %
OT5: MD16G1084300 (Chr16)	4	gRNA: GCTG T A TT CCGCATGAAT C C OT5: GCTG G A AA CCGCATGAAT G CAGG	0.05	0 %

Figure 7. Summary of the CRISPR/Cas9 off-targets analysis. The putative off-target genomic sites of the guide RNA were predicted with the CRISPOR Software (http://crispor.tefor.net - Haeussler et al., 2016), using the apple genome assembly GDDH13 v1.1 (Daccord et al., 2017) as reference parameter. Five predicted off-targets (OT1-5) were selected according to the annealing features with the guide RNA and relative CFD scores. Genomic DNA fragments containing selected OT sites were amplified in 6 apple mutant lines and screened by high-throughput sequencing. No editing was detected in any of the tested lines. The percentage of editing is representative of all the analyzed six lines. Sequences of the guide RNA, off-target region and PAM site are shown respectively in red, black and green. Primers sequences are listed in Table S4.

amino acid exchange (C156S) in the protein (Vogt et al., 2013; Broggini et al., 2014). In general, inherited resistance has been shown to be potentially overcome within years through pathogen mutation, hence requiring constant production of apple plants with new resistance traits for long-lasting disease management (Aldwinckle and Beer, 1997; Lespinasse and Aldwinckle, 2000; Mundt, 2014). The knock-out of susceptibility genes, which are required for compatible plant-pathogen interaction and for successful infection, has recently been considered a promising alternative strategy to breeding resistant plants (Pavan et al., 2010; Zaidi et al., 2018), potentially leading to more durable plant protection compared to that based on resistance genes. Campa et al. (2018) silenced the susceptibility *HIPM* gene and obtained around 50% of reduction of

fire blight symptoms. In our study we knocked out *MdDIPM4* in two commercial apple cultivars to confirm the hypothesis that this gene is associated with fire blight susceptibility. A highly significant reduction of symptoms was observed in edited plants compared to control (Figure 4), highlighting the importance of *MdDIPM4* in the onset of the disease. Our data confirmed the preliminary results of Borejsza-Wysocka and colleagues (2004) who used RNA interference to silence DIPM family with the difference that, while in all cases these authors cross-silenced two or more *DIPM* genes, in our work the CRISPR/Cas9 system targeted a specific region of *MdDIPM4* with no homology to other members of the family, ensuring a gene-specific knock-out (Figure S1). Overall, the knock-out of *MdDIPM4* resulted in a highly significant reduction of susceptibility, on average 50% in both cultivars. This is a valuable result in view of managing this disease in the future allowing to reduce chemical treatments for a sustainable agriculture. Even if the percentage of symptoms reduction is slightly lower than those obtained by Broggini et al. (2014) and Kost et al. (2015) by exploiting resistance genes (almost 80% and 50%-80% respectively), the approach we proposed may be more long-term effective. Moreover, considering the work of Campa et al. (2018) a promising strategy would be the simultaneous knock-out with CRISPR/Cas9 of the susceptibility genes *HIPM* and *DIPM4*, involved in different cellular processes.

Genome editing is a revolutionary technology in molecular biology, able to introduce mutations into a plant gene in a rapid and highly specific manner. Recently, the CRISPR/Cas9 system was successfully applied to gene functional studies and molecular breeding in both woody and non-woody plants (Andersson et al., 2017, Gil-Humanes et al., 2017, Jia and Wang, 2014, Liang et al., 2017, Malnoy et al., 2016, Nishitani et al., 2016, Pan et al., 2016, Ren et al., 2016, Ueta et al., 2017, Wang et al., 2018). Nevertheless, especially in apple, the potentiality of this editing system has to be further explored. Following the *A. tumefaciens*-mediated transformation, many plant lines were obtained, almost all with the T-DNA cassette integrated, indicating that the method used is efficient (Table 1). To estimate the editing performance, we sequenced the

MdDIPM4 target site by both Sanger and high-throughput sequencing (Figure S2, Figure 2). Our data demonstrated that HTS is a more effective, rapid and cost-efficient tool compared to Sanger sequencing for characterizing the profile of the target gene in edited plants. Nishitani et al. (2016) sequenced by Sanger method an average of more than 40 clones/putatively edited plant with great expense in terms of time and costs, while Charrier et al. (2019) only sequenced a mean of 4 clones/plant, a narrow spectrum to detect all possible mutation variants. On the contrary, our methodology, based on a HTS, allowed the simultaneous visualization of thousands of virtual clones for a single plant for less than $40. By using our HTS-based approach, new editing profiles were found for the lines V1-6 and V4-7 compared to previous data obtained with Sanger method (5 clones/plant), supporting the need of high-throughput sequencing for genotyping CRISPR/Cas9-edited plants.

Previous studies have shown that the editing efficiency and mutation types associated with CRISPR/Cas9 can vary widely, depending on the transformation method, plant species, target sequence, Cas9 promoter and guide RNA (Ma et al., 2016). In our work, the obtained editing efficiency and mutations (Figure 2) were not cultivar-dependent, as no important variations were observed between the two cultivars used. The editing efficiency we obtained (around 70% for both cultivars) is definitively higher compared to those found by Malnoy et al. (2016) (3.3%) and Nishitani et al. (2016) (31.8%). In the first case, it could be due to the low editing efficiency of protein-RNA complexes introduced into plant cells by polyethylene glycol (PEG) compared to classical methods (Metje-Sprink et al., 2019). In the second case, the use of a different promoter regulating the expression of the Cas9 protein or of a different guide RNA could be the cause. In addition, our data regarding the kinds of mutations were in agreement with the previous work of Malnoy and colleagues (2016), who tested the editing produced in *DIPM* genes by Cas9-guide RNA delivered as ribonucleoproteins in protoplasts of the apple cultivar 'Golden Delicious'. In fact, small deletions (-1, -2 and -3 nts) were mostly identified with only few cases of small insertions (+1, +2 and +3 nts). The types of mutations detected in our

work were also in agreement with those reported in the study of Nishitani and colleagues (2016), who targeted the *PDS* gene of the apple rootstock 'JM2'. On the contrary, in the work of Charrier et al. (2019), who edited *PDS* and *TFL1* genes of the apple cultivar 'Gala', a small insertion of 1 nt was the most abundant mutation in the two target sites of *PDS* gene. However, small deletions (-1, -2, and -4 nts) were mostly found in the two CRISPR/Cas9-targeted regions of *TFL1* gene. According to our results, it can be supposed that in apple, the cell repair system for double strand break (DSB) tends to preferentially produce deletions regardless of the CRISPR/Cas9 system variants (e.g. delivery methods, guide RNA, elements of the binary vectors). On the other hand, these variants are crucial to determine the efficiency of editing.

The CRISPR/Cas9 system has become a universal powerful tool for targeted gene manipulation. However, its application is associated with off-targeting, i.e. the generation of unwanted mutations in off-target genomic sites. In *Arabidopsis* (Zhang et al., 2018), barley (Lawrenson et al., 2015), *Brassica oleracea* (Lawrenson et al., 2015) rice (Tang et al., 2018), apple (Charrier et al., 2019) and pear (Charrier et al., 2019) the occasionally off-targeting has been reported to be a potential issue when CRISPR/Cas9 was applied, especially if the guide RNA shared significant similarity (complete homology or 1 mismatch) with OT sequences. On the contrary, in apple (Charrier et al., 2019) and grape (Wang et al., 2018) no editing activity was found by the analysis of OT regions showing 3 or 4 mismatches with the guide RNAs. In our work, the guide RNA was highly specific as only OT sequences with 4 or more mismatches were predicted. Five OTs were selected and screened by high-throughput sequencing in six candidate apple lines. As hypothesized, no mutations were detected in any of the sample tested (Figure 7) confirming that off-targeting tightly depends on the number of mismatches. These data suggest that a right selection of the target sequence, based on the genome information available for that particular plant species, is the first requirement to avoid the CRISPR/Cas9 off-target activity.

Among applications of the CRISPR/Cas9 system, the transformation mediated by *A. tumefaciens* is an effective system for achieving targeted mutations. However, this method leads to the production of transgenic edited plants. In Europe, genome edited organisms are to be considered GMO and must be subject to GMO legislation even if free of exogenous DNA (according to European Court of Justice sentence, July 2018). Many other countries such as USA, Argentina, Australia, and Brazil have established that genome edited cultivars that do not contain foreign DNA, will not be subject to additional regulatory oversight and risk assessment as required for GMO (Eriksson et al., 2019). The approach used by Charrier et al. (2019), based on *A. tumefaciens* transient transformation and on a high throughput screening of the T-DNA-free edited plants, may not be feasible for the editing of target sites that do not lead to a visual plant phenotype. These authors, in an experiment aimed at knocking out *PDS* gene in apple, had to regenerate 747 shoots to observe three albino events. Another strategy is based on a site-specific excision mechanism to remove a region of DNA. Several systems exist, such as Cre/loxP, R/Rs or FLP/FRT, based on recombinase enzymes that recognize two directly repeated Recombinase Recognition Sites (RRS) and excise the region within leaving in the plant genome a single 34 bps RRS. In apple, the application of these systems for the removal of selection marker genes was successfully reported (Herzog et al., 2012, Kost et al., 2015, Righetti et al., 2014). Other systems may be used like the insect PiggyBack transposon, which do not leave exogenous scars in the plant genome after excision. However, PiggyBack transposon has been poorly exploited in plant, proving a good efficiency only in rice (Nishizawa-Yokoi et al., 2014, Nishizawa-Yokoi et al., 2015).

In our study, we focused on the removal of the T-DNA cassette in those CRISPR/Cas9-edited lines with reduced fire blight susceptibility by using the FLP/FRT system, inducible with a heat-shock stimulus. The T-DNA cassette removal in six selected lines treated with heat-shock was preliminarily evaluated by estimating the copy number (CN) of *nptII*, the selection marker gene placed inside the T-DNA cassette (correlating its CN reduction with the removal of the

cassette itself) (Figure 5). Following the heat-shock inductions, 10 regenerated plants/line were screened and the removal was shown to occur in all analyzed lines with percentages that reached also 100% (Figure 5). These data, compared with those of previous studies (Herzog et al., 2012, Kost et al. 2015, Righetti et al., 2014), demonstrated that our method is efficient and rapid, as it does not require the treatment of thousands of leaf explants as well as callus regeneration which are laborious and time-consuming. To further validate the T-DNA excision and characterize the corresponding residual sequence after the FLP/FRT-mediated recombination event, the apple line V4-28 was investigated by sequencing for the identification of the predicted two T-DNA insertion sites (Figure 6). Insertions were found on Chr13 and Chr6, respectively. Thus, the insertion on Chr13 was selected and sequenced in *nptII*-free clones V4-28.4 and V4-28.6. Compared to the expected sequence, 135 bps of T-DNA ends were trimmed away (79 bps on left side and 56 bps on right side) and a residual exogenous DNA sequence of 267 bps was confirmed. In cisgenic apples, similar trimming patterns were already reported (Kost et al., 2015, Würdig et al., 2015). These results show that occasional border region truncations occur when the T-DNA is translocated into the plant genome and their effect must be considered during the construction of transformation vectors. The system we propose does not totally eliminate the whole T-DNA sequence (in the case of line V4-28 it leaves a scar of 267 bps in Chr 13 instead of the 402 bps predicted) and this aspect is still going to clash with strict legislations in many countries worldwide. However, successful deletion of a big region of T-DNA (more than 97% of its length) containing the CRISPR-Cas9 editing machinery may be promising for this important fruit crop where available tools for efficient genome engineering are still limited.

In conclusion, we have developed and applied a CRISPR/Cas9-FLP/FRT gene editing system to produce edited apple cultivars with reduced fire blight susceptibility and carrying a minimal trace of exogenous DNA. Overall, our data confirm that *MdDIPM4* is involved in apple susceptibility to fire blight and that the inactivation of this single gene of the DIPM family is sufficient to

significantly reduce disease symptoms. Moreover, T-DNA removal allows to eliminate the CRISPR/Cas9 from the genome in view of protecting plants from any effect due to the presence of an exogenous endonuclease and, simultaneously, to repeat gene transfer rounds on the same plants using the same selection marker gene. This methodology could represent a promising alternative strategy to the classical breeding for transgene introgression, especially for those plant species (such as apple) which require long maturation and crossing times. Plants produced in this work could be further investigated to better understand how *MdDIPM4* is involved in the onset of fire blight disease.

EXPERIMENTAL PROCEDURES

Construction and mechanism of action of the p9-C-HS-D4 binary vector

The 17 kb p9-C-HS-D4 binary vector (abbreviation of p9-CRISPR/cas9-Heat Shock-*MdDIPM4*) (Figure 1), was designed by us and assembled by the 'DNA Cloning Service e.K.' (Hamburg, Germany). The T-DNA cassette, flanked by the left and right borders (grey boxes - Figure 1), incorporated three distinct molecular systems respectively for antibiotic resistance, gene editing and T-DNA excision. The antibiotic resistance system (red boxes - Figure 1) was characterized by the *Neomycin phosphotransferase II* gene (*nptII*), driven by the *Cauliflower Mosaic Virus 35S* promoter, which conferred kanamycin resistance to apple transformants during the antibiotic-assisted selection following transformation (step 1 - Figure 1). The gene editing system (blue boxes - Figure 1) was based on the *wild-type CRISPR-associated protein 9* gene from *Streptococcus pyogenes*, controlled by the *Arabidopsis thaliana Ubiquitin-10* promoter, and the 20 bps guide RNA (5'-GCTGTATTCCGCATGAATCC-3' - Malnoy et al., 2016) for the target of exon 2 of *MdDIPM4* (yellow box, step 2 - Figure 1; Figure 2a, Figure S2), driven by the *Arabidopsis thaliana U6* promoter. Finally, the T-DNA excision system (green boxes - Figure 1) consisted of the FLP/FRT recombinase of *Saccharomyces cerevisiae* combined to the heat-shock

inducible promoter of the soybean gene *Hsp17.5-E* (Czarnecka et al., 1989). The system was designed with the *Flippase* gene under the control of the heat-shock inducible promoter and the two *Flippase Recognition Target* sites next to the left and right borders in order to remove the entire T-DNA cassette (leaving in the apple genome a predicted exogenous DNA sequence of 402 bps) (step 3 - Figure 1).

Apple transformation and identification of transgenic plants

Competent cells of *Agrobacterium tumefaciens* strain EHA105 (Hood et al., 1993) were transformed by electroporation with the p9-C-HS-D4 binary vector and used to transform plantlets of *Malus x domestica*, cultivars 'Gala' and 'Golden Delicious', as described by Joshi et al. (2011). Transformations were performed in duplicate (V1 and V3 for 'Gala'; V2 and V4 for 'Golden Delicious' - Table 1) using from 300 to 1000 leaf explants (Table 1). Regenerated plants (obtained after 6-7 months from co-culture with *Agrobacterium*) were screened to detect the presence of the T-DNA cassette (Table 1). For each plant, genomic DNA was extracted from 2 leaves using the Illustra™ Nucleon DNA Extraction Kit PHYTOPURE™ (GE Healthcare), quantified on the NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific), diluted to 50 ng/µL and used for PCRs using the thermocycle-3000 (Biometra), the GoTaq® Green Master Mix 1X (Promega, Fitchburg, MA) and primers Cas9, VirG and MdTOPO6 (0.4 µM) listed in Table S1.

Detection of the *MdDIPM4* editing by High-Throughput Sequencing

The *MdDIPM4* CRISPR/Cas9-targeted region of 57 transgenic apple lines (27 for 'Gala' and 30 for 'Golden Delicious') and *wild-type* plants was massively screened by high-throughput sequencing (Figure 2a). The *MdDIPM4* region containing the target site was amplified with primers MdDIPM4(2) (0.4 µM, Table S1) and overhang Illumina adapters to generate the Illumina library amplicons and sequenced on an Illumina MiSeq (PE300) platform (MiSeq Control Software 2.0.5 and Real-Time Analysis Software 1.16.18) as reported

by Quail et al. (2012). The CRISPResso pipeline (http://crispresso.rocks/ - Pinello et al., 2016) was used to process (with default parameters) the raw paired-end reads, contained into 'fastq' files, and to visualize the mutations profiles in *MdDIPM4* target sequence (Figure 2a).

Quantification of the *nptII* copy number by Taqman real-time PCR

The quantification of the *nptII* copy number was used to quantify T-DNA insertion (Table S2) and subsequently to assess the removal of exogenous DNA (Figure 5) in selected candidate apple lines following heat-shock inductions. The experimental procedure was conducted according to the Taqman real-time PCR method developed by Dalla Costa et al. (2019) and primers and probes for the endogenous gene *MdTOPO6* and for the marker gene *nptII* are listed in Table S1.

Plants resistance test to *Erwinia amylovora*

Resistance to *E. amylovora* was determined according to the scissor inoculation method described by Desnoues et al. (2018). From 3 to 15 biological replicates for each plant line were inoculated with *E. amylovora* strain Ea273 (10^9 CFU mL^{-1}) in each of the three independent experiments performed. Only actively growing plants that showed a shoot length of at least 13.0 cm were considered for the experiments. Data collecting was performed according to Campa et al. (2018). Statistical analysis was performed using the Dell™ Statistica™ Software version 13.1, considering 'Gala' and 'Golden Delicious' datasets separately (Figure 4). As the three experiments showed the same trend, measures of each plant line were merged and analyzed as a single experiment. Measures which differed more than ± 1.5 interquartile ranges from the mean of the relative group were considered outliers (Figure 4). Nonparametric Kruskal-Wallis test was used to compare groups as data did not show a normal distribution. Subsequently, all groups were compared simultaneously by multiple comparisons of mean rank. Statistical analysis was performed with α=0.05.

Heat-shock induction of the FLP/FRT recombination system

The induction of the FLP/FRT recombination system was based on Herzog et al. (2012) and Dalla Costa et al. (2016). From six to ten 2-week old plants for each line were incubated three times at 42°C for 6-h with a 48-h interval between consecutive incubations in a hybridization oven 'hybridizer HB-1000' (UVP, Upland, CA, USA). At the end of the heat-shock inductions, leaves, the vegetative apex (in most of the cases necrotic) and first 1-2 basal internodes of each plant were cut and discarded. The 2 central nodes of the stem were collected and placed horizontally onto a fresh propagation medium to promote the regeneration of new shoots. After 1 month, the first 2 leaves of 10 regenerated shoots for line were collected for DNA extraction and *nptII* quantification (Figure 5), according to the method previously described. Apple clones V1-6.12, V2-3.8 and V4-34.11 were micropropagated twice and the new shoots were re-tested for *nptII* copy number.

Identification of the T-DNA genomic insertion site

Genomic DNA (1 μg extracted from 1 unheated biological replicate of the plant line V4-28) was subjected to 3 low-intensity sonication cycles of 30-s with 90-s interval on a Bioruptor® NGS (Diagenode). Sonicated DNA was purified according to 1.8x AMPure XP Beads protocol (Agencourt) and subsequently checked on a D1000 ScreenTape (Agilent) to confirm the DNA fragmentation between 200 and 1000 bps. DNA fragments ends were repaired with the NEBNext® End Repair Module E6050S (New England Biolabs), following the manufacture's instructions, and the resulting DNA solution was again purified and checked, as mentioned above. The purified genomic DNA fragments were ligated to GenomeWalker adaptors, according to the procedure of the Universal GenomeWalker™ 2.0 kit (Takara Bio), and subsequently used in a selective PCR with primers GW1_F and 35S-P_R (0.4 μM) (Table S3) to amplify those fragments containing the junction between the genomic DNA and the left end of T-DNA (Figure 6a). The PCR product was purified with 0.8x AMPure XP Beads and checked on a D1000 ScreenTape to validate the removal of DNA

fragments smaller than 200 bps. Thus, the amplicon library product was sequenced by MiSeq Illumina platform, as previously mentioned. The obtained reads were visualized with the Unipro UGENE Software v1.31.1 (Okonechnikov et al., 2012) and checked to identify those containing the 35S-P_R primer sequence. From the selected reads, all the recognizable vector sequence was removed and the remaining flanking unknown sequence (putative genomic DNA) was blasted against the apple genome assembly GDDH13 v1.1 (Daccord et al., 2017) in order to identify putative T-DNA genomic insertion sites (Figure 6a). Following the identification, one genome-specific forward primer/insertion site (C13_F and C06_F - Table S3) was designed to anneal a genomic DNA sequence ca. 100 bps upstream the identified T-DNA insertion site. Thus, the genomic DNA of the unheated plant V4-28, of the heat-induced clones V4-28.4 and V4-28.6 and of *wild-type* control was amplified by PCR with primers C13_F + 35S-P_R or C06_F + 35S-P_R (0.4 µM) (Figure 6b) and PCR products were checked by 1% agarose gel electrophoresis. The obtained DNA bands (Figure 6c) were gel purified with the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel) and directly sequenced by the method of Sanger, as mentioned before. The obtained reads were visualized with UGENE Software to confirm the previous HTS results.

Validation of the T-DNA removal

The validation of the T-DNA removal was conducted in plants V4-28.4 and V4-28.6 which proved to be free from *nptII*. A genome-specific reverse primer/insertion site (C13_R - Table S3) was designed to anneal a genomic DNA sequence ca. 100 bps downstream the T-DNA insertion site. Thus, the genomic DNA of plants in object, relative V4-28 and *wild-type* controls was amplified by PCR using the couple of primers C13_F + C13_R (0.4 µM) (Figure 6d) and the corresponding PCR products were electrophoretically checked, gel purified and Sanger sequenced (Figure 6e), as described in the previous paragraph.

Off-target analysis

Genomic DNA was extracted, as previously reported, from one biological replicate of each plant line analyzed and the corresponding *wild-type* plant. DNA fragments containing the off-target sites (Figure 7) were amplified by PCR, with primers OT1-5 (0.4 μM) listed in Table S4, and the amplicon libraries were sequenced by MiSeq Illumina Platform according to the *MdDIPM4* on-target analysis. Raw paired-end reads were analyzed with CRISPResso using default parameters.

ACCESSION NUMBERS

Genes of *Malus x domestica* can be found in the GDR Database under accession numbers: MDP0000948404, MDP0000229861, MD01G1037700, MD08G1095900, MD11G1083100, MD13G1085100, MD16G1084300. Genes of *Arabidopsis thaliana* can be found in the TAIR Database under accession numbers: AT4G05320, AT3G13855. Additional genes can be found in the EMBL/ENA Database under accession numbers: AAB59340.1, M28070.1, X62885.1. The *SpCas9* nucleotide sequence is available at www.dna-cloning.com.

AUTHOR CONTRIBUTIONS

V.P. designed the experiments, conducted the experiments and wrote the paper. L.D.C. contributed to designing the binary vector, the experiments and revised the paper. S.P. contributed to designing the experiments, performing the procedure for validating the T-DNA removal and revised the paper. M.P. contributed to performing the sequencing analysis. M.M. designed the project, contributed to designing the experiments and revised the paper. All authors read and approved the paper.

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CONFLICT OF INTEREST DECLARATION

No conflict of financial interest declared.

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SUPPORTING INFORMATION

SUPPORTING EXPERIMENTAL METHODS

Experimental workflow, plant material and growth conditions

The experimental workflow was designed and conducted as described in Figure S3. For blocks 1, 2, 3, and 5 (respectively apple transformation, detection of CRISPR/Cas9-induced mutations, quantification of exogenous DNA, excision of exogenous DNA and off-target analysis - Figure S3), *in vitro* shoots of *M. x domestica* cultivars 'Gala' and 'Golden Delicious' were used. Baby jars containing apple plantlets in a shoot propagation medium (Pessina et al., 2016) were maintained in a growth chamber at 24 ± 1°C with a 16/8-h light/dark period (100 mmol/m2/s). For block 4 (*in vivo* plant resistance test to *E. amylovora* - Figure S3), soil-acclimatized apple plants of the same cultivars were used. To promote rooting, 2-week-old *in vitro* apple shoots were transferred from the shoot propagation medium to a Murashige and Skoog medium supplemented with indole-3-butyric acid (Pessina et al., 2016), maintained in a growth chamber in the dark for 2 weeks and subsequently under 16/8-h light/dark conditions, as described above, until complete root formation. Rooted plants were acclimatized in soil ('Terriccio Vegetal Radic' - TerComposti S.p.a., Brescia, Italy) and, by progressively reducing humidity for 3 weeks. Well-acclimatized plants were maintained at greenhouse conditions (24 ± 1°C, 16/8-h light/dark period, relative humidity of 70% ± 5%).

Detection of the *MdDIPM4* editing by Sanger Sequencing

The *MdDIPM4* genomic sequence containing the target site was screened in 10 transgenic apple lines and respective *wild-type* plants by Sanger Sequencing (Figure S2). DNA was extracted from leaves and amplified with primers MdDIPM4(1) (0.4 µM) (Table S1), as described in the previous paragraph. PCR products were purified with Nucleospin® Gel and PCR Clean-up Kit (Macherey-Nagel GmbH & Co.), quantified on the 2200 TapeStation System (Agilent Technologies) and cloned into *Escherichia coli* JM109 competent cells using a pGEM®-T Easy Vector (Promega), as in manufacturer's instructions. Five positive colonies were selected for each plant line and corresponding PCR

products were sequenced according to the method described by Sanger et al. (1977). Reactions were carried out on the ABI 3730xl DNA Analyzers (Applied Biosistems) following manufacturer's instructions. Produced 'SEQ' files containing raw single-end reads generated by sequencing were processed and visualized with Unipro UGENE Software v1.31.1 (Okonechnikov et al., 2012), using default parameters for the quality of reads, to detect NHEJ mutations (Figure S2).

SUPPORTING FIGURES

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MdDIPM1 (Chr 02)  GATGTAGCTCAAGGCCTTGCTTACATTCACCAATCGTCGT---------CCCTCATCCATG
MdDIPM2 (Chr 13)  GGAGCCGCTCGAGGCTTGGCCTGCATTCACGACTCGTGCAGGCCGCTTAAGCTCACCCACG
MdDIPM3 (Chr 15)  GACATAGCACAAGGCCTCTCTTACATCCACCAAGCATGGA---------GACTTGTCCATG
MdDIPM4 (Chr 12)  GGTGCGGCTCGTGGCCTCGCCAGGATTCATGCGGAATACAGCTCTGCTAAAGTACCGCATG
                  *     ** *  *** *  *    ** **       *               *    ** *
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Figure S1. Alignment of *MdDIPM* genes fragment corresponding to the target site of the CRISPR/Cas9 machinery. Related to the section "Generation of edited transgenic apple lines" (Results). In the *MdDIPM4* genomic sequence, the PAM and the CRISPR/Cas9-targeted region are shown in green and blue, respectively. Asterisks indicate common bases between all the four genes.

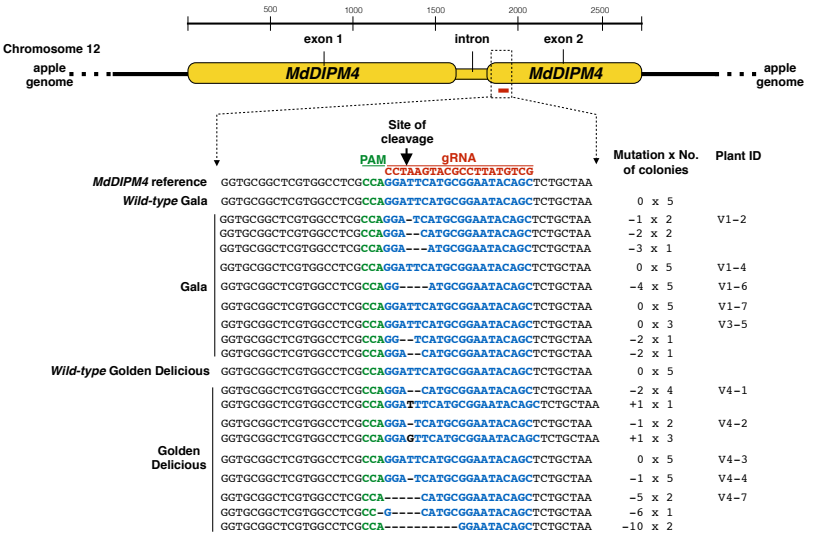

Figure S2. CRISPR/Cas9-editing in *MdDIPM4* gene identified by Sanger Sequencing. Related to the section "Detection of the *MdDIPM4* editing by Sanger Sequencing" (Experimental procedures in Supporting Information). A total of 5 colonies for each transgenic and *wild-type* plant tested were screened in the analysis. In addition to *wild-type* plants, the *MdDIPM4* genomic sequence of the apple genome assembly GDDH13 v1.1 (Daccord et al., 2017) was used as control. The *MdDIPM4* target sequence is colored in blue. Within the aligned sequences, deletions and insertions are represented respectively with traits and black bold letters. Mutations, related number of colonies and plant ID are reported on the right. Guide RNA (gRNA); Protospacer Adjacent Motif (PAM). Primers sequences are listed in Table S1.

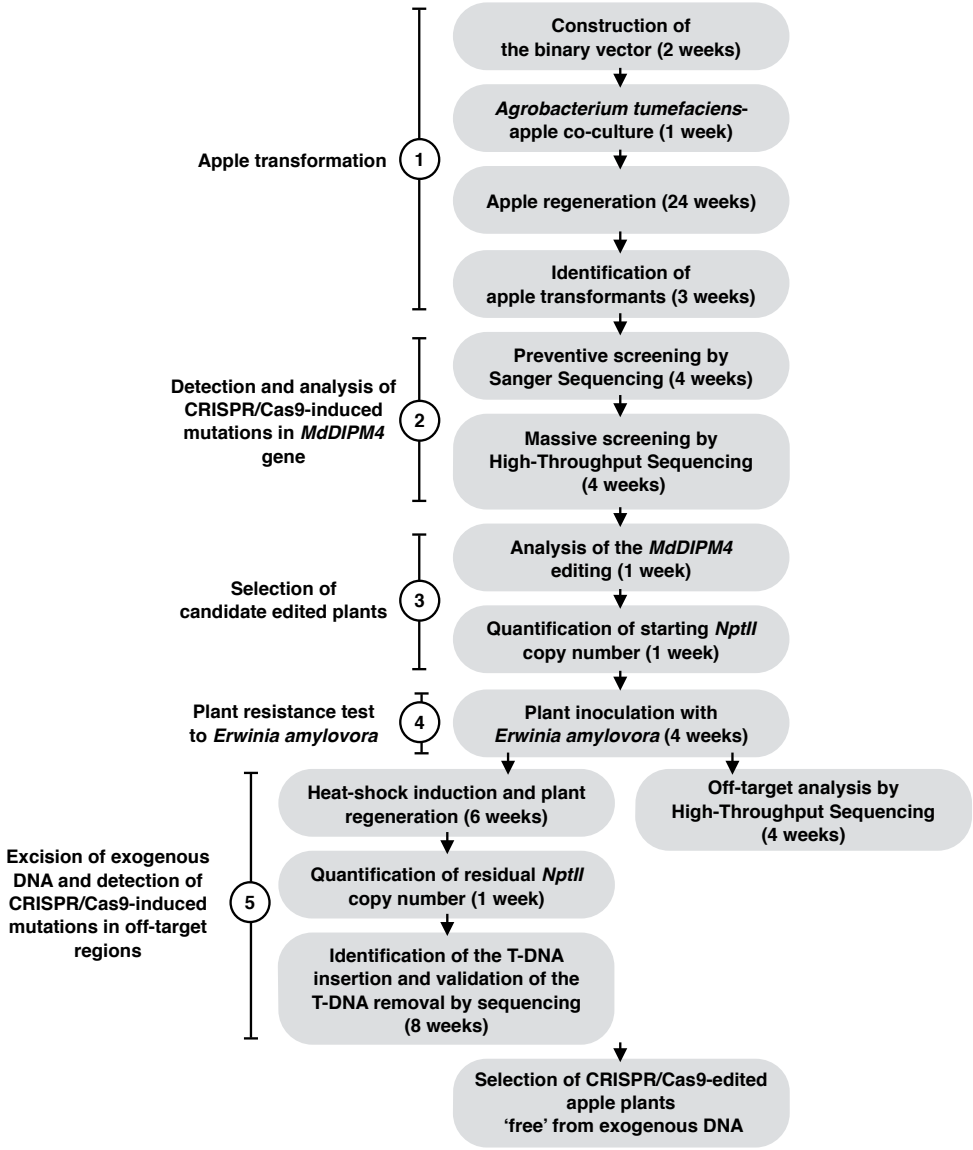

Figure S3. Experimental workflow for the *Agrobacterium tumefaciens*-mediated production of CRISPR/Cas9-edited apple plants with reduce susceptibility to fire blight and 'free' from exogenous DNA. Related to the section "Experimental workflow, plant material and growth conditions" (Experimental procedures in Supporting Information). The procedure requires almost 1.5 years to be completed. The five main steps are indicated with numbers 1 to 5 on the left.

SUPPORTING TABLES

Table S1. Sequences of primers and probes used for the PCR-based screening of apple transformants, the detection of CRISPR/Cas9 on-target mutations and the quantification of *NPTII* copy number by Taqman real-time PCRs. Related to Table 1, Figure 2, Figure S2, Figure 5 and Table S2.

Primer and probe name	Sequence (5′-3′)	Amplicon length (bps)	Annealing temperature (°C)
Cas9	F: AGATCCTCACTTTTAGAATCCC R: TGTCCTTGATAATCTTCAGGAG	486	56
VirG	F: GCCGGGGCGAGACCATAGG† R: CGCACGCGCAAGGCAACC†	605	56
MdTOPO6	F: TGTGGAAGGAGATCAAAGCGCA‡ R: CGCGTTGCTTCTTTGCTGCA‡	196	58
MdTOPO6_probe	FAM-5′-ACATGCCAACAGGAACAATCACA-3′-TAMRA‡		
MdDIPM4(1)	F: CAATGTGGTGAGGCTGAGTG R: GCTCGGTATCCTCCCAATCT	598	61
MdDIPM4(2)	F: GTGTTCAGTTTGGGGCACAT R: GGAGGTTCTAACGGGGAGAG	399	61
NPTII	F: CTTGCCGAATATCATGGTGGAA§ R:GGTAGCCAACGCTATGTCCTGA§	100	58
NPTII_probe	FAM-5′-TTCTGGATTCATCGACTGTGGC-3′-TAMRA§		

† (Herzog et al., 2012)
‡ (Dalla Costa et al., 2019)
§ (Dalla Costa et al., 2009)

Table S2. Quantification by Taqman real-time PCR of *nptII* copy number in CRISPR/Cas9-edited apple lines. Related to the section "Quantification of the *nptII* copy number by Taqman real-time PCR" (Experimental procedures). The *nptII* copy number, quantified in 1 biological replicate for each plant line analyzed, was used for the selection of candidate apple lines investigated in further analysis of plants resistance test to *E. amylovora* and heat-shock inductions. Copy number (CN). Primers sequences are listed in Table S1.

Gala		**Golden Delicious**	
Apple lines tested	***NptII* CN**	**Apple lines tested**	***NptII* CN**
V1-2	0.8	V2-1	3.4
V1-4	1.1	V2-3	1.1
V1-5	0.7	V4-1	4.9
V1-6	1.4	V4-2	1
V1-7	1.8	V4-3	0.4
V1-8	0.2	V4-4	1.8
V1-10	0.9	V4-5	0.6
V1-11	0.9	V4-7	3
V1-12	1	V4-12	1.5
V1-13	2.3	V4-13	0.8
V1-14	0.6	V4-14	2.8
V1-15	1.2	V4-15	0.8
V1-16	1.5	V4-17	1.5
V1-17	0.8	V4-19	2.1
V1-18	0.9	V4-20	0.3
V1-20	2.1	V4-21	1.1
V1-21	1.3	V4-22	2.7
V1-22	0.9	V4-24	0.8
V1-23	0.7	V4-25	1.6
V1-24	1.3	V4-26	1.5
V3-1	2	V4-27	0.8
V3-2	0.8	V4-28	2
V3-3	0.7	V4-29	0.7
V3-5	2.1	V4-30	1
V3-6	1	V4-31	2.4
V3-9	4.5	V4-32	0.7
V3-10	0.8	V4-33	1.1
		V4-34	1.7
		V4-35	0.9
		V4-36	0.9

Table S3. Sequences of primers used in the identification of the T-DNA genomic insertion sites and validation of the T-DNA removal. Related to Figure 6.

Primer name	Sequence (5'-3')	Amplicon length (bps)	Annealing temperature (°C)
GW1 35S-P	F: GTAATACGACTCACTATAGGGC† R: GCTGGGCAATGGAATCCGAG	400-700	59
C13	F: AGGTGGATTTTGTGGAAGGGA R: AATAGCCACCGGATGAAGGG	206	59
C06	F: CTGGGATTTGCGTTTCGAGT		

† (Universal GenomeWalker™ 2.0 kit, Takara Bio)

Table S4. Sequences of primers used for the off-target analysis. Related to Figure 7.

Primer name	Sequence (5'-3')	Amplicon length (bps)	Annealing temperature (°C)
OT1	F: CGAACACGAGGGCTTTTCAT R: AGATCGCACCACTTCTTCCA	189	58
OT2	F: CAGGTAGTCGCCGGGATTTT R: TTTCTATCTCCGGGGCCAGA	89	60
OT3	F: TGATGTCTACAATGTGTACTTTGCT R: GAGGCTATCCAGACCCAACG	107	60
OT4	F: TCTGTAACAGCAATTTGGGAAAA R: ATCCAGGTCGTACTTTGTACC	191	58
OT5	F: TGCTTGTGCATTTCCTCTCG R: CGAACTCGAGGGCTGTTCAT	146	60

ONGOING ANALYSIS

PART 1

Transcriptomic and metabolomic analyses of apple (*Malus x domestica* cv. 'Golden Delicious') clones in which the DspA/E-Interacting Protein of *Malus* 4 (MdDIPM4) is knocked-out

Valerio Pompili[1,2], Stefano Piazza[1], Salvatore Samuele Multari[1], He Jieqiang[3], Alessandro Cestaro[1], Stefan Martens[1] and Mickael Malnoy[1]

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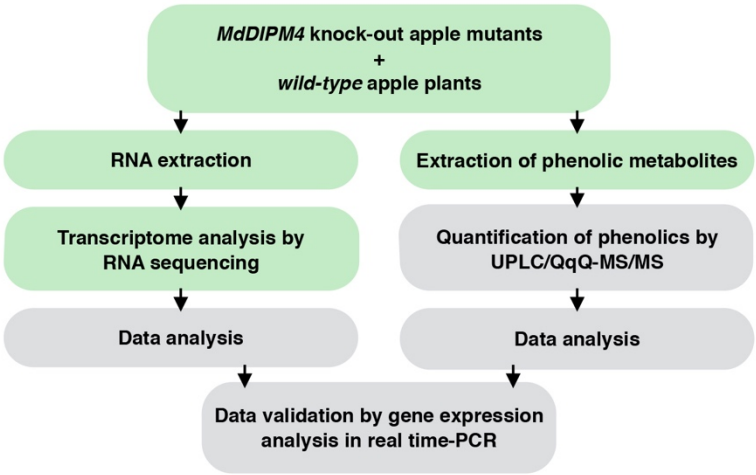
[3] College of Horticulture; Northwest A&F University; Shaanxi; 712100; China

Schematic overview



Experimental workflow. Green and grey boxes indicate concluded and ongoing analysis, respectively.

PART 2

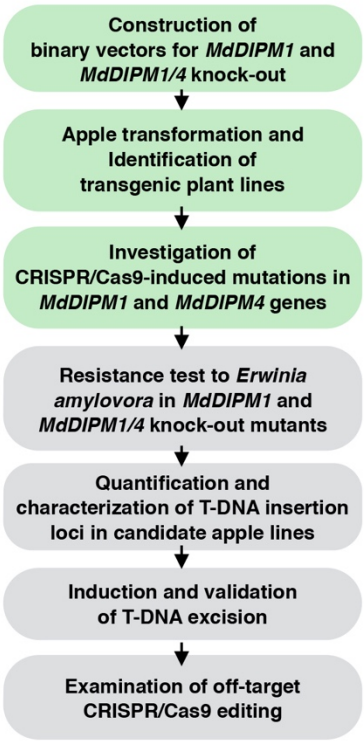
Investigation of *MdDIPM1* and *MdDIPM1/4* knock-out apple (*Malus x domestica* cv. 'Golden Delicious') mutants as additional sources of fire blight resistance

Valerio Pompili[1,2], Lorenza Dalla Costa[1], Stefano Piazza[1] and Mickael Malnoy[1]

[1] Research and Innovation Centre; Fondazione Edmund Mach; San Michele a/Adige; 38010; Italy

[2] Department of Agricultural, Food, Environmental and Animal Sciences; Università degli Studi di Udine; Udine, 33100; Italy

Schematic overview



Experimental workflow. Green and grey boxes indicate concluded and ongoing analysis, respectively.

PART 3

Evaluation of the molecular diversity associated with susceptibility to fire blight disease in apple collections

Valerio Pompili[1,2], Diego Micheletti[1], Jugpreet Singh[3], Mickael Malnoy[1] and Awais Khan[3]

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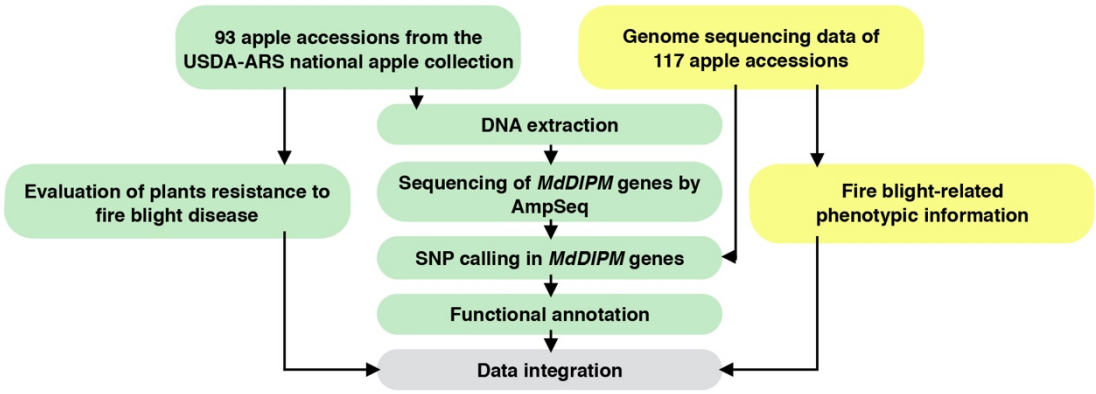
[3] Plant Pathology and Plant-Microbe Biology section; Cornell University; Geneva, NY; United States of America

Schematic overview



Experimental workflow. Green and yellow boxes indicate concluded analysis and data retrieved by external sources, respectively. The grey box indicates the ongoing analysis.

CHAPTER 3

Physiological and pathogen-induced transcriptional regulation of *MdmiR285N* microRNA in apple (*Malus x domestica*) and the heterologous plant system *Arabidopsis thaliana*

CHAPTER 3

Physiological and pathogen-induced transcriptional regulation of *MdmiR285N* microRNA in apple (*Malus x domestica*) and the heterologous plant system *Arabidopsis thaliana*

Valerio Pompili[1,2], Stefano Piazza[1], Mingai Li[1], Claudio Varotto[1] and Mickael Malnoy[1]

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Running title: *MdmiR285N* transcriptional regulation in *Malus* and *Arabidopsis*

ABSTRACT

Malus x domestica microRNA MdmiR285N is a potential key regulator of plant immunity, as it is predicted to target 35 RNA transcripts coding for different disease resistance proteins involved in plant defense to pathogens. In this study, the promoter region of *MdmiR285N* was isolated from the apple genome and analyzed *in silico* to detect potential regulatory regions controlling its transcription. A complex network of putative regulatory elements involved in plant growth and development, and in response to different hormones and stress conditions, was identified. Activity of the *β-Glucoronidase* (*GUS*) reporter driven by the promoter of *MdmiR285N* was examined in transgenic apple, demonstrating that *MdmiR285N* was induced during the vegetative growth phase. Similarly, in transgenic *Arabidopsis thaliana*, spatial and temporal patterns of *GUS* expression revealed that *MdmiR285N* is differentially regulated during seed germination, vegetative phase change, and reproductive development. Moreover, to elucidate the role of *MdmiR285N* in plant immunity, *MdmiR285N* expression in *wild-type* apple plants and *GUS* activity in transgenic apple and *Arabidopsis thaliana* plants were monitored in response to *Erwinia amylovora* and *Pseudomonas syringae* pv. *Tomato* DC3000. A significant decrease of MdmiR285N levels and *GUS* expression was observed during pathogen infections. Overall, these data suggest that MdmiR285N is a multifunctional microRNA involved in many plant biological processes, such as biotic stress response, plant growth, and reproductive development.

KEYWORDS

Malus x domestica, *Arabidopsis thaliana*, MdmiR285N, promoter, microRNA

INTRODUCTION

Plant microRNAs (miRNAs) are a large subclass of the category of endogenous non-coding RNAs with 20-22 nucleotides taking part in Post Transcriptional Gene Silencing (PTGS; Bartel, 2004; Jones-Rhoades et al., 2006). The biogenesis of plant miRNAs occurs in the cell nucleus and involves transcription

of *MIRNA* (*MIR*) genes, processing of primary miRNA (pri-miRNA) transcripts by DICER-LIKE (DCL) proteins into miRNA:miRNA* duplexes, and loading of mature miRNA strands into ARGONAUTE (AGO)-containing RNA-induced silencing complexes (RISC) (Bartel, 2004; Jones-Rhoades et al., 2006). After RISC loading, the mature miRNA guides the RISC machinery to complementary target sequences on messenger RNAs (mRNA) leading to miRNA-related RNA degradation or translational repression (Bartel, 2004; Jones-Rhoades et al., 2006).

By functioning in RNA silencing and post-transcriptional regulation of gene expression, plant miRNAs coordinate a wide range of biological processes in different cells, tissues and organs. Since their initial discovery, several functional analyses elucidated the importance of these bio-regulators in almost all aspects of plant growth and development (D'Ario et al., 2017; Liu et al., 2018), in the crosstalk between phytohormone signaling pathways (Curaba et al., 2014), and in response to environmental stimuli (Song et al., 2019), abiotic stresses (Koroban et al., 2016) and pathogen invasions (Brant and Budak, 2018). Besides their relevance in fundamental research, miRNAs are also very important from an applicative point of view to manipulate specific agricultural traits by modulation of plant gene expression (Djami-Tchatchou et al., 2017; Zheng and Qu, 2014; Zhou and Luo, 2013). Over the last decades, miRNA-mediated crop improvement was successfully achieved by the use of different molecular strategies, including constitutive, stress-induced, or tissue-specific expression of miRNAs (Eamens et al., 2008), RNA interference (Eamens and Waterhouse, 2001) and artificial miRNAs (Ossowski et al., 2008). However, most of these studies have focused on the analysis of miRNAs especially in non-woody plant species, such as *A. thaliana*, rice, wheat, and tomato, while limited investigations have been performed on miRNAs in agronomically and economically important woody plant species.

Apple (*M. x domestica*) is one of the most widely cultivated woody plant species in the world, with a total worldwide production of 83 million tonnes and a global value of 45 billion dollars in 2017 (FAOSTAT, 2017). As a result, the scientific

attention on this fruit crop has drastically grown in the last years, focusing not only on different aspects of apple horticulture, but also on its fundamental biology, such as the study of miRNAs. To date, approximately three hundred apple miRNAs are deposited in miRBase (www.mirbase.org, release 22.1: October 2018). Some studies were performed to identify apple miRNAs involved in the regulation of plant tissue development (Gleave et al., 2008; Varkonyi-Gasic et al., 2010; Xia et al., 2012), shoot growth (Song et al., 2017; Xing et al., 2016), flower induction (Fan et al., 2018; Xing et al., 2016), and fruit production (Qu et al., 2016; Yao et al., 2015). Others focused on the identification of miRNAs associated with apple response to different diseases, such as apple ring rot (Yu et al., 2014), *Alternaria* leaf spot (Ma et al., 2014; Zhang et al., 2018), *Glomerella* leaf spot (Zhang et al., 2019), *Valsa* canker (Feng et al., 2017), and fire blight (Kaja et al., 2015). One study reported a series of miRNAs involved in the response of the plant to drought stress (Niu et al., 2019). Although a considerable amount of data is now available, many gaps still exist for apple miRNAs research. Indeed, most of the above studies exploited microarray and next-generation sequencing to screen for putatively novel or stress-responsive miRNAs, but very few studies have been published on the functional characterization of the plethora of candidate miRNAs identified. More efforts are thus required to better characterize miRNAs and their functions in this important plant species.

In this study, as part of a long-term goal to identify promising miRNAs for potential genetic improvement of apple, we focused our attention on MdmiR285N, a novel apple miRNA of 21 nucleotides in length, which is predicted to target thirty-five RNA transcripts (Kaja et al., 2015). The mRNAs putatively regulated by this miRNA code for different disease resistance proteins belonging to the families of Toll Interleukin 1 Receptor-Nucleotide Binding Site-Leucine Rich Repeat (TIR-NBS-LRR), SUPPRESSOR of NPR-1 CONSTITUTIVE (SNC1), and Calcium-Dependent Protein Kinase (CDPK). These resistance proteins are well-known to play key roles in plant response to pathogen infections (Li et al., 2001; DeYoung and Innes, 2006; Nagamangala

Kanchiswamy et al., 2013). Within this framework, MdmiR285N was thus hypothesized to act as a crucial regulator of plant immunity. Here, a first characterization of the *MdmiR285N* promoter region was carried out *in silico* to identify putative cis-acting regulatory elements (cREs) and their cognate transcription factors. After isolation from the *M. x domestica* genome, a 2 Kbp promoter region of *MdmiR285N* was analyzed *in vivo* both in *M. domestica* and *A. thaliana* to examine putative roles of *MdmiR285N* in plant growth, development and especially pathogen resistance. In particular, tissue- and organ-specific expression patterns of *β-glucoronidase* (*GUS*) driven by the promoter of *MdmiR285N* were analyzed in transgenic apple and *A. thaliana* plants. With the aim of elucidating the function of *MdmiR285N* in plant immunity, *MdmiR285N* expression in *wild-type* apple plants and GUS activity in transgenic apple and *A. thaliana* plants were investigated in response to *Erwinia amylovora* (*E. amylovora*) and *Pseudomonas syringae* (*P. syringae*) pv. *Tomato* DC3000 infections.

RESULTS

Generation and selection of transgenic apple and *Arabidopsis thaliana* plants

In this study, *Prom_MdmiR285N::GFP-GUS* transgenic apple (*M. x domestica* cultivar 'Gala') and *A. thaliana* (ecotype Columbia-0) plants lines (PMd and PAt, respectively) were produced by *Agrobacterium tumefaciens* (*A. tumefaciens*)-mediated transformations using the pKGWFS7 binary vector shown in Figure 1.

For apple, the summary of transformation results is shown in Table S1. By infecting 770 leaf explants, five plants were regenerated approximately five months after transformation and culture on selective medium, thus screened by PCR for T-DNA integration. Four transgenic apple lines were obtained with the T-DNA integrated into the genome, as demonstrated by PCR amplification of *NptII* (the selectable marker of the T-DNA cassette) and lack of *VirG* amplification (therefore free from *A. tumefaciens* contamination), resulting in a transformation efficiency of 0.5%. Among the obtained lines, PMd1 and PMd2

were selected for further analyses. The other two lines showed a severe vitrified phenotype compared to *wild-type* plants, most likely due to the transformation event (data not shown) and were discarded from further analyses. The two selected lines were characterized for the number of T-DNA integration events by quantifying the copy number (CN) of the *NptII* marker gene. The line PMd1 showed a *NptII* CN mean of 2.01±0.12 which corresponded to two T-DNA integration events (Table S1). The line PMd2 showed a *NptII* CN mean of 1.00±0.45, which reflected the presence of a remarkable T-DNA chimeric profile. In fact, for this line the presence of T-DNA chimeric tissues was attributed to a *NptII* CN value lower than 1 in some biological replicates tested.

For *A. thaliana*, the summary of transformation results is shown in Table S2. After transformation, the T2 offspring of 40 T1 independent transgenic lines was screened under selective conditions to determine the ratio of resistant to susceptible seedlings and to calculate the number of segregating T-DNA loci. Eight lines showed a single T-DNA insertion event as characterized by a germination ratio $Kanamycin^{Resistant}$:$Kanamycin^{Susceptible}$ significantly not different from 3:1 (X^2<3.84, P>0.05, Table S2). Among those lines, T4 offspring of lines PAt6 and PAt28 was used for further experiments.

In silico **prediction of a putative transcription factor binding site based-gene regulatory and functional profile of** ***MdmiR285N*** **gene**

To identify the likely Transcription Factor (TF) Binding Sites (TFBSs) and corresponding TFs of *MdmiR285N* gene, its promoter region was analyzed by the Plant Promoter Analysis Navigator PlantPAN 2.0 (Chi-Nga et al., 2016). When using as reference the database of *M. x domestica* species, 24 unique TFBSs distributed fairly evenly along the *MdmiR285N* promoter sequence were identified (Figure 2a; Table S3.). Overall, the TFBSs fell into 17 different TFs families. Interestingly, among them C2H2, CSD, HD-ZIP, NAC and WRKY families, which are known to play key roles especially in plant development and stress responsiveness (Ariel et al., 2007, Chen et al., 2019, Ciftci-Yilmaz and

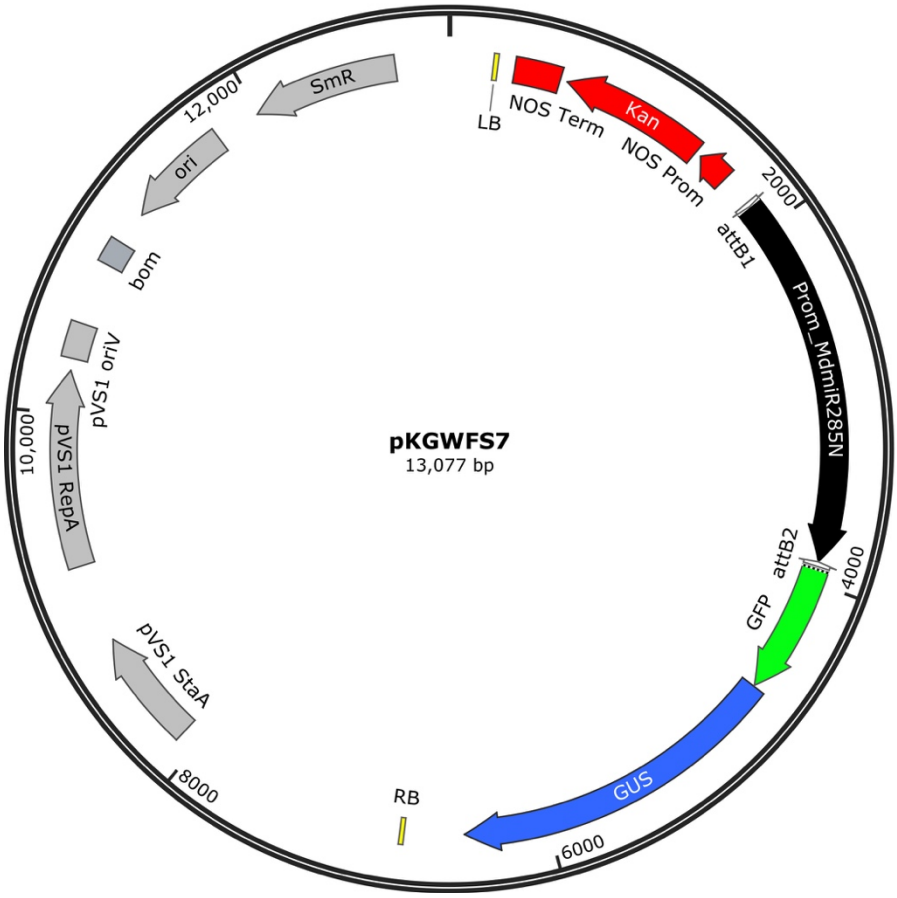

Figure 1. Map of the pKGWFS7 binary vector used for *Agrobacterium tumefaciens*-mediated transformations of apple and *Arabidopsis thaliana*. Vector backbone is shown by grey boxes. Left (LB) and right (RB) borders (yellow boxes) delimits the T-DNA. Within the transgenic cassette: the kanamycin (Kan) resistance system for plant selection after transformation is shown by red boxes; the *MdmiR285N* gene promoter (ca. 2 kb) regulating the GFP-GUS reporter system is shown by the black box; *Green Fluorescent Protein* (GFP) and *β-Glucuronidase* (GUS) reporter genes are shown respectively by green and blue boxes. AttB1-2 sites resulting from the GATEWAY™ Cloning Technology are indicated with white boxes.

Mittler, 2003, Sasaki and Imai, 2012, Yuan et al., 2019), were the most frequent, being characterized by multiple TFBS sequences (Figure 2a). Moreover, consistent results were found when the presence of putative TFBSs was investigated in the heterologous database of *A. thaliana* (Figure 2 and Table S3). However, being *A. thaliana* a model plant species for which the availability of information is significantly greater compared to other plants, the number of putative TFBSs identified (n=40) was higher than that reported in

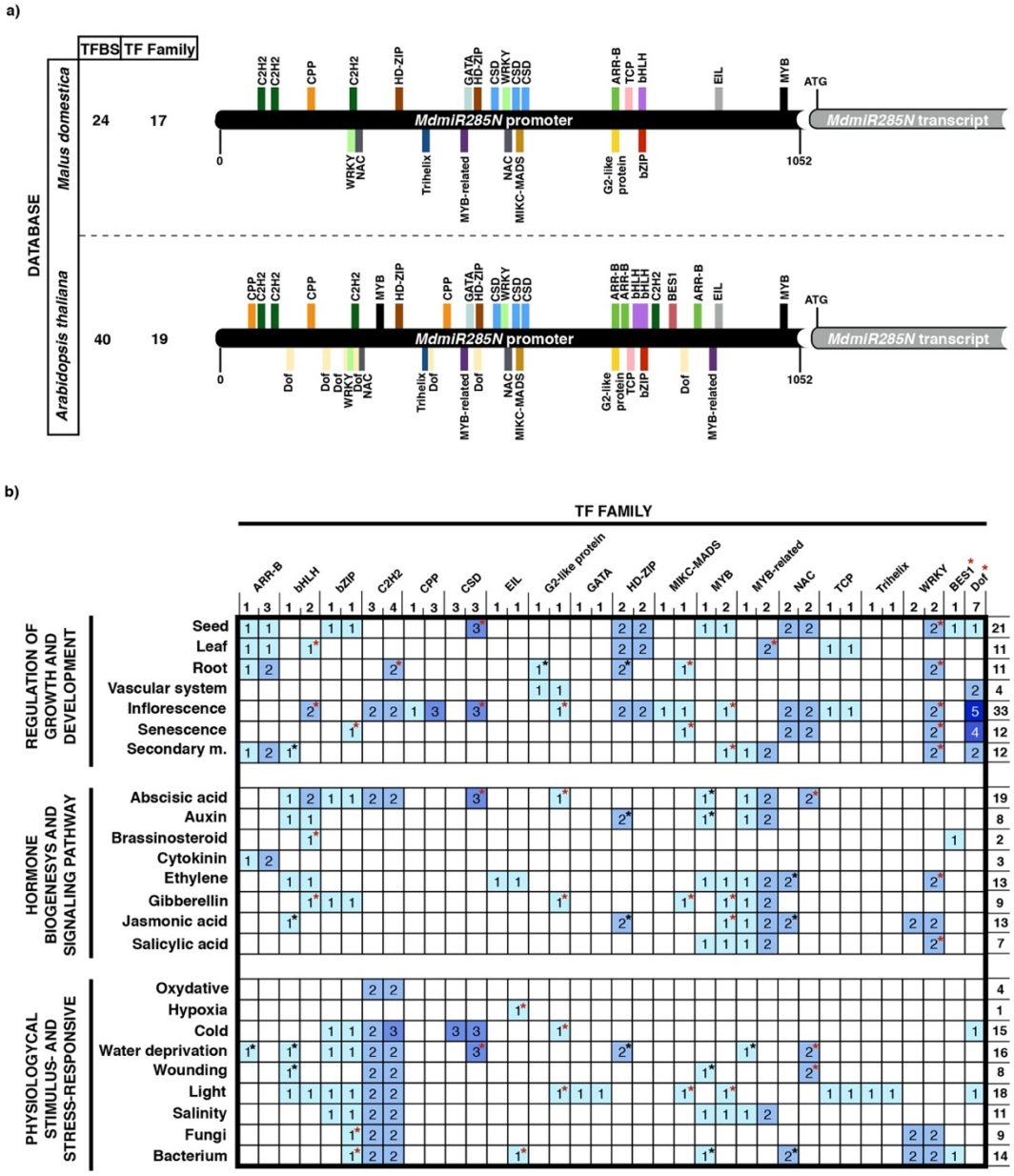

Figure 2. Putative TFBS-based regulatory and functional profile of *MdmiR285N* gene. a) Summary of TFBSs and corresponding TFs families identified in the promoter region of *MdmiR285N* by PlantPAN 2.0 (http://PlantPAN2.itps.ncku.edu.tw), using *M. x domestica* and *A. thaliana* databases. The spatial distribution of TFBSs along approximately 1 kb of genomic DNA sequence upstream of the transcription start site (ATG) is reported. Each TFBS is highlighted with a different color based on the corresponding TFs family. b) Heat-map showing the putative TFBS-based functional profile of *MdmiR285N* gene. According to the identified TFBSs and related TFs, TFs families and corresponding biological processes annotated (retrieved by comparing information of both PlantPAN 2.0 and PlantTFDB 5.0 (planttfdb.cbi.pku.edu.cn) databases) are reported respectively on the upper and left sides of the heat map. Below each TFs family, the total number of TFBS detected in *M. x domestica* (left column) and *A. thaliana* (right column) is reported. BES1 and Dof families were identified only in *A. thaliana*. For each TFs family, the number of TFBSs recognized by at least one TF associated with a certain biological process is reported within boxes. Black and red asterisks indicate data obtained

exclusively in *M. x domestica* or *A. thaliana*, respectively. For each biological process, the total number of associated TFBSs is reported on the right side of the heat map.

M. x domestica species (Figure 2a). Nevertheless, almost all TFBSs clustered into the same TFs families previously predicted for *M. x domestica*. Indeed, only two TFs families namely BES1 and Dof, which are involved in several plant physiological processes and stress responses (Li et al., 2018, Noguero et al., 2013), were identified only with *A. thaliana* matrixes but not apple (Figure 2a). Using the available gene ontology information concerning the biological processes associated with each TF detected (Table S3), a putative functional profile of *MdmiR285N* gene promoter was generated (Figure 2b). Results were consistent using either *M. x domestica* or *A. thaliana* matrixes and only few discrepancies, mostly due to the previously mentioned lack of information in apple, were identified. In both cases, *MdmiR285N* promoter was found to be potentially regulated during several biological processes linked to plant growth and development, especially seed formation, vegetative (leaf and root) and reproductive (inflorescence) development, organs senescence and secondary metabolism (Figure 2b). In addition, a putative functional profile was associated with phytohormones biogenesis and signaling pathways, particularly to those of abscisic acid, ethylene and jasmonic acid (Figure 2b). Finally, potential responses to multiple physiological stimulus and stress conditions, especially light intensity, water availability, temperature conditions and bacterial infections, were also predicted (Figure 2b). Although interesting, information obtained from this *in silico* analysis should be validated by experimental evidences.

Tissue- and organ-specific expression pattern of *MdmiR285N* gene in apple and *Arabidopsis thaliana* plants

According to the *in silico* analysis, to test the hypothesis that *MdmiR285N* is associated with plant growth and development (Figure 2b), its tissue- and

organ-specific expression pattern was investigated in transgenic apple and *A. thaliana* plants by histochemical GUS assay. Since apple was maintained *in vitro* by clonal propagation, the tissue- and organ-specific expression pattern of *MdmiR285N* was analyzed only during the vegetative growth phase (Figure 3). However, by using *A. thaliana* as heterologous plant system the activity of *MdmiR285N* promoter was investigated during the entire plant life cycle, including seed germination, juvenile-to-adult vegetative phase change and reproductive development (Figure 4, 5).

Histochemical expression pattern of *MdmiR285N* during vegetative development in apple

In apple, the histochemical GUS staining revealed that *MdmiR285N* promoter is expressed during the plant vegetative development (Figure 3). In the shoot system, GUS expression was detected in the shoot apical meristem (SAM) and in the stem (Figure 3a). Moreover, all leaves were stained (Figure 3a), although a stronger GUS signal was observed in young leaves (Figure 3a, n=9-14; Figure 3b) compared to adult leaves (Figure 3a, n=1-8; Figure 3c). Indeed, while in adult leaves GUS staining was detected only in the vascular system (Figure 3c), in young leaves also parenchyma cells proximal to vessels appeared stained (Figure 3b). In general, the vascular system was always stained with no intensity variation between different regions of the leaf (Figure 3d, 3e). In the root system, strong GUS expression was detected in the tip of primary and secondary roots and in the lateral root buds, in the root vascular system, and in the root elongation zone up to the root maturation region (Figure 3f-3h). Despite the transgenic lines used (PMd1 and PMd2) showed different T-DNA (or *NptII*) copy number values (Table S1), no significant discrepancy was found in the pattern of GUS activity (data not shown).

Overall, these results were consistent with the previous *in silico* analysis, according to which the *MdmiR285N* gene promoter appeared to be potentially regulated by a complex network of TFs involved in plant growth and development. Indeed, several of the identified TFBSs, namely those belonging

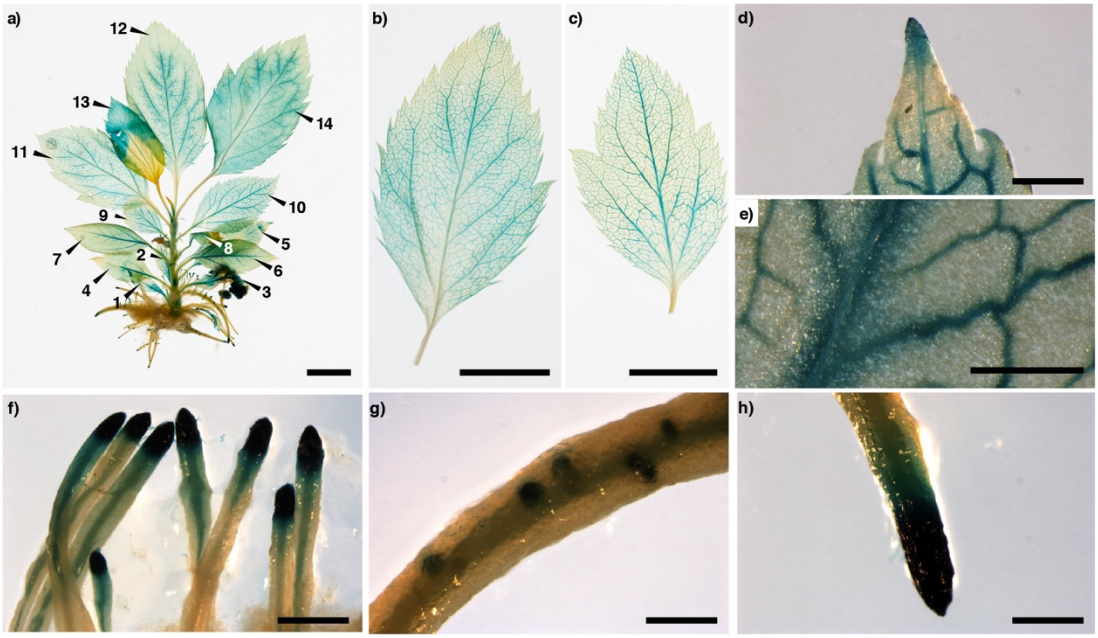

Figure 3. Promoter activity of *MdmiR285N* during vegetative growth in apple. Pictures show the histochemical GUS staining in different tissues and organs of 3-week-old apple plants carrying the construct *Prom_MdmiR285N::GFP-GUS*. a) Developed apple plant. Numbers indicate the order of leaf appearance. b, c) Young and adult leaf, respectively. d, e) Vascular system in the apical and medial region of the leaf, respectively. f) Secondary roots g) Root buds. h) Primary root tip. Results (a-h) were obtained by observations conducted after 3 independent experiments and are representative of both PMd1 and PMd2 transgenic lines used. In each experiment, 5 biological replicates/plant line were investigated. *Wild-type* plants were used as negative control and were never stained following the histochemical GUS protocol (data not shown). Black unit bars indicate 1 cm in "a-c", 1 mm in "d-h".

to the ARR-B, G2-like protein, HD-ZIP and TCP TFs families, were associated with TFs linked to the formation of leaf and root meristems, the morphogenesis of shoot organs, and the development of the vascular system through the regulation of xylem and phloem differentiation (Table S3).

Histochemical expression pattern of *MdmiR285N* during seed germination and vegetative development in *Arabidopsis thaliana*

In *A. thaliana*, the histochemical GUS assay showed that *MdmiR285N* expression was specifically and differentially regulated during different stages of seed germination and vegetative development. No *MdmiR285N* promoter-driven

GUS expression was observed in imbibed seeds (1 dap: day after plating, Figure 4a). In the later steps of seed germination, GUS staining was evident in the root apical meristem (RAM) of emerging seedlings (2 dap, Figure 4b; 3 dap, Figure 4c). Similarly, RAM appeared strongly stained in fully germinated and elongating seedlings (4 dap, Figure 4d; 5 dap, Figure 4e), although GUS signal was also detected in the root elongation and maturation zones and partially in the root vascular system. The same expression pattern was maintained in the primary and secondary roots of seedlings at the juvenile phase of vegetative growth (7 dap, Figure 4f). Besides roots, the vegetative system was never stained at any of the developmental stages mentioned above (1-7 dap).

A significant correlation was found between the observed results and the *in silico*-predicted gene regulatory and functional profile of *MdmiR285N*. Indeed, among the TFs putatively involved in the regulation of *MdmiR285N* promoter, multiple members of the ARR-B, C2H2, MIKC-MADS, and WRKY TFs families were associated with biological processes linked to the regulation of root growth and development (Table S3). On the contrary, no functionality of *MdmiR285N* was associated with seed activation and cotyledons development (Table S3).

In the later phases of vegetative growth (14 dap, Figure 4g; 21 dap, Figure 4h), in multiple organs of the seedling a gradual increase of GUS signal was observed. Its maximum intensity was reached at the late stage of vegetative development (Figure 4h). Roots (Figure 4g, 4h, 4k, 4l) were strongly stained according to the pattern previously described, however strong GUS staining was also visible in the SAM (Figure 4g, 4h), in the parenchyma cells of leaves (Figure 4g-4j), and in the leaf vascular system (Figure 4g-4j). As for apple, at each stage (14 and 21 dap) the intensity of GUS signal was reduced in adult leaves (Figure 4g, n=1, 2; Figure 4h, n=1-4; Figure 4j) compared to young leaves (Figure 4g, n=3-5; Figure 4h, n=5-8; Figure 4i).

Overall in *A. thaliana*, especially in the late phase of vegetative growth (21 dap, Figure 4h), the tissue- and organ-specific expression pattern of *MdmiR285N* was consistent with that reported in apple (Figure 3a). This result was not

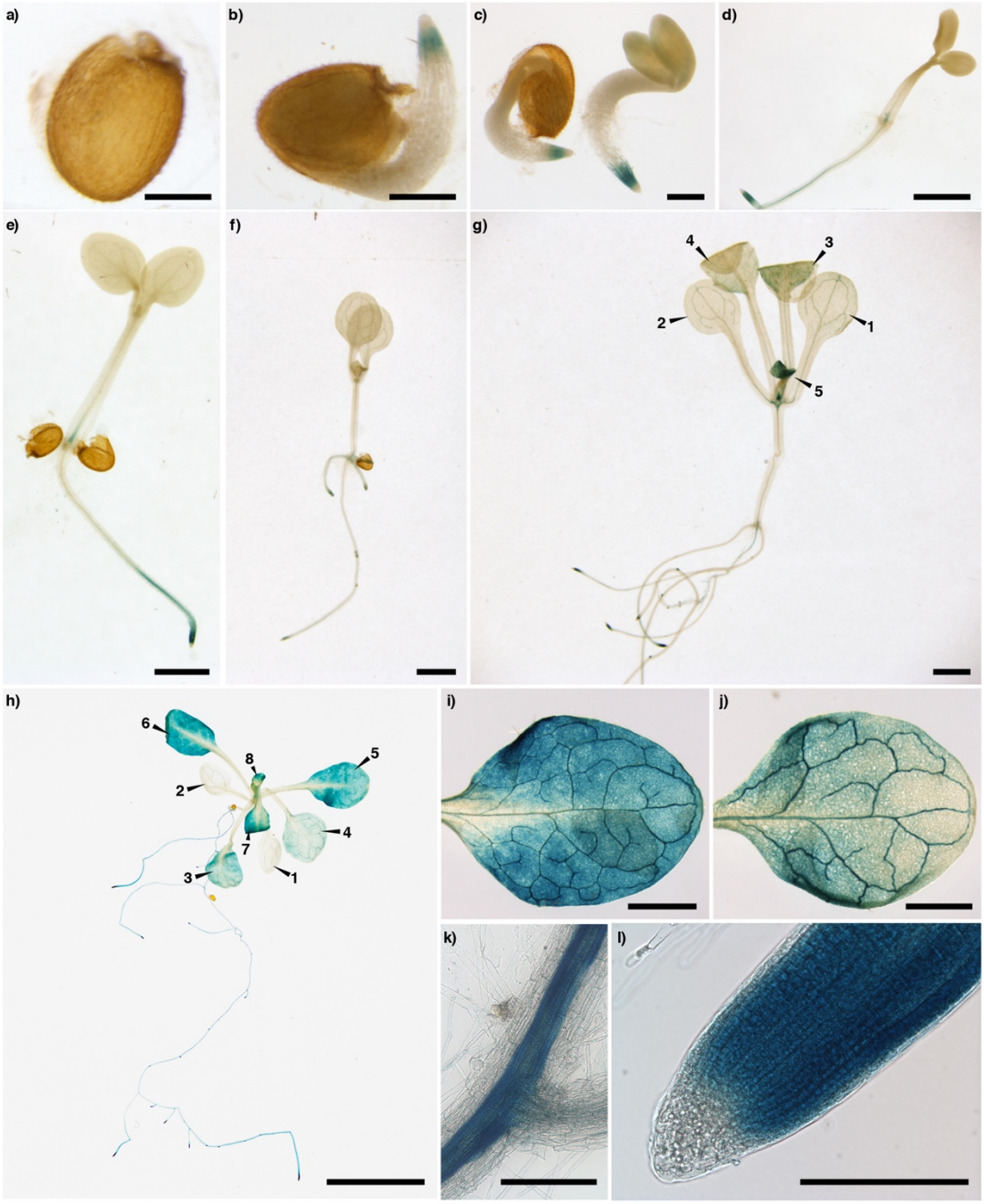

Figure 4. Promoter activity of *MdmiR285N* during seed germination and vegetative development in *Arabidopsis thaliana*. Pictures show the histochemical GUS staining of *A. thaliana* plants carrying the construct *Prom_MdmiR285N::GFP-GUS*. a) Imbibed seed (1 dap). b) Emerging radicle from seed coat (2 dap). c) Emerging hypocotyl and cotyledons from seed coat (3 dap). d) Germinated seedling (4 dap). e) Seedling in elongation (5 dap). f-h) Seedling at the juvenile, intermediate and late phase of vegetative development, respectively (7, 14, 21 dap). In "g" and "h", numbers indicate the order of leaf appearance. i-l) Different tissues of *A. thaliana* seedling at the late phase of vegetative development (h): young leaf (i), adult leaf (j), root vascular system (k), root tip (l). Results (a-l), representative of both transgenic lines used (PAt6, PAt28), were obtained by observations conducted after 3 independent experiments. In

each experiment, 10 biological replicates/plant line/time point were investigated. *Wild-type* plants used as negative control were never stained following the histochemical GUS protocol (data not shown). Black unit bars indicate 200 μm in "a-c, k, l", 1 mm in "d-g, i, j", 1 cm in "h".

unexpected, as a significant similarity in the regulatory and functional profile of *MdmiR285N* promoter using TFBSs specific for the two plant species was previously observed *in silico* (Figure 2). As for apple, also in *A. thaliana* multiple TFs belonging to the ARR-B, G2-like protein and TCP TFs families were involved in the morphogenesis of shoot and root organs, and the histogenesis of the vascular system (Table S3). However, in *A. thaliana* the same biological functions were also observed for members of the C2H2, Dof, MYB-related, and WRKY TFs families (Table S3). Interestingly, some TFs of the CSD and MIKC-MADS families were associated with the vegetative to reproductive phase transition of meristems (Table S3). This data supported the tremendous increase of *MdmiR285N* expression during the later phases of vegetative growth in *A. thaliana* (Figure 4g, 4h).

Histochemical expression pattern of *MdmiR285N* during reproductive development in *Arabidopsis thaliana*

In *A. thaliana*, *MdmiR285N* expression is regulated also during the reproductive development (Figure 5). In the upper part of the primary inflorescence, strong *MdmiR285N* promoter-related GUS expression was observed in the stalk and flower sets (Figure 5a, 5b). A close-up examination of close floral buds and fully open flowers revealed that GUS expression was particularly evident in the organ abscission zone, and the veins of flower petals and sepals (Figure 5c-5e). The *MdmiR285N* promoter was also active during the initial developmental stage of the silique. Indeed, GUS signal was observed in the elongating stigma (Figure 5f), particularly in the abscission zone (Figure 5f), the ovule (Figure 5g), the stigma apex (Figure 5h), the pollen grains on stigma apex (Figure 5i), and the guard cells of stigma cover (Figure 5j). However, as the silique became mature, the promoter activity of *MdmiR285N* was drastically reduced to an invisible

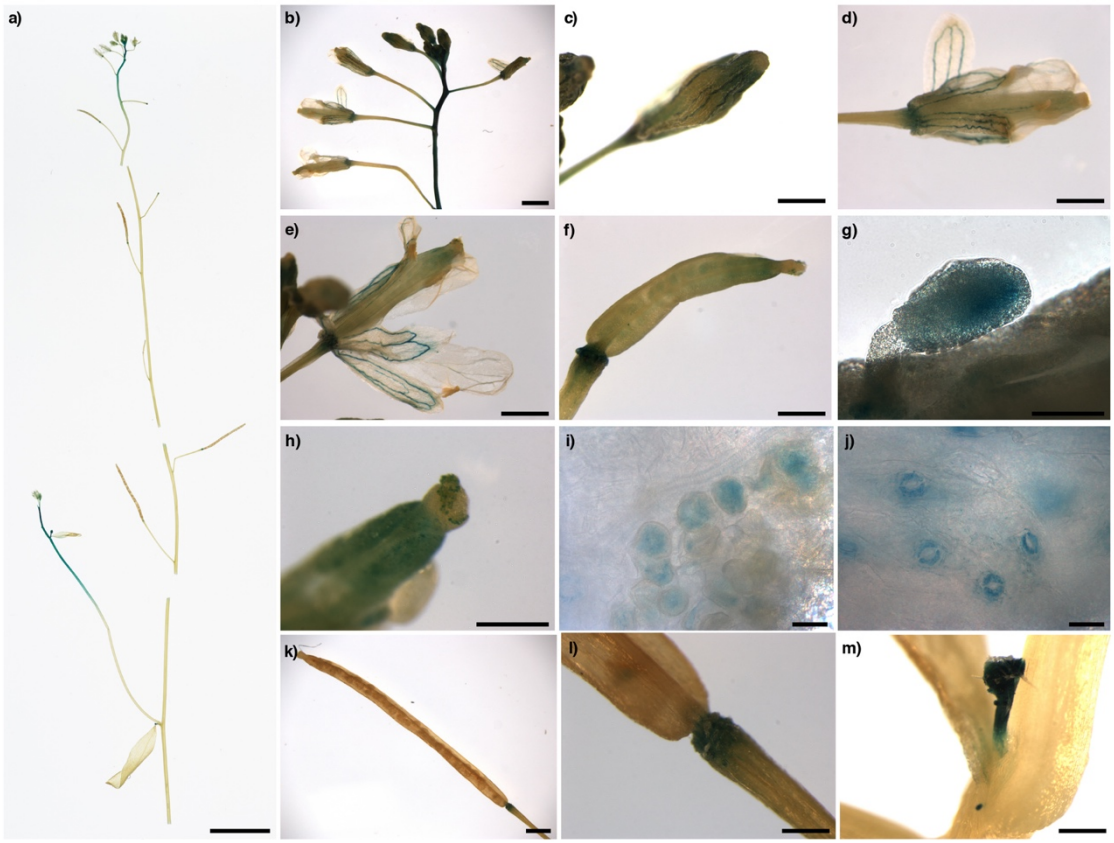

Figure 5. Promoter activity of *MdmiR285N* during reproductive development in *Arabidopsis thaliana*. Pictures show the histochemical GUS staining in reproductive organs of 5-week-old *A. thaliana* plants carrying the construct *Prom_MdmiR285N::GFP-GUS*. a) Primary inflorescence. b) Flower set. c) Closed floral bud. d, e) Mature flowers. f) Elongating stigma after pollination. g) Ovule. h) Stigma apex. i) Pollen grains on stigma apex. j) Guard cells of stigma. k) Mature silique. l) Abscission zone of mature silique. m) Axillary bud. Results (a-m), representative of both AtP6 and AtP28 transgenic lines, were obtained by observations conducted after 2 independent experiments. In each experiment, the primary inflorescence of 5 biological replicates/plant line was analyzed. *Wild-type* plants used as negative control were never stained following the histochemical GUS protocol (data not shown). Black unit bars indicate 1 cm in "a", 1 mm in "b-f, k", 200 μm in "h, l, m", 20 μm in "g, i, j".

level, remaining evident only in the abscission zone (Figure 5k, 5l). Finally, strong GUS signal was also observed in axillary buds (Figure 5m).

The obtained results were clearly supported by the previous *in silico* analysis. Indeed, in *A. thaliana*, except for ARR-B, EIL, GATA, MYB-related and Trihelix, all the identified TFs families were characterized by TFs associated with the morphogenesis of the inflorescence, the maturation of pollen, the formation of

plant ovule, and the development of seeds (Table S3). Many other TFs, by acting as regulators of cell aging, were also correlated to the regulation of leaf senescence and the floral organs abscission (Table S3).

Expression profile of *MdmiR285N* gene after host-pathogen infection in apple and *Arabidopsis thaliana* plants

Besides histological experiments, the investigation of putative changes in the expression profile of *MdmiR285N* in response to environmental stimuli, such as bacterial infections, may provide insights into the biological roles of this novel apple miRNA. As *MdmiR285N* is predicted to target many disease resistance RNA transcripts, and given that from our *in silico* analysis *MdmiR285N* promoter was shown to be potentially regulated by several TFs of BES1, bZIP, C2H2, EIL, MYB, NAC, and WRKY families associated with plant immunity and systemic acquired resistance (Table S3), in the present work the expression pattern of *MdmiR285N* gene was examined in apple and *A. thaliana* plants following inoculation with *E. amylovora* strain Ea273 and *Pst* DC3000, respectively.

In apple, when soil-acclimatized *wild-type* plants used as control were mock-inoculated by leaf wounding, no significant fluctuation of mature *MdmiR285N* transcripts was detected by real-time PCR 12, 24, 36 and 48 hours after the lesion (Figure 6a). Differently, if plants experienced the bacterium, the abundance of mature *MdmiR285N* transcripts decreased significantly and specifically 24, 36 and 48 hours after the application of the stress (Figure 6a). Consistent results were obtained when the stimulatory effect of *E. amylovora* on *MdmiR285N* expression was investigated in the transgenic apple lines PMd1 and PMd2 (Figure 6b). A decrease of GUS activity was confirmed 24 and 48 hours after imposition of the infection. In *A. thaliana*, similar results were obtained when the heterologous regulation of *MdmiR285N* promoter was qualitatively and quantitatively monitored in the transgenic lines PAt6 and PAt28 throughout *Pst* DC3000 infection (Figure 6c, 6d).

Overall, in both plant species examined, *MdmiR285N* is negatively regulated during the plant response to pathogen infection. These data suggest that

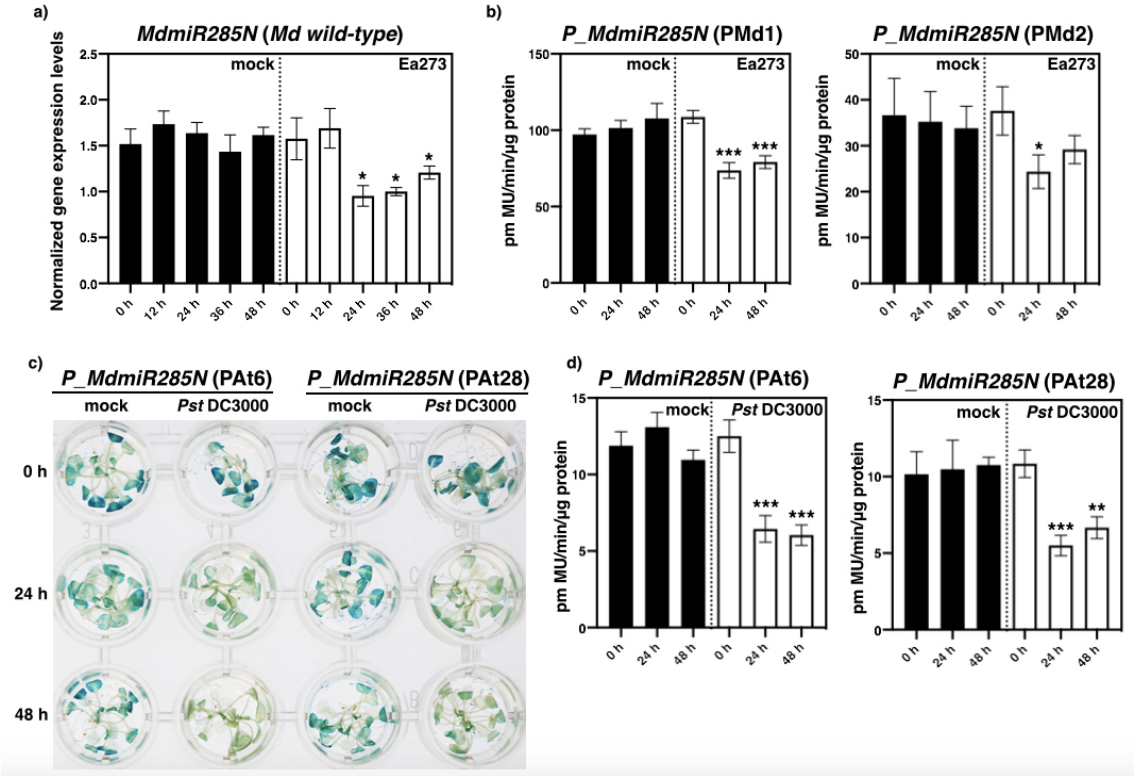

Figure 6. Expression pattern of *MdmiR285N* gene after host-pathogen infection in apple and *Arabidopsis thaliana* plants. a) Transcripts levels of mature *MdmiR285N* quantified by real-time PCR in *M. x domestica wild-type* (cv. 'Gala') at different time points (0 hours, 12 h, 24 h, 36 h, 48 h) after treatment (mock and *E. amylovora* strain Ea273). b) Fluorometric *MdmiR285N* Promoter-driven GUS activity in transgenic apple lines (PMd) at different time points (0 h, 24 h, 48 h) after treatment (mock and Ea273). c, d) Histochemical and fluorometric *MdmiR285N* Promoter-driven GUS activity in transgenic *A. thaliana* lines (PAt) at different time points (0 h, 24 h, 48 h) after treatment (mock and *Pst* DC3000). Experiments were performed *ex vitro* (a) or *in vitro* (b-d), in duplicate (a, b, d) or triplicate (c). For each experiment, 4 (a) and 3 (b-d) plant biological replicates/treatment/time point were used. In "a" and "b-d" 1 biological replicate was made by pooling 3 leaf strips and 3 plantlets, respectively. Apple and *A. thaliana wild-type* plants used as negative control did not show appreciable histochemical or fluorometric GUS activity (data not shown). In graphs "a, b, d", bars indicate the mean values ± SE. Considering mock- and pathogen-treatments separately, asterisks indicate statistically significant differences of datasets from the corresponding dataset at time zero (0 h), according to one-way ANOVA followed by post-hoc Dunnett's test (α=0.05).

MdmiR285N may participate to the post-transcriptional regulation of its target transcripts may be involved in the biological processes underlying compatible plant-pathogen interactions. Further gene functional analysis may better elucidate the role of this microRNA in plant immunity.

DISCUSSION

miRNAs have been identified as post-transcriptional regulators in many eukaryotic plants and coordinate a broad range of biological processes associated with plant growth, development, and response to environmental stimuli, such as abiotic and biotic stresses (Brant and Budak, 2018; Curaba et al., 2014; D'Ario et al., 2017; Koroban et al., 2016; Liu et al., 2018; Song et al., 2019). As a result, a significant progress has been made to identify and characterize plant miRNAs, with an increasing number of research reports especially in agronomically important plant species (Djami-Tchatchou et al., 2017). For apple, miRNAs research remains, however, still poorly investigated. Here, we studied the novel apple miRNA *MdmiR285N* which was predicted to target 35 RNA transcripts coding for resistance proteins TIR-NBS-LRR, SNC1 and CDPK (Kaja et al., 2015). Many of these proteins accumulate within the cell after pathogen attacks and are pivotal for the activation of defense responses, while their decrease attenuates the activation of downstream defense signaling (Burch-Smith et al., 2007; Cheng et al., 2009; Shen et al., 2007; Wirthmueller et al., 2007). The presence of a complex MdmiR285N-resistance gene regulatory module able to control the plant immune system was thus hypothesized. Here, as initial characterization of *MdmiR285N* in *M. x domestica* and in the heterologous plant species *A. thaliana*, we investigated its promoter region *in silico* (Figure 2, Table S3), by histological assays (Figure 3-5) and functional gene expression analysis in response to the bacterial pathogens *E. amylovora* and *Pst* DC3000 (Figure 6).

Information regarding the presence of putative Transcription Factor (TF) Binding Sites (TFBSs) in a given gene promoter and their corresponding TFs is valuable for understanding potential gene regulation and biological functions. Over the past few years, different computational approaches have been developed to identify and feature DNA sequences regulating the transcription of genes (Koryachko et al., 2015; Meng and Wang, 2015). In our work, the promoter region of *MdmiR285N* was scanned by the Plant Promoter Analysis Navigator PlantPAN 2.0 (Chi-Nga et al., 2016), using both *M. x domestica* and

A. thaliana TFBSs databases as reference. In both plant species examined, a considerable series of putative TFBSs and corresponding TFs was identified regulating the *MdmiR285N* gene promoter during different stages of plant growth and development, and in response to multiple phytohormones signaling pathways and environmental stresses (Figure 2, Table S3). Taking into account that *MdmiR285N* is an endogenous miRNA of apple, our results show that its putative TFBS-based gene regulatory profile is conserved in the heterologous system *A. thaliana*, thus suggesting also that the regulation of genes involved in defense responses may be comparable between the two plant species examined. Moreover, such a heterogeneous network of gene regulatory elements indicates that the post-transcriptional activity of MdmiR285N on its target resistance transcripts is differentially regulated during various phases of the plant life cycle and thus it is not only limited to the molecular mechanisms triggered by plant-pathogen interactions.

To validate by experimental evidences whether *MdmiR285N* expression was regulated during the plant development, GUS histochemical observations were conducted in *Prom_MdmiR285N::GFP-GUS* transgenic apple and *A. thaliana* plants and the obtained results correlated with the *in silico* data. At first, GUS analysis conducted in germinating *A. thaliana* seedlings (Figure 4b-4f) revealed that *MdmiR285N* is expressed only in roots, thus suggesting that *MdmiR285N* has function limited to root formation or nutrients up-take during the early phase of vegetative development. Differently, the expression of *MdmiR285N* drastically increases in multiple organs of *A. thaliana* during the juvenile-to-adult vegetative phase change (Figure 4g, 4h). To date, it is well-documented that miRNAs play an important role in regulating vegetative phase change in plants (Poethig, 2009; Wang et al., 2011). In *A. thaliana* (Figure 4g), two weeks after plant germination such a precise increase in the expression of *MdmiR285N* suggests that this miRNA may be key during the transition phase of meristems. Subsequent GUS analysis showed that *MdmiR285N* is expressed in almost all tissues of fully developed apple and *A. thaliana* plantlets (Figure 3; Figure 4h-4l), especially in newly and growing tissues of both root and shoot

systems, suggesting important roles of *MdmiR285N* in the molecular mechanisms underlying actively dividing tissues. Finally, strong expression of *MdmiR285N* was also observed in *A. thaliana* reproductive tissues and in floral organ abscission zones (Figure 5). The role of miRNAs in the control of flowering time, floral organ identity and abscission is now reported (Damodharan et al., 2016; Teotia and Tang, 2015). Based on this information, *MdmiR285N* may be likely involved also in cellular processes responsible for plant reproduction.

In general, a close up examination of histochemical results may propose that the vascular system is the main source of MdmiR285N. Within this context, miRNAs localized in the vascular system have often roles in plant long-distance signaling. Different studies reported the presence of specific miRNAs moving over long distances in grafts (Buhtz et al., 2010; Pant et al., 2008). Moreover, miRNA movement from shoots to roots could be correlated with long-distance signaling during nutrient starvation responses in *A. thaliana* (Buhtz et al., 2010; Pant et al., 2008) or the regulation of specific developmental events in potato (Bhogale et al., 2014; Martin et al., 2009). It is therefore possible that MdmiR285N acquired a long-distance signaling role. However, it is also known that many bacterial pathogens are specialized parasites of plant vascular systems (Bendix and Lewis, 2016; Bové and Garnier, 2003; Yadeta and J. Thomma, 2013). Based on these observations, and given that MdmiR285N post-transcriptionally regulate several disease resistance proteins, its localization in the vascular system could also reflect the presence of a putative defense mechanism mediated by MdmiR285N against plant vascular pathogens.

To confirm the putative role of *MdmiR285N* in response to host-pathogen infection, *MdmiR285N* expression in *wild-type* apple plants and GUS activity in transgenic apple and *A. thaliana* plants were analyzed in response to *E. amylovora* (for apple) and *Pst* DC3000 (for *A. thaliana*) (Figure 6). Overall, *MdmiR285N* appeared down-regulated in both plant species examined thus suggesting an increase of its targeted disease resistance transcripts during pathogen infection. To date, many studies reported that plants are able to

induce expression of disease resistance genes by suppression of the miRNA-mediated gene silencing pathway upon pathogen attack (Li et al., 2012; Shivaprasad et al., 2012; Yang et al., 2015; Zhai et al., 2011; Zhu et al., 2013). Within this context, a fine regulation of disease resistance proteins is also mandatory for a correct plant growth and development. Disease resistance proteins were indeed shown to have a cost to plants (Tian et al., 2003) because if unregulated they can trigger autoimmunity in the absence of pathogen infection and inhibit plant growth (Li et al., 2012). Plants have thus evolved miRNA-disease resistance proteins regulatory loops as counter mechanisms to minimize the cost of over-expression of disease resistance genes in the absence of a pathogen, and to ensure rapid induction of disease resistance proteins during pathogen invasion. This information supports our findings, suggesting a similar mechanism of action for MdmiR285N on its putative resistance transcripts, and that *MdmiR285N* may act as positive regulator of plant defense response upon plant-pathogen interactions. These observations could also explain the tissue and organ-specific expression patterns of *MdmiR285N*, according to which this miRNA was shown to be strongly induced in juvenile or developing plant tissues. The activity of MdmiR285N in those tissues is thus probably required to suppress basal defense mechanisms and allow growth and development of actively dividing tissues.

In conclusion, in this study we provide *in silico* and histological information regarding how *MdmiR285N* is regulated during the growth and development of *M. x domestica* and the heterologous plant species *A. thaliana*. Moreover, we demonstrate that *MdmiR285N* is down-regulated in response to plant-pathogen interactions, thus probably promoting an up-regulation of its target resistance transcripts upon pathogen infection. This study opened up insights into the role of *MdmiR285N* in apple and *A. thaliana* species, however deeper analysis must be performed for a better understanding of its functions and to facilitate the designing of putative *MdmiR285N*-based strategies in a view of genetic engineering of apple.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

All experiments were performed with apple (*M. x domestica*) cultivar 'Gala' plants and *A. thaliana* ecotype 'Columbia-0' plants grown in a growth chamber at 24 ± 1°C with a 16/8-h light/dark period.

In apple, *in vitro* propagation, *in vitro* roots stimulation and acclimation to soil were performed as described by Pessina et al. (2016). Before *in vitro* experiments (pathogen inoculation followed by Bradford and fuorometric assays, Figure 6b; and histochemical GUS analysis, Figure 3), to reduce putative effects of medium ingredients on the regulation of *MdmiR285N* gene and to minimize any difference with *A. thaliana* culturing, *in vitro* rooted plants were transferred to a Murashige and Skoog basal medium (MS) supplemented with 0.5% (w/v) sucrose and acclimatized for 5 days. For *ex vitro* experiments (pathogen inoculation followed by gene expression analysis, Figure 6a), soil-acclimatized plants were grown at growth chamber conditions to the stage of interest.

Regarding *A. thaliana*, the maintenance of plants lines was ensured by sowing seeds in a 3:1 soil:perlite mixture and growing plants to mature stage for seed harvesting. Before *in vitro* experiments (see above, Figure 4, 6c, 6d), harvested seeds were sterilized using 70% (v/v) ethanol x 10 min followed by 100% (v/v) ethanol x 2 min, suspended in 0.1% (w/v) agar and vernalized for 3 days at 4°C in the dark. Thus, seedlings were germinated and grown to the stage of interest in liquid ½MS basal medium supplemented with 0.5% (w/v) sucrose, using 24-well plates. For *ex vivo* histochemical GUS analysis of the inflorescence (Figure 5), seeds were germinated in a soil:perlite mixture as previously mentioned and plants were grown to the stage of interest.

Construction of the transformation vector

To produce the binary vector (Figure 1) used for apple and *A. thaliana* transformations (Table S1, S2), genomic DNA was extracted from apple leaf tissue using the Illustra™ Nucleon DNA Extraction Kit PHYTOPURE™ (GE

Healthcare). Extracted DNA was quantified on the NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific) and then used in a PCR aimed at amplifying 2 kb of intergenic genomic DNA sequence upstream of the transcription start site of *MdmiR285N* gene. PCR was performed on 40 ng of starting DNA using the thermocycle-3000 (Biometra), the Phusion® High-Fidelity DNA Polymerase (Thermo Fisher Scientific) and the pair of primers attB-MdmiR285N_Prom reported in Table S4. The PCR product was directly cloned into a pENTR/D TOPO vector (Invitrogen), and subsequently the *MdmiR285N* promoter region was inserted by LR reaction (Invitrogen) into the GATEWAY™ binary vector pKGWFS7 (Karimi et al., 2002) in-frame with the downstream GFP-GUS gene fusion system (Figure 1).

Plant transformation and identification of transgenic lines

For the production of *Prom_MdmiR285N::GFP-GUS* transgenic apple and *A. thaliana* plants, *A. tumefaciens* strain GV3101-pMP90RK (Koncz and Schell, 1986) competent cells were transformed by electroporation with the previously generated pKGWFS7 binary vector. The resulting transformed *A. tumefaciens* was conserved as stock in 40% (v/v) glycerol at -80°C, and subsequently used for plants transformations.

In apple (Table S1), *in vitro*-propagated *wild-type* plantlets were transformed as described by Joshi et al. (2011), using 770 leaf explants for infections. After transformation, regenerated plants were screened for T-DNA. Genomic DNA was extracted from leaves and quantified, as previously mentioned. Thus, genomic DNA was amplified by PCR using the thermocycle-3000 (Biometra), the GoTaq® Green Master Mix 2X (Promega, Fitchburg, MA) and the pairs of primers NptII (used to detect T-DNA), MdUBQ (used as endogenous control for genomic DNA amplification) and VirG (used to verify the presence of residual *A. tumefaciens*) listed in Table S4. The identified transgenic plants were collected and propagated *in vitro*.

In *A. thaliana* (Table S2), soil-grown *wild-type* plantlets were transformed by the *A. tumefaciens*-mediated floral dip transformation method (Bent, 2006).

Following transformation, T1 independent transgenic lines were retrieved using ½MS medium supplemented with 0.5% (w/v) sucrose and 50 ng/µL kanamycin. Thus, the germination profile of the T2 offspring of the identified transgenic lines was screened on selective medium and only those lines that showed a germination ratio Kanamycin^{Resistant}:Kanamycin^{Susceptible} significantly near to 3:1 were collected. Candidate lines selected in this study were grown to the T4 generation and the obtained T4 seeds were used for the experiments.

Quantification of *NptII* copy number by Taqman real-time PCR

In apple, the investigation of the *NptII* CN (Table S1) was performed to quantify the number of T-DNA insertion events in *in vitro* transgenic plants obtained by *A. tumefaciens*-mediated transformation. The experimental procedure was conducted according to the TaqMan real-time PCR method described by Dalla Costa et al. (2019). Primers and probes used for the amplification of *MdTOPO6* (endogenous gene) and *NptII* (marker gene) are listed in Table S4.

***In silico* analysis of *MdmiR285N* gene promoter sequence**

To detect putative TFBSs and corresponding TFs involved in the regulation of *MdmiR285N* (Figure 2, Table S3), its promoter sequence (approximately 1 kb upstream of the translation start site) was scanned by the 'Promoter Analysis' tool of PlantPAN 2.0 (http://PlantPAN2.itps.ncku.edu.tw; Chi-Nga et al., 2016), using both '*M. x domestica*' and '*A. thaliana*' databases as reference. The similarity score for TFBSs calling was set to 0.95. Results were downloaded and manually checked to remove putative inconsistencies. For each TFs detected, corresponding biological functions based on gene ontology information were retrieved by using both PlantPAN 2.0 and PlantTFDB 5.0 (planttfdb.cbi.pku.edu.cn) databases.

Histochemical GUS assay

The histochemical GUS staining of apple and *A. thaliana* samples (Figure 3-5, 6c) were carried out following the procedure described by Jefferson et al. (1987) with some variations. Samples were immersed in 90% (v/v) acetone, kept at -20°C for 30 min, then transferred into a GUS staining solution containing 1 mM X-Gluc, 2.5 mM $K_3Fe(CN)_6$, 2.5 mM $K_4Fe(CN)_6$, 0.2% (v/v) Triton X-100 (Sigma-Aldrich) and 50 mM sodium phosphate buffer (pH 7.0). Thus, samples were vacuum infiltrated (2 min and 1 min for apple and *A. thaliana,* respectively) and incubated for 12 h at 37 °C. After staining, the GUS reaction was stopped by immersing samples in a 3:1 ethanol:acetic acid solution for 6 h. Finally, samples were washed two times with 100% (v/v) ethanol for 12 h to remove the chlorophyll, and subsequently conserved in 70% (v/v) ethanol. Imaging of stained tissues was performed using a full-frame DLSR camera with a 100 mm macro lens (Nikon), a Axio Imager 2 microscope (ZEISS), and a MZ16 F stereomicroscope (LEICA).

Pathogen inoculation

For *ex vivo* inoculations of apple (Figure 6a), *wild-type* plantlets (grown for 3 weeks after acclimation to soil) were inoculated according to the scissor inoculation method described by Desnoues et al. (2018), using *E. amylovora* strain Ea273. *E. amylovora* was grown at 28°C x 24 h in liquid KADO medium (Kado and Heskett, 1970) supplemented with 0.3 g/L $MgSO_4$. Following growth, the bacterial cell density was measured with a BioPhotometer (Eppendorf, Hamburg, Germany), thus the inoculum solution was prepared by adjusting bacterial concentration to 1 x 10^9 CFU/mL with 0.05 M potassium phosphate buffer (pH 6.5). The three youngest leaves of plants actively growing were transversally cut using scissors dipped in the bacteria suspension or potassium phosphate buffer (mock) as mechanical damage control. After treatment, plants were maintained at growth chamber conditions and subsequently sampled. Approximately 5 mm wide leaf strips, parallel to the inoculation cut, were

collected at 0, 12, 24, 36 and 48 h post-inoculation. Samples were frozen in liquid nitrogen and kept at -80°C for the further RNA extraction.

For *in vitro* inoculations of apple and *A. thaliana* (Figure 6b-6d), the procedure was carried out according to the flood-inoculation technique described by Ishiga et al. (2011) with some modifications. For *A. thaliana*, inoculations were performed using *Pst* DC3000 (Buell et al., 2003). The bacterial pathogen was grown at 28°C on Luria-Bertani (LB) medium x 24 h. After growth, bacterial was suspended in sterile distilled H_2O and the bacterial cell density (OD_{600}) was measured as previously mentioned. Thus, bacterial inoculation solution (1 x 10^6 CFU/mL), prepared in sterile distilled H_2O containing 0.005% Silwet L-77 (Sigma-Aldrich), was poured into 24-well plates containing 3-week-old *A. thaliana* seedlings. Plants used as control were treated using a mock solution prepared according to the previous inoculation solution without the bacteria. After 3 minutes of immersion and low agitation at 50 rpm, inoculation solutions were discarded and the liquid culture medium was replaced. Treated plants were maintained at growth chamber conditions and sampling was performed at 0, 24 and 48 h post-inoculation. Collected plants were directly used in the histochemical GUS procedure as previously described, or frozen in liquid nitrogen and conserved at -80°C for the further Bradford and fluorometric MUG assays. For apple, *E. amylovora* was grown as previously described and the inoculum solution was prepared by adjusting bacterial concentration to 1 x 10^6 CFU/mL with 0.05 M potassium phosphate buffer (pH 6.5) and 0.005% Silwet L-77. For control experiments, a mock solution was made as the inoculum solution without the bacteria. The treatment was performed by pouring inoculation or mock solutions into baby jars containing 3-week-old apple plantlets. After 6 minutes of flood-treatment with low agitation at 50 rpm, solutions were discarded and corresponding treated plants were kept at growth chamber conditions. As for *A. thaliana*, plants were sampled at 0, 24 and 48 h post-inoculation and conserved at -80°C for the subsequent Bradford and fluorometric MUG assays.

Real-time PCR

For the expression analysis of mature *MdmiR285N* transcripts (Figure 6a), the experimental procedure was conducted according to the protocol of Varkonyi-Gasic et al. (2007) with minor variations. Samples were ground with a mortar and pestle chilled with liquid nitrogen, and the resulting powder was used for total RNA extraction using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich). Extracted RNA was quantified on the NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific) and subsequently treated with DNase I (Sigma-Aldrich) to remove genomic DNA contamination. 1 μg of DNase-treated RNA was applied for the *MdmiR285N*-specific cDNA synthesis using the Superscript III RT kit (Invitrogen), the MdmiR285N-stemloop primer (Table S4), and a pulsed reverse transcription (1 cycle of 16°C for 30 min; 60 cycles of 30°C for 30 s, 42°C for 30 s, and 50°C for 1 s; and 1 cycle of 70°C for 15 min). In parallel, *MdU6* and *MdACT2* genes (used as internal controls) were retrotranscribed according to the manufacture's instructions of the Superscript III RT kit (Invitrogen). After RT reaction, the produced cDNA was diluted ten times and then used in real-time PCR reactions conducted in a 96-well plate with 5 ng of starting cDNA, the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) and the couples of primers MdU6, MdACT2 and MdmiR285N (F, R) reported in Table S4. Real-time PCRs were performed on a C1000 thermal cycler (Bio-Rad) equipped with CFX96 real-time PCR detection system (Bio-Rad) and a data analysis software CFX Maestro (Bio-Rad).

Bradford and fluorometric MUG assays

Collected apple and *A. thaliana in vitro* plantlets were ground with a mortar and pestle chilled with liquid nitrogen. 100 mg of the resulting powder was used for the quantification of GUS activity (Figure 6b, 6d) according to the experimental procedure described by Dalla Costa et al. (2014).

Statistical analysis

Regarding the statistical analysis of segregation T-DNA loci data in *A. thaliana* (Table S2), the Chi-square (X^2) test was used to assess the differences between the observed values and the expected values.

For gene expression and GUS activity quantitative data (Figure 6), the statistical analysis was conducted with the Dell™ Statistica™ Software version 13.1, considering datasets of mock- and pathogen-treatments separately. A one-way ANOVA followed by post-hoc Dunnett's test was used to assess differences between datasets and the corresponding control dataset (0 h). Statistics was performed with α=0.05.

AUTHOR CONTRIBUTIONS

V.P. designed the experiments, conducted the experiments and wrote the manuscript. S.P. and M.L. contributed to designing and performing the experiments, and revised the manuscript. C.V. and M.M designed the project, contributed to designing the experiments and revised the manuscript. All authors read and approved the manuscript.

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DECLARATIONS

No conflict of interest declared.

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SUPPORTING INFORMATION

SUPPORTING TABLES

Table S1. Summary of transformation results and selection of transgenic lines in apple (*Malus x domestica*). The transformation efficiency was calculated by dividing the number of regenerated plants positive for *NptII* and negative for *VirG* by the number of leaf explants infected. *MdUBQ* gene was amplified to test the integrity of genomic DNA used in PCRs. The *NptII* copy number (CN) of each selected plant line is the mean ± SD of 4 biological replicates. *Neomycin phosphotransfare II* gene (*NptII*); *Ubiquitin* gene (*MdUBQ*); *A. tumefaciens Virulence G* gene (*VirG*).

Apple cultivar	**Transformation vector**	**No. of leaf explants infected**	**No. of regenerants collected**	**PCR screening**				**Transformation efficiency**	**Selected lines: Line ID (*NptII* CN)**
				No. of regenerants tested	**No. of positive regenerants**				
					NptII	***MdUBQ***	***VirG***		
Gala	pKGWFS7	770	6	5	5	5	1	0.5	PMd1 (2.01±0.12) PMd2 (1.00±0.45)

Table S2. Summary of transformation results and selection of transgenic lines in *Arabidopsis thaliana*. Chi-square (X^2) test was used to determine the probability (*P*) of which the deviation of the observed value (ratio tested) from the expected value (ratio R:S=3:1) was due to chance. Kanamycin (Kan); Resistant (R); Susceptible (S).

***Arabidopsis thaliana* ecotype**	**Transformation vector**	**Selection of transformed plants with single T-DNA insertion event**					X^2	**Signification level (*P*)**
		No. of analyzed T1 lines	**Screening of T2 generation**					
			Line ID	**Kan^R (No. seeds)**	**Kan^S (No. seeds)**	**Ratio tested (R:S=3:1)**		
Col-0	pKGWFS7	40	PAt2	135	54	2.5:1	1.286	0.25
			PAt6	112	46	2.4:1	1.426	0.23
			PAt13	140	61	2.3:1	3.066	0.08
			PAt26	186	48	3.9:1	2.513	0.11
			PAt27	106	44	2.4:1	1.502	0.22
			PAt28	140	39	3.6:1	0.985	0.32
			PAt32	153	47	3.3:1	0.240	0.62
			PAt36	146	63	2.3:1	2.949	0.08

Table S3. List of TFBSs identified in the promoter region of *MdmiR285N* gene. TFBSs calling was performed in 1 kb of *MdmiR285N* promoter sequence upstream of the transcription start site (ATG), using separately the *Malus x domestica* and *Arabidopsis thaliana* databases of PlantPAN 2.0 (http://PlantPAN2.itps.ncku.edu.tw). A sequence similarity score higher than 0.95 was used. Information regarding TFs families, TFs and the associated biological process were retrieved by comparing the results obtained from both PlantPAN 2.0 and PlantTFDB 5.0 (planttfdb.cbi.pku.edu.cn) databases.

***MALUS x DOMESTICA* DATABASE**			
TFBS sequence	**Position (0..1052-ATG)**	**TFs Family**	**TF gene ID (Biological Process)**
gAGATTcgag	724	ARR-B	MDP0000124301 (response to cytokinins); MDP0000290818 (response to cytokinins and water deprivation; regulation of root meristem growth, chlorophyll biosynthetic process and anthocyanin metabolic process; development of shoot, primary root and seed)
ccCACGTgt	778	bHLH	MDP0000029168 (response to dessication, wounding, abscisic acid, jasmonate signaling pathway and chitin; regulation of flavonoid biosynthetic process); MDP0000138721 (phytochrome signaling pathways); MDP0000198404 (photrotransduction; ethylene biosynthetic process; response to light stimulus; regulation of auxin biosynthetic and signaling pathways)
ccCACGTgtg	778	bZIP	MDP0000144105 and MDP0000177486 (response to cold, water deprivation, salt stress, abscisic acid, gibberellin, chitin; seed germination and development); MDP0000286846 (response to light stimulus)
tAGTGTt	101	C2H2	MDP0000762756 (response to oxidative stress, cold, water deprivation, wounding, high light intensity, salt stress, abscisic acid, chitin; photoprotection)
tACACTa	244		
aTAACTc	88		MDP0000225209 (response to hormone-mediated signaling pathways; specification of floral organ identity; regulation of cell

			proliferation)
gtTTAAAa	177	CPP	MDP0000182903 and MDP0000233007 (regulation of development of both male and female reproductive tissues)
aATAAAag	509	CSD	MDP0000155077 and MDP0000202739 (response to cold-shock)
tTATTTtg	544		
aATAAAaa	561		
tagTGCATca	912	EIL	MDP0000144922 (involved in the ethylene response pathway)
ggaGATTCgag	723	G2-like protein	MDP0000202657 (phosphate signaling in roots); MDP0000229587 (regulation of vascular development; phloem and xylem differentiation)
gAGATCaaag	469	GATA	MDP0000137305 (circadian rhythm and seed germination); MDP0000220844 (response to light stimulus)
ttcATTAAtt	327	HD-ZIP	MDP0000046392 (seed gemination; epidermal cell differentiation; maintenance of floral organ identity; cotyledon development); MDP0000125182 and MDP0000125184 (response to drought stress; regulation of jasmonate and auxin biosynthesis and signaling; lateral root formation)
gacATTAAaa	483		
aaacAAAAAgaaaaataaaaa	548	MIKC_MADS	MDP0000013331 (meristem structural organization; maintenance of floral meristem identity and determinancy)
agTAGGTa	1025	MYB	MDP0000135594 (response to ethylene, abscisic acid, auxin and biotrophic phatogens); MDP0000175918 (response to wounding, salt stress, abscisic acid and salicylic acid; seed trichome elongation)
catTATCCc	448	MYB-related	MDP0000140324 and MDP0000230601 (regulation of drought-responsive genes; stomatal closure; response to abscisic acid); MDP0000758053 (response to salt stress, ethylene, auxin, abscisic acid, gibberellin, salicylic acid and jasmonate; regulation of anthocyanin biosynthetic process)

aaGTCAAtc	253	NAC	MDP0000120881 (anther and pollen development; seed development and morphology); MDP0000132623 (jasmonate-mediated response to biotrophic pahtogens); MDP0000180683 (regulation of ethylene biosynthesis; seed development and morphology); MDP0000481448 (fruit ripening; flower development; leaf senescence); MDP0000802924 (stress-induced signaling); MDP0000868419 (response to jasmonate; seed morphogenesis and embryonic development)
acTTGACtg	525		
acttGCACCa	766	TCP	MDP0000120671 (leaf morphogenesis and cell differentiation); MDP0000130524 (ovule development; control of shoot organs morphogenesis; response to light stimulus)
ttTAACCa	386	Trihelix	MDP0000257669 (response to light signals)
aaaGTCAAtc	252	WRKY	MDP0000119590 (regulation of elicitor-responsive genes); MDP0000121669 (jasmonic acid mediated signaling pathway; defense response to fungus); MDP0000128464 (defense reponse to necrotrophic and biotrophic pathogens); MDP0000130400 (regulation of jasmonic acid signaling pathway; regulation of systemic resistance)
cTTGACtg	526		
***ARABIDOPSIS THALIANA* DATABASE**			
TFBS Sequence	**Position (0..1052-ATG)**	**TF Family**	**TF gene ID (Biological process)**
acGGATCcac	758	ARR-B	AT1G67710 (response to cytokinin; cytokinin-activated signalin pathway; regulation of root growth); AT2G01760 (cytokinin-activated signaling pathway)
ctgcAATCTc	867		AT4G18020 (regulation of circadian rhythm)
gaGATTCgag	724		AT3G16857 (response to water deprivation; regulation of root growth, chlorophyll biosynthetic process, anthocyanin metabolic process, cytokinin-activated

			signaling pathway and seed growth; shoot system development; primary root development); AT2G01760 (cytokinin-activated signaling pathway)
ttcccACGTGtgaa	776	BES1	AT1G19350 (brassinosteroid mediated signaling pathway; defense response to bacterium); AT1G75080 (brassinosteroid mediated signaling pathway; regulation of growth; seed development; plant ovule development)
cccACGTGt	778	bHLH	AT1G10120 (response to light; regulation of growth); AT1G18400 (positive regulation of shade avoidance); AT1G25330 (brassinesteroid mediated pathway); AT1G26260 (response to light; positive regulation of flower development; regulation of growth); AT1G59640 (petal morphogenesis); AT1G68920 (regulation of growth); AT1G73830 (response to brassinosteroid, auxin, ethylene and abscisic acid); AT3G23690 (response to light; positive regulation of flower development); AT5G50915 (response to gibberellin); AT5G08130 (brassinosteroid mediated signaling; positive regulation of shade avoidance); AT1G09530 (phototransduction; de-etiolation; gibberellic acid mediated signaling pathway); AT2G46970 (shade avoidance; light signaling pathway)
tccACTTGc	763		AT1G05805 (regulation of stomatal movement); AT1G35460 (cuticle development); AT1G51140 (regulation of stomatal movement; cuticle development; regulation of flowering); AT2G42280 (regulation of flowering); AT2G43140 (response to abscisic acid stimulus)

cccACGTGtg	778	bZIP	AT1G49720 (response to abscisic acid); AT3G19290 (response to water deprivation and salt stress; abscisic acid-activated signaling pathway; regulation of leaf senescence and chlorophyll catabolic process); AT1G45249 (response to water deprivation and salt stress; abscisic acid-activated signaling pathway); AT2G46270 (response to abscisic acid); AT4G36730 (regulation of hydrogen peroxide metabolic process; regulation of cell aging); AT4G01120 (response to light); AT2G36270 (response to cold, water deprivation, salt stress, abscisic acid and gibberellin; pollen and seed maturation; response to chitin); AT3G54620 (seed maturation)
agCAGCTgcc	798	C2H2	AT2G17180 (pollen sperm cell differentiation)
aTAACTc	88		AT5G06070 (response to cold; regulation of meristem structural organization; petal development)
tACACTa	244		AT1G27730 (response to oxidative stress, cold, water deprivation, wounding, high light intensity, salt stress and chitin; abscisic acid mediated signaling); AT5G04340 (response to chitin; regulation of root development); AT5G43170 (response to chitin, cold and salt stress); AT3G19580 (response to water deprivation, salt stress and chitin; abscisic acid mediated signaling); AT5G04340 (response to chitin; root development); AT5G43170 (response to chitin, cold and salt stress)
tAGTGTt	101		
gcaAATTTcaaag	74	CPP	AT4G14770 (development of both male and female reproductive tissues)
gtTTAAAa	177		
ttcttAAATTtac	410		
aATAAAaa	509	CSD	AT2G21060 (embryo and fruit development; response to cold stress); AT4G38680 (response to cold and dessication; abscisic acid mediated signaling; seed development; vegetative to reproductive phase transition of meristem)
aATAAAaa	561		
aATAAAac	544		
aaAAAAGgac	125	Dof	AT5G39660 (flower development)

aaaTCTTTtt	379		AT5G65590 (guard cell differentiation; stomatal movement; regulation of cell wall pectin metabolic process)
aCTTTAacg	192		AT4G38000 (floral organ abscission)
aCTTTAacg	221		
aCTTTTaac	818		
taAAAAGtca	249		AT2G46590 (response to cold; response to light stimulus; seed germination); AT5G39660 (flower development); AT4G38000 (floral organ abscission); AT5G62940 (procambium histogenesis; phloem or xylem histogenesis); AT3G47500 (flower development)
taAAAGAgat	464		AT5G65590 (guard cell differentiation; stomatal movement; regulation of cell wall pectin metabolic process)
tagTGCATca	912	EIL	AT3G20770 (response to hypoxia and iron ion; ethylene-activated signaling pathway; defense response to bacterium); AT5G21120 and AT5G65100 (response to iron ion; ethylene-activated signaling pathway)
ggaGATTCgag	723	G2-like protein	AT2G40970 (response to cold); AT3G46640 (regulation of circadin rhythm); AT5G59570 (regulation of circadian rhythm and flower development); AT2G03500 (response to temperature stimulus; gibberellic acid mediated signaling pathway; flower development; negative regulation of long-day photoperiodism; flowering); AT1G68670 (response to abscisic acid); AT4G28610 (regulation of circadian rhythm; response to high light intensity; response to phosphate starvation); AT1G79430 (development of flower, phloem, and xylem); AT3G24120 (cellular response to phosphate starvation)
gAGATCaaag	469	GATA	AT2G45050 and AT3G51080 (response to light stimulus); AT5G25830 (circadian rhythm; response to light stimulus)
gacATTAAaa	483	HD-ZIP	AT1G05230 and AT1G17920 (trichome morphogenesis; maintenance of floral organ

ttcATTAAtt	327		identity); AT2G32370 (cotyledon development); AT3G61150 (maintenance of floral organ identity); AT4G21750 (seed germination; cotyledon development); AT5G46880 (maintenance of floral organ identity)
aaacAAAAAgaaaaataaaaa	548	MIKC-MADS	AT4G22950 (flower development; vernalization response); AT4G24540 (response to gibberellin; maintenance of floral and inflorescence meristem identity; vernalization response); AT5G51860 and AT5G51870 (regulation of flower development); AT5G60910 (positive regulation of flower development; maintenance of inflorescence meristem identity; fruit development); AT5G62165 (regulation of flower development; abscission; leaf and floral organ senescence); AT1G26310 (positive regulation of flower development; floral meristem determinancy); AT2G14210 (response to nutrient and nitrate; lateral root development); AT2G22630 (positive regulation of long-day photoperiodism; flowering); AT2G45650 (embryo sac development; positive regulation of flower development; specification of carpel identity; vegetative to reproductive phase transition of meristem; floral meristem determinancy; floral organ development; stamen formation; floral whorl structural organization; specification of floral organ number; integument development; seed growth); AT2G45660 (response to cold; response to gibberellin; positive regulation of flower development; maintenance of inflorescence meristem identity); AT3G57230 (stomatal lineage progression; long-day photoperiodism; flowering); AT3G57390 (pollen development; negative regulation of flower development; negative regulation of short-day photoperiodism; flowering); AT3G61120 (pollen and plant ovule development);

			AT4G09960 (seed development; plant ovule development; regulation of double fertilization forming a zygote and endosperm; seed trichome differentiation); AT4G11880 (flower development; maintenance of floral meristem identity; regulation of root meristem growth; vegetative to reproductive phase transition of meristem; regulation of auxin polar transport)
tccTTATCat	292	MYB	AT5G04760 (regulation of flower morphogenesis)
agTAGGTa	1025		AT2G16720 (response to salt stress and salicylic acid; regulation of flavonol biosynthetic process); AT4G09460 (response to gibberellin, salicylic acid and jasmonic acid; seed trichome elongation); AT4G34990 (response to salt stress, ethylene, abscisic acid, salicylic acid and jasmonic acid); AT4G38620 (response to salicylic acid, jasmonic acid and UV-B; secondary cell wall biogenesis involved in seed trichome differentiation)

caTTATCccc	448	MYB-related	AT1G70000 (response to salt stress, ethylene, auxin, abscisic acid, gibberellin; salicylic acid and jasmonic acid; regulation of anthocyanin biosynthetic process); AT3G16350 (response to salt stress, ethylene, auxin, gibberellin and jasmonic acid); AT5G47390 (response to salt stress, ethylene, abscisic acid, gibberellin; salicylic acid and jasmonic acid; leaf development)
aaCCCTAg	899		AT3G49850 and AT5G67580 (response to salt stress, ethylene, auxin, gibberellin, salicylic acid and jasmonic acid)
aaGTCAAtc	253	NAC	AT1G01720 (response to wounding; negative regulation of abscisic acid-activated signaling pathway); AT1G52880 (regulation of embryonic development; seed morphogenesis; integument development); AT1G52890 (response to water deprivation); AT1G69490 (fruit ripening; flower development; leaf senescence); AT3G15500 (jasmonic acid mediated signaling pathway; response to water deprivation); AT3G15510 (response to jasmonic acid; regulation of embryonic development; seed morphogenesis; integument development); AT4G27410 (response to water deprivation; response to abscisic acid)
acTTGACtg	525		
acttGCACCa	766	TCP	AT1G30210 (leaf morphogenesis; regulation of development); AT1G67260 (flower development); AT1G68800 and AT3G18550 (regulation of secondary shoot formation); AT3G02150 (leaf morphogenesis; regulation of development; regulation of light stress response)
tTAACCaact	387	Trihelix	AT1G13450 (response to light signal)

aaGTCAAtcc	253	WRKY	AT1G18860, AT1G29280 and AT1G29860 (regulation of elicitor-induced response); AT1G55600 (regulation of elicitor-induced response, endosperm development); AT1G62300 (ethylene-activated signaling pathway; response to chitin and phosphate starviation); AT2G34830 (pollen development; embryo development ending in seed dormancy); AT2G40750 (response to salicylic acid; regulation defense response to pathogens; regulation of leaf senescence); AT2G46400 (response to chitin; lateral root development); AT3G56400 (systemic acquired resistance, salicylic acid mediated signaling; induced systemic resistance, jasmonic acid mediated signaling; response to chitin; ; regulation of leaf senescence); AT4G23810 (response to salicylic acid; defense response to bacterium, incompatible interaction; leaf senescence); AT4G39410 (regulation of lignin biosynthetic process); AT5G15130 and AT5G22570 (salicylic acid mediated signaling pathway and defense response to bacterium); AT5G26170 (jasmonic acid mediated signaling pathway; defense response to fungus); AT5G45050 (defense response to biotic stress); AT5G45260 (cell death; defense response to bacterium, incompatible interaction)
cTTGACtg	526		

Table S4. List of primers and probes used during the analysis. AttB-MdmiR285N_Prom primers were used to isolate the promoter sequence (2 kb) of *MdmiR285N* gene. NptII, MdUBQ and VirG primers are related to the PCR-based screening of putative transgenic plant lines. NptII and MdTOPO6 primers and probes sets were used for the quantification of T-DNA copy number by Taqman real-time PCR. MdU6, MdACT2 and MdmiR285N primers sets were used for the expression analysis of mature *MdmiR285N* by real-time PCR.

Primers and probes	Sequences (5'-3')	Annealing temperature (°C)
MdUBQ	F: CATCCCCCCAGACCAGCAGA R: ACCACGGAGACGAAGCACCAA	60
VirG	F: GCCGGGGCGAGACCATAGG[a] R: CGCACGCGCAAGGCAACC[a]	56
MdTOPO6	F: TGTGGAAGGAGATCAGCGCA[b] R: CGCGTTGCTTCTTTGCTGCA[b]	58
MdTOPO6_probe	FAM-5'-ACATGCCAACAGGAACAATCACA-3'-TAMRA[b]	
NptII	F: CTTGCCGAATATCATGGTGGAA[c] R: GGTAGCCAACGCTATGTCCTGA[c]	58
NptII_probe	FAM-5'-TTCTGGATTCATCGACTGTGGC-3'-TAMRA[c]	
attB-MdmiR285N_Prom	F: CCATCTTAGGCTCCAAAACTCGCAATCAAG R: CTCCTACCTACTAGCTTTAGCCAAAGAG	58
MdmiR285N	Stemloop: GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACTATGGT F: GTTTGGGTAAGTTCATCCAAAC Universal R: GTGCAGGGTCCGAGGT	60
MdU6	F: GATAAAATTGGAACGATACAG[d] R: GGACCATTTCTCGATTTATGCG[d]	60
MdACT2	F: TGGTGAGGCTCTATTCCAAC R: TGGCATATACTCTGGAGGCT	60

[a] (Herzog et al., 2012)
[b] (Dalla Costa et al., 2019)
[c] (Dalla Costa et al., 2009)
[d] (Kaja et al., 2015)

ONGOING ANALYSIS

PART 1

Characterization and functional analysis of *MdmiR285N* and its target transcripts in transgenic apple (*Malus x domestica* cv. 'Gala') and *Arabidopsis thaliana*

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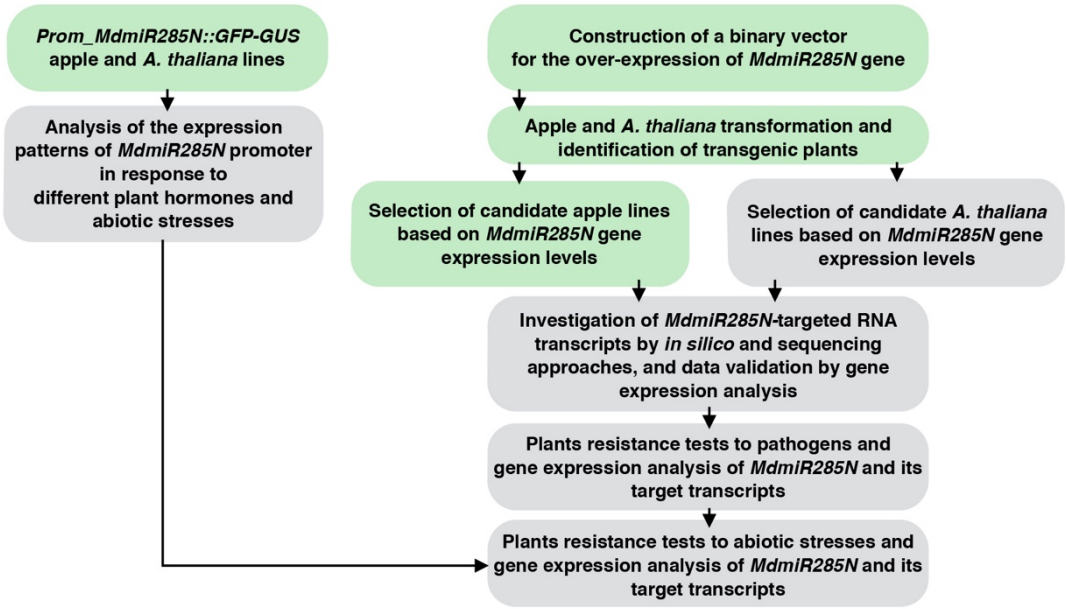
Schematic overview



Experimental workflow. Green and grey boxes indicate concluded and ongoing analysis, respectively.

CHAPTER 4

Development of a Taqman Real-time PCR method to quantify *nptII* in apple lines obtained with 'established' or 'new breeding' techniques of genetic modification

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Development of a Taqman Real-time PCR method to quantify *nptII* in apple lines obtained with 'established' or 'new breeding' techniques of genetic modification

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ABSTRACT

Cisgenic plants must be free of exogenous genetic elements such as antibiotic or herbicide resistance genes commonly used in the selection phase of a gene transfer protocol. However, the use of a selection marker is essential for the transformation of many fruit crops, including apple (*Malus x domestica*), for which the efficiency of DNA integration is very low. Currently, the approach with the highest chances of success relies on the removal of undesired exogenous genes by means of an inducible site-specific recombinase enzyme and its recognition sites. We developed a quantitative, rapid and cost-effective method based on real-time PCR to quantify the copy number of *nptII* marker gene in apple lines and to evaluate its elimination after the activation of the recombinase system. *MdTOPO6* gene was chosen as endogenous reference gene for apple due to the single copy presence in the haploid genome and to the species-specificity. A recombinant plasmid harboring specific sequences of both the reference gene and the target gene *nptII* was used as calibrator to build the standard curves. The limit of quantification of the method was evaluated and precision and trueness of the quantification performances proved to be valid according to international reference criteria. Finally, this method was applied to characterize transgenic and cisgenic apple lines and to investigate the possibility of removing an entire T-DNA cassette from the genome of edited apple plants.

KEYWORDS

Real-time PCR; Copy number; Marker-free; NPBT; *Malus x domestica*

INTRODUCTION

The new breeding techniques (NBT) in agriculture represent an evolution of the genetic engineering technology for crop improvement allowing to introduce the desired agronomic/qualitative traits with a minimal modification of the plant genome. The term NBT comprises several techniques having different purposes and methodologies – the two best known ones are 'cisgenesis' and 'genome

editing' - however, a common requirement is the absence of exogenous genetic elements in the genome of deriving organisms. Indeed, the definition of cisgenesis states that: "A cisgenic plant is a crop plant that has been genetically modified with one or more genes (containing introns and flanking regions such as native promoter and terminator regions in a sense orientation) isolated from a crossable donor plant. In contrast, transgenic plants contain genes from non-crossable organisms (e.g., a selection marker gene originating from a microorganism), synthetic genes or artificial combinations of a coding gene with regulatory sequences, such as a promoter, from another gene" (Schouten et al. 2006).

Despite avoiding the use of a selection marker is a feasible strategy in the case of species for which highly efficient transformation protocols are available (de Vetten et al. 2003), for fruit crops the screening process would be excessively demanding and laborious (Yau et al. 2013). For these reasons, alternative approaches are being followed mainly based on segregation of selectable marker genes in the next generations by crossing or auto-fertilization (for seed propagated species) or by site specific-recombination mechanisms to remove the undesired exogenous DNA (for vegetatively propagated species).

Cisgenic apples, resistant to *Venturia inaequalis* the causal agent of apple scab (Vanblaere et al. 2011; Schouten et al. 2014; Krens et al. 2015) or *Erwinia amylovora* the causal agent of fire blight (Kost et al. 2015), were produced. In these cisgenic plants the elimination of the selectable marker gene *nptII,* coding for an aminoglycoside phosphotransferase which confers resistance to kanamycin and other antibiotics, relies on the presence of a recombinase enzyme which recognizes two directly repeated sites (i.e. *Flp* recombinase/FRT sites or R recombines/Rs sites) and produces the excision of the DNA in between. The application of these excision mechanisms to obtain cisgenic apples has been preceded by studies focused on their development and optimization which tested different induction strategies. Two systems have been successfully set up in apple: the heat-shock induction (Herzog et al. 2012;

Würdig et al. 2013) and the treatment based on dexamethasone followed by 5-fluorocytosine (Righetti et al. 2014).

An important aspect in the application of NBT to crops, especially to vegetatively propagated species like apple, is the availability of analytical tools for selecting individual lines with the required features. The copy number (CN) quantification of the selectable marker gene and the evaluation of its subsequent removal under a specific induction has been mainly based on end-point PCR and Southern Blot hybridization Herzog et al. 2012; Würdig et al. 2013; Kost et al. 2015; Krens et al. 2015). However, both techniques are qualitative while, for the optimization of an induction treatment, a quantitative assay able to detect slight variations of *nptII* CN should be preferred. In addition, Southern blot is impractical for examining large population of plants as it requires many laborious and time consuming steps (Collier et al. 2017). Real-time PCR methods for CN determination have also been developed in other plants such as grapevine (Dalla Costa et al. 2009), citrus (Omar et al. 2008), maize (Ingham et al. 2001; Song et al. 2002; Shou et al. 2004), tomato (Mason et al. 2002; Huang et al. 2013), soybean (Schmidt et al. 2001; Takabatake et al. 2013), rice (Prior et al. 2006; Wang et al. 2015), wheat (Li et al. 2004; Wang et al. 2015) and others. Real-time PCR shows certain technical advantages, such as rapidity, requirement of low quantity DNA, high repeatability and reproducibility, and the possibility of applying statistical tests to the quantitative results (Dalla Costa et al. 2009). Moreover, official methods for GMO detection and quantification validated by the JRC (Joint Research Center) are based on real-time PCR (http://gmo-crl.jrc.ec.europa.eu/gmomethods/).

In order to accurately estimate the copy number of an exogenous gene integrated in the plant genome, it is crucial to detect and quantify an endogenous reference gene with high species-specificity, low intraspecific variability and a known copy number, preferentially single-copy (prEN ISO 2426 and 21569; Collier et al. 2017). According to literature, *TOPO6* proved to be a single-copy gene in 30 out of 30 tested species including *Malus x domestica*

Borkh (Blattner 2016). *TOPO6* is a nuclear gene homolog of archean topoisomerase VI subunit B and codes for a topoisomerase involved in handling supercoiled DNA and in inducing meiotic DNA double strand breaks during Prophase I.

In this study we set up a method based on Taqman quantitative real-time PCR to assess *nptII* CN in apple using *MdTOPO6* as endogenous reference gene. *NptII* in fact was the first selection marker gene to be tested in plants and has become the most widely used (Miki and McHugh, 2004). Determination of method performances parameters was carried out and cisgenic and transgenic apple lines were evaluated. Finally, this method was applied for quantifying *nptII* in genome-edited apple lines where the T-DNA cassette was designed to be removed by an inducible site-specific recombination mechanism.

MATERIALS AND METHODS

Plant material

Genetically modified plants of *Malus x domestica* were obtained by gene transfer experiments via *Agrobacterium tumefaciens* using *nptII* as selectable marker gene. A complete list of the analyzed plants is reported in Table 1. The scab resistant apple lines Vf2 and Vr2C were developed by using the binary vector pMF1 (Schaart et al. 2011) designed for a cisgenic approach which allows the removal of the marker gene *nptII* under the induction with 10 µM dexamethasone solution (Vanblaere et al. 2011). Line (Vr2C+Vf2)_1 was produced by re-transforming the cisgenic Vr2C_1 line with the binary vector *pMF1* carrying the *HcrVf2* gene. V25 lines were produced using the vector pBINplus (van Engelen FA et al. 1995). The plants Ga2_8 and Ga2_21 used to evaluate method accuracy were produced by Belfanti et al. (2004), while the *ad-hoc* samples 1 (AHS_1) was prepared by mixing 500 ng of genomic DNA from a 'Gala' wild type plant (WT) with 500 ng of T_1 genomic DNA and AHS_2 by mixing 500 ng of 'Gala' WT genomic DNA with 500 ng of T_2 genomic DNA, where T_1 and T_2 where transgenic plants previously obtained in our laboratory. Lines G-6 and GD-7, used for heat-shock experiments were

Table 1 List of the plants analyzed for the quantification of *nptII* CN.

Plant ID	Cultivar	Gene of interest	Binary Vector	Method for inducible *nptII* removal	Cultivation	Reference
Vf2_1	'Gala'	*HcrVf2* (*Rvi6*)	pMF1	Recombinase-LBD/Rs Dexamethasone	*in-vitro*	Vanblaere et al. 2011
Vr2C_1 Vr2C_2	'Gala'	*Vr2C* (*Rvi15*)	pMF1	Recombinase-LBD/Rs Dexamethasone	*in-vitro*	Schouten et al. 2014; Vanblaere et al. 2011
(Vr2C+Vf2)_1	'Gala'	*Rvi15* + *Rvi6*	pMF1	Recombinase-LBD/Rs Dexamethasone	*in-vitro*	Malnoy, personal comm.
V25_1 V25_2 V25_3	'Gala'	V25 (*Rvi18*)	pBINplus	--	*in-vitro*	Schouten H, personal comm.
Ga2_8 Ga2_21	'Gala'	*HcrVf2* (*Rvi6*)	pCambia-2301	--	green-house	Belfanti et al. 2004
T_1 T_2	'Gala'	35SCaMV-*gus*-*gfp*	pKGWFS7	--	*in-vitro*	Piazza S, personal comm.
AHS_1 AHS_2					*in-vitro*	
G-6 GD-7	'Gala' 'Golden Delicious'	[a]	p9[a]	Recombinase Flp/FRT Heat-shock	*in-vitro*	Pompili et al. (in preparation)

[a]Further details in Pompili et al. (in preparation).

transformed following the protocol of Pessina et al. (2016) and using a binary vector containing the *Flp* recombinase gene under the control of the soybean heat-shock promoter Gmhsp17.5-E and the *Flp* recognition sites (FRT) next to the Left and Right borders of T-DNA (further details in Pompili et al. in preparation). *In-vitro* apple plantlets were maintained in shoot propagation medium (Pessina et al. 206) in baby food jars.

DNA extraction

DNA was extracted from 100 mg leaf tissue of different *Malus x domestica* cultivars ('Gala', 'Idared', 'Golden Delicious', 'Fuji' and 'Pinova') and wild *Malus* species (*floribunda* clone 821, *sieversii* and *sylvestris*) cultivated in field following CTAB extraction protocol (Doyle and Doyle, 1987) while for Gala *in-vitro* plants DNA was extracted from 10 mg leaf tissue (1-2 leaves) using "Nucleospin Plant II" (Macherey-Nagel, Düren, Germany*).* DNA concentration was quantified with Nanodrop 8000 (Thermo Scientific, Wilmington, USA). All the DNA samples were diluted to 20 ng/µl with sterile water before the real-time PCR reactions.

***MdTOPO6*:*nptII* plasmid calibrator**

The fragment of *MdTOPO6* (accession number: MDP0000229861, source: malus_x_domestica_v3.0.a1 contig in www.rosaceae.org) used for plasmid calibrator preparation was amplified with specific primers designed with restriction sites for *ApaI* and *NcoI* at the 5' end: *MdTOPO6_ApaI*_Fw: 5'-AATGGGCCCTGTGGAAGGAGATCAAAGCGCA-3'; *MdTOPO6_NcoI*_Rv: 5'-AAACCATGGCGCGTTGCTTCTTTGCTGCA-3'. The PCR was carried out in 20 µl final volume containing 1x GoTaq green mix (Promega, Madison, Wisconsin, USA), 0.5 µM of each primer and 50 ng of DNA extracted from 'Gala' WT plant. The thermal protocol used was as follows: a first step of 2 min at 95 °C followed by 35 cycles of denaturation, annealing and extension of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C respectively, with a final extension of 5 min at 72 °C. PCR product (6 µl) was electrophoresed on a 1% agarose gel with

ethidium bromide and compared to the 1 kb Plus DNA ladder (Fermentas, Waltham, Massachusetts, USA). The DNA was gel purified using "PCR gel clean up" kit by Macherey-Nagel (Düren, Germany) and the fragment was then cloned in a *pGEM-T easy* vector containing an *nptII* cassette (Dalla Costa et al. 2009) by following the subsequent steps: (i) digestion of the vector and the fragment with *ApaI* and *NcoI* restriction enzymes according to the supplier instructions (Fermentas, Waltham, Massachusetts, USA), (ii) ligation reaction overnight at 4 °C using *T4 ligase* (Fermentas, Waltham, Massachusetts, USA) with a insert:vector molar ratio 3:1 and (iii) heat-shock transformation into competent *E.coli* DH5α. The plasmid was purified by "Nucleospin Plasmid" kit (Macherey-Nagel, Düren, Germany) from a 3 ml overnight bacterial culture derived from a selected colony and eluted in 50 µl nuclease-free water. Plasmid concentration was measured with Nanodrop 8000 (Thermo Scientific, Wilmington, USA) and molarity in the solution was calculated using a web tool which allows to convert the weight concentration into molar concentration (http://molbiol.edu.ru/eng/scripts/01_07.html). Plasmid solution was diluted in water to a concentration of 10^8 molecules/µl in 100 µl aliquots and stored at -20 °C.

Real-time reaction

The real-time PCR reaction to amplify *nptII* and *MdTOPO6* genes was performed in a 96-wells plate on a C1000 thermal cycler (Bio-Rad, Hercules, USA) equipped with CFX96 real-time PCR detection system (Bio-Rad, Hercules, USA). Primers and TaqMan probes for *MdTOPO6* were designed using Primer3 online software (http://primer3.ut.ee/): *MdTOPO6*_fw:5'-TGTGGAAGGAGATCAAAGCGCA-3'; *MdTOPO6*_rv:5'-CGCGTTGCTTCTTTGCTGCA-3'; *MdTOPO6*_Probe: FAM-5'-ACATGCCAACAGGAACAATCACA-3'-TAMRA. Primers and TaqMan probes for *nptII* were the ones reported in Dalla Costa et al. (2009): *nptII*_fw:5'-CTTGCCGAATATCATGGTGGAA-3'; *nptII*_rv: 5'-GGTAGCCAACGCTATGTCCTGA-3'; *nptII*_Probe: FAM-5'-TTCTGGATTCATCGACTGTGGC-3'-TAMRA. The real-time PCR singleplex reaction

was carried out in a 10 µl final volume containing 1 x SsoAdvanced™ Universal Probes Supermix (Bio-Rad, Hercules, USA), 40 ng of genomic DNA, 0.3 µM primers (Sigma, Haverhill, UK) and a 0.2 µM specific Taqman probe (Sigma, Haverhill, UK). The thermal protocol used was as follows: polymerase activation for 3 min at 95 °C followed by 40 cycles of denaturation of 10 s at 95 °C, annealing of 5 s at 58 °C and 5 s at 60 °C and an elongation of 30 s at 72 °C. The standard curves for both *nptII* and *MdTOPO6* were built with the *MdTOPO6*:*nptII* plasmid calibrator using four points and a serial dilution of 1:5, starting from 4 x 10^5 plasmid molecules. For each sample, the *nptII* CN was calculated using the following formula: (*nptII* total copies/*MdTOPO6* total copies) X 2. The total copies of *nptII* and *MdTOPO6* were calculated as mean values of the quantification cycles (Cq) of two technical replicates on the basis of the standard curves obtained.

Heat-shock induction for the removal of the T-DNA cassette

Ten biological replicates of G-6 and GD-7 lines were divided in three groups and incubated respectively 1, 2 or 3 times at 42 °C for 6 h with a 24 h interval between consecutive incubations. Heat incubations of baby food jars containing plantlets were carried out in hybridization oven hybridizer HB-1000 (UVP, Upland, CA, USA). Three leaves were collected from each biological replicate 16 h after the first incubation (replicates A, B, C), 16 h after the second (replicates D, E, F) and 16 h after the third one (replicates G, H, I, L) and DNA was extracted for *nptII* CN analysis. At the end of the heat-shock induction, the remaining leaves of each biological replicate were removed and the resulting stem was placed horizontally on the propagation medium to promote the regeneration of new shoots from the nodes. After one month, leaves of the regenerated shoots were sampled and used for the quantification of the *nptII* CN.

RESULTS AND DISCUSSION

Evaluation of a suitable endogenous reference gene for apple

For a real-time PCR assay, accurate quantification of exogenous gene copy number relies on the concurrent evaluation of a suitable endogenous reference gene with a known copy number -possibly a single copy- and peculiar features like species-specificity and low intraspecific variability (prEN ISO 2426 and 21569). The gene *MdTOPO6* (MDP0000229861) proved to be a single copy gene in apple, composed of 20 exons and 19 introns (Blattner 2016) with an additional intron (no. 18) close to the 3' end of the gene which is not present in the other angiosperm taxa analyzed. This peculiar region has been exploited for the choice of the primers/probe set for the Taqman real-time PCR assay, in order to find a species-specific system for apple. An amplicon length of 80-100 bp within intron 18 has been sought being short fragments recommended for real-time PCR (Zhang et al. 2017). However, in that specific region it was not possible to find a suitable primer pair due to the intron sequence rich in A and T bases. Therefore, the fragment size has been enlarged and primers and probe with proper features have been found. This set amplifies a 196 bp region covering intron 18 and its flanking exons (Fig. 1a). Restrictions on the PCR amplicon size (maximum 150 bp) are useful to detect target genes in highly processed food or feed matrices where the likelihood of DNA degradation is high (Dooley et al. 2004). However, our goal is to analyze fresh tissues from *in-vitro* or greenhouse plants to characterize and select the plant materials produced for functional genomics studies or for commercial purpose. DNA degradation may be considered minimal in fresh tissues compared with processed products where many factors may affect the state of the DNA, the most important of which are high temperature and low pH (Gryson 2010).

In order to investigate the intra-specific variability of *MdTOPO6*, the selected region was amplified in different cultivars of *Malus x domestica* and closely related wild species and fragments were sequenced and aligned. As shown in Fig. 1b, the amplified region is identical in all the analyzed genotypes. This means that the selected reference system may be used for the analysis of

a

5'-GTATCTTGATAATGCTTGTACTGTTTTTTATGATG**TGTGGAAGGAGATCAAAGCGCA**GAATTCGgtgagaatagaatgattttatttttcatctctcatagacg
tatataaactgattacaagaggtccttctaaataaggctaggaaacaataaaaggaaaagtgatt*acaacctaatgaaagttgtacatgccaacagGAACAATCACA***TGCAGCAA**
AGAAGCAACGCGATGAAATGTACAAGGAAATATCAAATCAAACAGTTACAAAAGCAATACTCAAGGAAAAGCTTTTACAACATGTTGAACAG-3'

b

'Gala'	**TGTGGAAGGAGATCAAAGCGCA**GAATTCGGTGAGAATAGAATGA----------TTACAACCTAATGAAAGTTGT*ACATGCCAACAGGAACAATCACA***TGCAGCAAAGAAGCAACGCG**
'Fuji'	**TGTGGAAGGAGATCAAAGCGCA**GAATTCGGTGAGAATAGAATGA----------TTACAACCTAATGAAAGTTGT*ACATGCCAACAGGAACAATCACA***TGCAGCAAAGAAGCAACGCG**
Malus floribunda	**TGTGGAAGGAGATCAAAGCGCA**GAATTCGGTGAGAATAGAATGA----------TTACAACCTAATGAAAGTTGT*ACATGCCAACAGGAACAATCACA***TGCAGCAAAGAAGCAACGCG**
'Golden Delicious'	**TGTGGAAGGAGATCAAAGCGCA**GAATTCGGTGAGAATAGAATGA----------TTACAACCTAATGAAAGTTGT*ACATGCCAACAGGAACAATCACA***TGCAGCAAAGAAGCAACGCG**
'Idared'	**TGTGGAAGGAGATCAAAGCGCA**GAATTCGGTGAGAATAGAATGA----------TTACAACCTAATGAAAGTTGT*ACATGCCAACAGGAACAATCACA***TGCAGCAAAGAAGCAACGCG**
'Pinova'	**TGTGGAAGGAGATCAAAGCGCA**GAATTCGGTGAGAATAGAATGA----------TTACAACCTAATGAAAGTTGT*ACATGCCAACAGGAACAATCACA***TGCAGCAAAGAAGCAACGCG**
Malus sieversii	**TGTGGAAGGAGATCAAAGCGCA**GAATTCGGTGAGAATAGAATGA----------TTACAACCTAATGAAAGTTGT*ACATGCCAACAGGAACAATCACA***TGCAGCAAAGAAGCAACGCG**
Malus sylvestris	**TGTGGAAGGAGATCAAAGCGCA**GAATTCGGTGAGAATAGAATGA----------TTACAACCTAATGAAAGTTGT*ACATGCCAACAGGAACAATCACA***TGCAGCAAAGAAGCAACGCG**

Fig. 1 a Partial sequence of *MdTOPO6* (Acc. No. MDP0000229861) and primers/probe position; exons 17 and 18 are reported in capital letters at 5' and 3' respectively, intron 18 is reported in lowercase letters, the primers sequences are indicated in bold and the Taqman probe sequence is underlined; the amplicon length is 196 bp; **b** Sequence amplified with the selected primer/probe set in several *Malus x domestica* cultivars and wild related species.

Malus x domestica varieties and related crossable wild species assuring same analytical performances.

To further confirm this result, the same quantity of DNA (about 40 ng) extracted from 12 apple genotypes (8 *Malus x domestica* cultivars - 'Empire', 'Fuji', 'Gala', 'Golden Delicious', 'Idared', 'Cripps Pink', 'Pinova', 'Stark Red Gold'- and 4 wild species -*Malus baccata*, *Malus floribunda* clone 821, *Malus sieversii*, *Malus sylvestris*-) was analyzed in real-time PCR for the amplification of the *MdTOPO6* reference gene (Table 2).

The DNA C-values (corresponding to plant haploid genome sizes), retrieved from the Royal Botanic Garden database, are comparable among genotypes and therefore there was no need of any adjustment in the initial DNA content. Similar Cq values were detected for all the genotypes, with a mean Cq of 22.90 and a Delta (Δ)Cq of 1.24 (ranging from 22.31 of *Malus sieversii* to 23.55 of *Malus sylvestris*). If only the *Malus x domestica* cultivars are considered, the ΔCq is slightly reduced (i.e. 1.09). Real-time PCR theory stipulates that a

Table 2 Evaluation of the intra-specific variability of *MdTOPO6* on genomic DNA (40 ng) amplified from 8 *Malus x domestica* cultivars and 4 wild species.For each sample four Cq values were produced; C -value is the plant haploid genome size (in picograms) retrieved from the Royal Botanic Garden database; [a] originally reported in Tatum et al. (2005); [b] originally reported in Höfer and Meister (2010); SD standard deviation.

	C-value	Cq				Cq mean	SD
		1	2	3	4		
'Empire'	-	22.51	22.39	22.50	22.42	22.46	0.06
'Fuji'	-	23.61	23.52	23.51	23.46	23.53	0.06
'Gala'	0.78[a]	23.09	23.03	22.97	23.04	23.03	0.05
'Golden delicious'	0.81[a]	22.90	22.76	22.73	22.61	22.75	0.12
'Idared'	0.79[a]	23.02	23.17	23.15	23.18	23.13	0.07
'Pink Lady'	-	23.08	22.84	22.90	22.90	22.93	0.10
'Pinova'	-	23.41	23.46	23.41	23.51	23.45	0.05
'Stark'	-	22.45	22.40	22.42	22.49	22.44	0.04
Malus baccata	0.72[b]	22.49	22.53	22.53	22.52	22.52	0.02
Malus floribunda	0.75 [b]	22.67	22.70	22.64	22.78	22.70	0.06
Malus sieversii	0.75 [b]	22.27	22.33	22.34	22.29	22.31	0.03
Malus sylvestris	0.74 [b]	23.50	23.57	23.51	23.61	23.55	0.05

ΔCq=1 corresponds to a two-fold difference in the starting amount of DNA (Hamalainen et al. 2001). This is true when precise serial dilutions of the same DNA are assessed. On the contrary, when different samples are compared, the DNA concentration estimated for each sample may be affected by technical errors associated to the spectrophotometric measure. For these reasons, the ΔCq value found among the analyzed genotypes is likely due to small deviation from the expected DNA content in the reaction.

Assessment of method performance parameters

A crucial element required by a real-time PCR method for copy number determination is the standard calibrator. This is used to build the standard curves for the endogenous and the exogenous/target sequences to derive the quantity values for each sample. The calibrator may be a genomic DNA extracted from a plant containing a known copy number of the target sequence (Biricolti et al. 2016) or, as alternative, a plasmid. The plasmid offers several

advantages compared to the first since it provides highly reproducible performances, may be cloned in *E. coli* and purified in high quantity at any time and, if suitably stored, it is stable in the long term (Dalla Costa et al. 2009).

We developed a plasmid calibrator*, p-MdTOPO6:nptII*, carrying two regions, one of the *MdTOPO6* gene and one of the *nptII* gene which simulates an ideal modified plant with an homozygous insertion of the selection marker gene *nptII*. Standard curves for both the endogenous gene *MdTOPO6* and the transgene *nptII* were generated and parameters such as coefficient of determination (R^2) and efficiency (E %) of the amplification reaction were evaluated. These resulted to be optimal according to the document published by the European network of GMO laboratories (ENGL 2015) 'Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing' which defines as acceptable a $R^2 \geq 0.98$ and an efficiency % between 110% and 90%. The R^2 and E % mean values calculated on 5 individual runs were respectively 0.99 and 98.8 % for the *MdTOPO6* standard curve and 0.99 and 99.4% for the *nptII* standard curve.

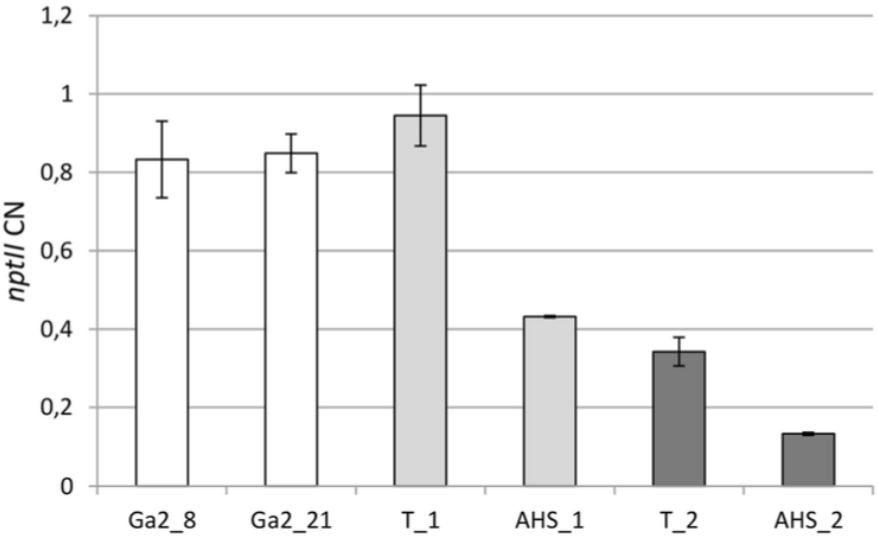
Another important parameter is the limit of quantification (LOQ) of the method, defined as the minimum copy number detectable among the various replicates with a sufficient level of accuracy and precision (Zhang et al. 2017) such that the relative repeatability standard deviation (RSDr) of the measurement should not be higher than 25%. In order to evaluate the LOQ of this method, serial dilutions 1:10 of plasmid DNA were estimated, starting from 100000 plasmid molecules to 1 (Table 3). The LOQ value for *MdTOPO6* resulted to be 220 CN, corresponding to a quantity of about 0.2 nanograms of apple DNA. Given that the yield of DNA extraction may range from 180 ng to more than 800 ng for 1 mg of fresh leaf tissue depending on the extraction protocol used (Aubakirova et al. 2014), and that generally the amount of tissue sampled for DNA extraction is around 10 mg, the average quantity of extracted DNA is abundantly above the LOQ. Besides, the calculated LOQ value for *nptII* is 78 CN. Since the amount of apple DNA used in the real-time PCR reaction is around 40 ng, corresponding to about 50000 copies of *MdTOPO6*, the lowest

Table 3 Determination of the limit of quantification (LOQ) of the method for the endogenous reference gene *MdTOPO6* and the selectable marker gene *nptII.* A serial dilution 1:10 of the plasmid calibrator was prepared, from 10^5 molecules to one. For each point, ten or nine technical replicates were run (Signal ratio). By plotting the Mean Cq value on the standard curve regression line, a Log_{10} value of input nucleic acid was derived (Log_{10}(CN); CN corresponds to the input nucleic acid calculated by the method for each dilution point; the LOQ value is the CN associated to a relative repeatability standard deviation (RSDr) of 25%.

	MdTOPO6 (standard curve y=-3.36x + 38.99)					***nptII*** (standard curve y=-3.17x + 37.57)				
Molecules	**Signal ratio**	**Mean Cq**	**Log_{10}(CN)**	**CN**	**RSDr (%)**	**Signal ratio**	**Mean Cq**	**Log_{10}(CN)**	**CN**	**RSDr (%)**
100000	10/10	22.12	5.01	102656.46	3	9/9	21.51	5.06	114750.2	4
10000	10/10	25.55	3.99	9893.20	5	9/9	24.97	3.97	9400.662	5
1000	10/10	28.98	2.97	951.67	14	10/10	28.27	2.93	863.8278	5
100	10/10	32.20	2.02	111.42	36	10/10	31.05	2.05	129.6398	22
10	10/10	34.43				10/10	33.76			
1	07/10	35.67				10/10	32.90			
LOQ (RSDr= 25%)	220 CN					78 CN				

quantifiable percentage of *nptII* in the sample is 0.16 % (78/50000*100). However, this value may be reduced by analyzing larger quantities of genomic DNA.

In order to evaluate precision and trueness of the method, samples with a known *nptII* CN were evaluated. These samples correspond to two transgenic lines, Ga2_8 and Ga2_21, transformed with the apple scab *HcrVf2* resistance gene which, according to Southern Blot analysis, resulted to have a single copy T-DNA integration in the diploid genome (Belfanti et al. 2004). In addition, *ad-hoc* samples (AHS_1 and AHS_2) have been prepared mixing the DNA extracted from a 'Gala' WT plant and a transgenic line (T_1 or T_2) in 1:1 ratio. The precision is evaluated with the relative repeatability standard deviation or coefficient of variation (RSDr) of test results under repeatability conditions and should be ≤ 25 % over the whole dynamic range. Trueness is defined as the closeness of agreement between the average value obtained from a series of test results and an accepted reference value and according to the ENGL document (2015) should be within ± 25 % of the accepted reference value. As shown in Fig. 2, the RSDr were < 25 % proving that the method can be considered precise. Regarding trueness, the average CN values obtained from a series of repeated measures were within ± 25 % of the accepted reference value for all the analyzed samples proving that the method can be considered accurate. We decided to focus on lines with single copy T-DNA integration since these minimally alter the plant genome and generally do not induce gene silencing effects in the host (De Bunk et al. 2004). Besides, due to the relevance of the occurrence of chimerism in apple transformation, evaluation of method trueness at CN levels lower than 1 was one of our purpose. Low percentages of the target gene, or in other words, chimeric tissues made up of mixtures of cells whether or not containing the target gene may be frequent in genetically modified plants (Dalla Costa et al. 2014), and even more in apple plants which are regenerated via organogenesis from leaf disks (Flachowsky et al. 2008). Therefore, an analytical tool for its evaluation is necessary in order to properly select the most interesting lines and discard the chimeric material.



	n	CN	SD	RSDr (%)	Accepted	Trueness (%)
Ga2_8	4	0.83	0.098	11.75	1.00	-16.69
Ga2_21	4	0.85	0.049	5.80	1.00	-15.11
T_1	2	0.95	0.078	8.23		
AHS_1	2	0.43	0.003	0.59	0.47[a]	-8.59
T_2	4	0.34	0.037	10.72		
AHS_2	4	0.13	0.003	2.56	0.17[a]	-22.41

Fig. 2 Evaluation of precision and trueness of the method on transgenic lines (Ga2_8, Ga2_21, T_1, T_2) and *ad-hoc* samples (AHS_1 and AHS_2); *nptII* copy number value (CN) is the mean value of repeated measures (n) obtained in different PCR sessions; SD standard deviation; RSDr (%) relative repeatability standard deviation; [a] T_1 and T_2 have not an accepted reference value; [b] the accepted reference values for AHS_1 and AHS_2 are expected to be half of CN calculated for T_1 and T_2 respectively; Trueness (%) is calculated with the following formula (accepted value – CN value)/accepted value x 100.

Method applications

The real-time PCR analysis has been performed to analyse *nptII* CN in cisgenic or transgenic apple plantlets carrying the following apple scab resistance genes: *HcrVf2* (*Rvi6*) isolated by Vinatzer et al. (2001), *Vr2C* (*Rvi15*) isolated by Galli et al. (2010) and *V25* (*Rvi18*) (Schouten, unpublished). Vf2 and Vr2C lines proved the success of a cisgenic approach for the *nptII* removal even if a tiny percentage of *nptII* has yet been detected for line Vrc2C_1, very close to the LOQ value. Vr2C_2 showed about 2 *nptII* copies indicating that the cassette

Table 4 Analysis of *in-vitro* apple lines transformed with a binary vector (Bv) designed for a cisgenic (C) or a transgenic (T) approach.For each line, mean *nptII* copy number (CN), relative repeatability standard deviation (RSDr (%) and confidence intervals (CI at 95%) were evaluated on the DNA extracted from 2 or 3 independent leaf samples (n).

Plants ID	Bv	n	CN	RSDr (%)	CI at 95%
Vf2_1	C	2	0.02	26.53	
Vr2C_1	C	2	0.16	107.67	
Vr2C_2	C	2	1.77	30.05	1.03 - 2.50
(Vr2C+Vf2)_1	C	3	0.94	8.20	0.86 - 1.03
V25_1	T	2	5.57	10.66	4.75 - 6.39
V25_2	T	2	3.77	8.34	3.33 - 4.20
V25_3	T	2	1.22	13.11	1.00 - 1.45

containing the selection marker has not been removed. The double transformants (Vr2C+Vf2)_1 and V25_3 line showed one copy of *nptII* while V25_1 and V25_2 presented multiple copies (Table 4).

Besides, this method has been used to evaluate the possibility to remove an entire transformation cassette (T-DNA) from the genome of "edited" apple lines. The use of agrobacterium for transferring the CRISPR/*Cas9* machinery in fact entails the stable integration of a big T-DNA cassette in the genome, which is no more needed after the desired mutation has been achieved. Site-specific recombination mechanisms may be an effective way to obtain plants with the trait of interest but "free" of foreign DNA. In the case here presented, the T-DNA was 10 Kb in length, had FRT sites at both ends and contained the *Flp* recombinase gene under the control an heat-shock promoter, the selectable marker gene *nptII* and the *Cas9*/single guide RNA elements. Its excision from the genome was expected to occur after an heat-shock induction. For both apple lines, G-6 and GD-7, after the first incubation at 42 °C no significant reduction of *nptII* CN was detected compared to the untreated plant (Table 5). On the contrary, after the second and the third stimulus a *nptII* decrease was observed even if not proportional to the number of inductions. The shoots regenerated by nodes of each induced biological replicate showed highly heterogeneous assets: those regenerated from replicate H of G-6 line and from

Table 5 Heat-shock induction for the removal of a 10 kb T-DNA cassette containing *nptII*.For each heat-shock treatments, HS1, HS2, HS3 (consisting of incubations at 42 °C for 6 h repeated for 1, 2 or 3 times respectively), the mean *nptII* copy number (CN) of 3 or 4 biological replicates ± standard deviation was evaluated. For each shoot regenerated (SR) from treated nodes, the *nptII* CN was reported as well as a mean value ± standard deviation associated with the treatment group (SR mean). A percentage of removal was calculated using the following formula (SR mean/starting CN x 100).

		CN					
Plant line	**Replicates**	**HS 1**	**HS 2**	**HS 3**	**SR**	**SR mean**	**Removal (%)**
G-6 Starting CN= 1.37±0.33	A	1.76±0.55			0.86	0.75±0.16	45
	B				0.83		
	C				0.56		
	D		1.18±0.19		--	0.55±0.18	60
	E				0.68		
	F				0.42		
	G			1.07±0.04	0.77	0.43±0.36	68
	H				0.00		
	I				0.68		
	L				0.28		
GD-7 Starting CN= 2.35±0.48	A	2.51±0.54			0.00	0.65±0.96	72
	B				1.75		
	C				0.19		
	D		1.67±0.13		0.43	0.67±0.21	72
	E				0.75		
	F				0.82		
	G			1.84±0.30	0.01	1.15±0.90	51
	H				1.98		
	I				0.85		
	L				1.75		

replicates A and G of GD-7 line proved a complete removal of *nptII* while the others showed a wide range of values. These indications may be very useful in an initial screening phase to discriminate those replicates which have lost the cassette. Afterward, the complete removal of the T-DNA needs to be confirmed by sequencing plant genomic DNA.

CONCLUSIONS

We provided an analytical tool to precisely and accurately quantify *nptII* CN in transgenic apple lines and to verify its removal in cisgenic apple lines. Moreover, this tool may also be used to explore the feasibility of a site-specific

elimination of the transformation cassette, leading the way for a "clean" genome editing approach. Our method relies on a quantitative Taqman real-time PCR and complies with the criteria established by ENGL. *MdTOPO6* proved to be a valid endogenous reference gene for apple assuring the same quantification performances for several cultivars and wild related species. Besides, one of the strength of the method is the reliability, cost-effectiveness and versatility of the calibrator plasmid which may be easily enriched with new gene sequences (e.g. hygromycin resistance gene -hpt-, the other marker gene commonly used in apple transformation, or specific resistance/quality traits) becoming a 'universal' calibrator.

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CONFLICT OF INTEREST

Lorenza Dalla Costa declares that she has no conflict of interest. Matteo Bozzoli declares that he has no conflict of interest. Valerio Pompili declares that he has no conflict of interest. Stefano Piazza declares that he has no conflict of interest. Giovanni AL Broggini declares that he has no conflict of interest. Andrea Patocchi declares that he has no conflict of interest. Mickael Malnoy declares that he has no conflict of interest.

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CHAPTER 5

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

In conclusion, this research aims to provide a solution to fire blight disease in apple through the state of the art technologies.

In Chapter 2, we present the development of a CRISPR/Cas9-FLP/FRT-based gene editing system mediated by *A. tumefaciens* for the production of edited apple cultivars carrying reduced susceptibility to fire blight disease and a minimal trace of exogenous DNA. Gene editing *via* CRISPR/Cas9 has emerged as a powerful tool to precisely and quickly modify the desired trait into a plant genome. However, especially in apple, the potential of this editing system has not been widely explored. This work, which aims at knocking out an apple gene (*MdDIPM4*) predicted to be involved in plant susceptibility to fire blight, could represent a breakthrough application to combat this devastating disease. In fact, the inactivation of a susceptibility gene required for compatible plant-pathogen interaction could be considered as a revolutionary tool to breed fire blight-resistant apples, since it is more durable compared to resistance gene-based strategies. Moreover, in order to eliminate the exogenous genes introduced in the genome, the FLP/FRT site-specific recombination system was exploited. The T-DNA removal from edited plants with reduced susceptibility to the disease, also allows to protect them from any effect due to the presence of an exogenous endonuclease and, simultaneously, to add other traits of interest by subsequent gene transfer rounds using the same selection marker gene. This methodology could represent a promising alternative strategy to the classical breeding, especially for those plant species (such as apple) which require long maturation and crossing times. With this work, we feel to have set a milestone in the field of genetic engineering of apple plant, providing a pipeline for a 'clean' gene editing approach.

In Chapter 3, we characterize the novel apple miRNA MdmiR285N which is predicted to be crucial in the control of plant immunity by post-transcriptionally regulating several disease resistance transcripts. In recent years, microRNAs have been found among the most important gene regulators, thus emerging as the next generation targets for genetic engineering of crops. Especially in apple, miRNAs are however still poorly investigated. Our study, which provides basic

information regarding the physiological and pathogen-induced transcriptional regulation of *MdmiR285N*, represents an initial part of a long-term study that aims at identifying a promising miRNA for potential genetic improvement of apple. Within this context, given that MdmiR285N resulted to be involved in several biological processes of plant growth, reproduction and response to pathogens, the manipulation of its expression levels could represent an effective strategy for improving a wide range of traits in apple. However, further analyses are needed to better clarify the mechanisms of MdmiR285N regulation of its target transcripts, thus facilitating the design of a suitable strategy to engineer the desired trait with the minimum trade-offs for the plant.

In Chapter 4, we set up a quantitative method based on real-time PCR to assess *nptII* CN in modified apple lines. Genetic engineering in apple trees mostly relies on gene transfer mediated by *A. tumefaciens* and on the use of *nptII* as selection marker gene. In this framework, the quantification of *nptII* may have different and valuable applications: (i) estimation of the number of T-DNA ectopic integrations; (ii) identification of chimeric plants; (iii) determination of the efficiency of marker-gene removal (produced by an inducible site-specific recombination mechanism) in cisgenic plants; (iv) evaluation of the removal of an entire T-DNA cassette (via site-specific recombination) in genome edited plants. These last applications have a great impact nowadays being "the new breeding technologies (NBT)" (i.e. cisgenesis and genome editing) one of the most important tools for a sustainable agriculture in the future. Unlike classical gmo, cisgenic and genome edited plants should be free of exogenous sequences, initially necessary for the transformation or editing process but no more desired when the expected modifications have been produced. This method represents a reliable, rapid and cost-effective quantitative screening for the characterization of NBT products (Chapter 2) or more in general transgenic plants (Chapter 3), and may be also used by those who handle 'gmo' and 'new gmo' traceability.

This research produced apple lines more tolerant to fire blight disease (Chapter 2) and laid the basis for identifying a key gene for *E. amylovora* resistance

(Chapter 3) leading to an advancement of the knowledge and of the technological tools and applications to combat this important disease in apple. However, at present in Europe, organisms obtained by mutagenesis (including the new mutagenesis techniques such as genome editing) are GMO within the scope of the GMO European Directive 2001/18/EC (see Discussion in Chapter 2). A hundred of European research centres (University and public and private institutes) have signed and submitted a letter to the President of the European Commission on January 2019. In this document, scientists claim that regulating genome editing as GMOs will have negative consequences for agriculture, society and economy in Europe and advocate that the Directive may be amended with the hope that proper policy measures could be adopted to promote technologies for sustainable development in agriculture.

GENERAL APPENDICES

APPENDIX 1

Poster and oral presentations at: 9th International Rosaceae Genomics Conference (RGC), June 26-30 2018, Nanjing, China.
This work won the 'Third Place Award' during the poster competition.

Genome edited and T-DNA-free apple plants resistant to fire blight

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Abstract

Fire blight, caused by the bacterium *Erwinia amylovora* (*E.a.*), is one of the most devastating diseases affecting members of the Rosaceae family, in particular apple (*Malus x domestica*) whose worldwide production is extremely hit by this disease. For this reason, many efforts have been made to understand the molecular mechanisms underlying both plant host resistance and *E.a.* pathogenesis, especially those induced by effector proteins during bacterial-host interaction. In this regard, the DspA/E effector, codified by a disease specific gene localized in the pathogenicity island of *E.a.*, is absolutely required for the pathogenesis and interacts specifically with four Disease Interacting Proteins of *Malus* (DIPM1-4). This interaction suggests that *DIPM* genes may act as susceptible genes during the infection, therefore their silencing could lead to the reduction of plant susceptibility. In this study, conducted on two *Malus x domestica* susceptible varieties, 'Royal Gala' and 'Golden Delicious', a genome editing approach based on CRISPR/Cas9 via

Agrobacterium tumefaciens (*A.t.*) was applied to mutate and silence *DIPM4*. The binary vector used contained FRT sites next to the *A.t.* left and right borders and the *Flp* gene under the control of an inducible promoter in order to remove the T-DNA cassette in those lines selected for the desired mutation. About sixty putative edited lines were analyzed using an high throughput screening approach by Next Generation Sequencing in order to verify the CRISPR/Cas9-induced mutations. Seventy percent of the plants was completely mutated and showed different types of mutations, especially deletions producing premature stop codons. Some of these lines were treated and the removal of the T-DNA cassette was proved. The selected lines are under *ex vivo* investigation to test their resistance to *Erwinia amylovora.*

Key words: *Malus x domestica*, fire blight, DIPM, CRISPR/Cas9, NGS, marker-free plants

APPENDIX 2

Poster presentation at: LXII Italian Society of Agricultural Genetics (SIGA) Annual Congress '*Plant development and crop productivity for sustainable agriculture*', September 25-28 2018, Verona, Italy.
This work won the Special Award 'Domenico Mariotti'.

Efficient and 'clean' CRISPR/Cas9 editing for fire blight resistance in apple

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Abstract

Fire blight, caused by the bacterium *Erwinia amylovora* (*E.a.*), is one of the most economically important and invasive diseases affecting apple (*Malus x domestica*). Nowadays, the *E.a.*-apple interaction is widely described and the main molecular mechanisms underlying both plant host resistance and *E.a.* pathogenesis are elucidated. Nevertheless, the management of the disease remains still arduous in apple orchard. Indeed, the use of genetically modified plants is forbidden by the current GMO legislation and the application of antibiotic-based pesticides is prohibited due to their effects on human health and environment. Therefore, one of the hardest challenges for plant biotechnology is to provide apple varieties that are simultaneously disease-resistant and public-acceptable. In this study, conducted on two *Malus x domestica* susceptible varieties, 'Royal Gala' and 'Golden Delicious', a genome

editing approach based on CRISPR/Cas9 via *Agrobacterium tumefaciens* (*A.t.*) was applied to produce the knock-out of *MdDIPM4*. In apple, evidences show that MdDIPM4 protein interacts specifically with the DspE effector, mandatory for the pathogenesis of *Erwinia amylovora*. About sixty transgenic lines were analyzed using an high throughput screening approach on the Illumina MiSeq platform in order to verify the CRISPR/Cas9-induced mutations. The eighty percent of the plants showed mutations, especially translation-terminated deletions resulting in *MdDIPM4* knock-out. Moreover, with the aim of producing public-acceptale engineered apple plants, our strategy allowed to remove the entire exogenous T-DNA in those lines selected for the desired mutation. This mechanism relies on the heat schock-inducible expression of the *Flp* gene which induces a site-specific recombination at the two FRT sites flanking the *A.t.* left and right borders. Some genome edited lines were heat-treated and the removal of a 10.5 kb T-DNA cassette was proved. Currently, the selected genome edited and T-DNA-free apple plants are under *in vivo* investigation to test their resistance to *Erwinia amylovora*.

Key words: *Malus x domestica*, fire blight, DIPM, CRISPR/Cas9, T-DNA-free plants

APPENDIX 3

Poster presentation at: International Society - Molecular Plant Microbe Interaction (IS-MPMI) XVIII Congress, July 14-18 2019, Glasgow, Scotland.

The inactivation of *MdDIPM4* by CRISPR/Cas9 editing reduces the susceptibility of apple to fire blight disease

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Abstract

Fire blight, caused by the bacterium *Erwinia amylovora* (*E.a.*), is one of the most economically important and invasive diseases affecting apple (*Malus x domestica*). The molecular mechanisms of *E.a.*-apple interaction are widely elucidated but the management of the disease remains still arduous. Apple protein MdDIPM4 interacts with the DspA/E effector, mandatory for the pathogenesis of *Erwinia amylovora*, but its biological function is still unknown. In this work, the knock-out of MdDIPM4 has been produced in two *Malus x domestica* susceptible varieties, by using the CRISPR/Cas9 system delivered via *Agrobacterium tumefaciens*. Fifty-seven transgenic lines were analyzed using a Next Generation Sequencing to identify the CRISPR/Cas9-induced mutations. Some edited plants with a loss of function mutation were selected and inoculated with the pathogen. An increased resistance was observed, demonstrating that *MdDIPM4* is involved in plant susceptibility to fire blight. Moreover, with the aim of producing plants 'clean' from exogenous DNA, we

used a heat shock-inducible FLP/FRT recombination system designed specifically to remove the entire T-DNA in those plants with an increased pathogen resistance. Our data demonstrated the possibility to produce apple varieties more resistant to fire blight by using the CRISPR/Cas9 technology, and containing a minimal trace of exogenous DNA. These plants may be used in further analysis to elucidate how *MdDIPM4* is involved in the onset of the disease.

Key words: *Malus x domestica*, fire blight, susceptibility gene, gene editing, FLP/FRT

APPENDIX 4

Poster presentation at: International Society - Molecular Plant Microbe Interaction (IS-MPMI) XVIII Congress, July 14-18 2019, Glasgow, Scotland.

Characterization and functional analysis of the *Md-miR285N* gene promoter in transgenic apple (*Malus x domestica*) and *Arabidopsis thaliana*

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Abstract

MicroRNAs are key regulators of plant physiological activities by functioning in RNA silencing and post-transcriptional regulation of gene expression. In apple, the uncharacterized Md-miR285N is predicted to target 35 RNA transcripts, mostly coding for TMV, SNC1 and CDPK proteins involved in plant defense to pathogens. In this work, ca. 2 Kb of the *Md-miR285N* promoter were isolated and cloned into an expression vector with its 3'-end fused to the *ß-glucoronidase* (GUS) reporter gene. The expression vector was used for *Agrobacterium*-mediated transformation of both *Malus x domestica* (cv. 'Gala') and *Arabidopsis thaliana* (ecotype Col-0). In transgenic apple plantlets a strong GUS activity was detected in stems, leaves and veins. In *Arabidopsis* seedlings the promoter was shown to be highly expressed in roots, shoot apex meristem, primary and secondary leaves and veins. To understand the functional response of *Md-miR285N* against pathogen infections, apple and *Arabidopsis* plantlets

were inoculated, respectively, with *Erwinia amylovora* strain Ea273 and *Pseudomonas syringae* pv. tomato DC3000. The GUS activity was qualitatively and quantitatively evaluated in treated plants at 12, 24, 36 and 48 hours post inoculation. A differential regulation of promoter expression was detected compared to untreated plants. These data suggest that Md-miR285N may act as important regulator of plant defense after bacteria infections.

Key words: *Malus x domestica*, *Arabidopsis thaliana*, microRNA, *Md-miR285N*, plant defense

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