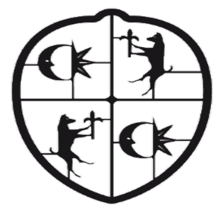
Transcriptional regulation of *MdmiR285N* microRNA in apple (*Malus x domestica*) and the heterologous plant system *Arabidopsis thaliana*



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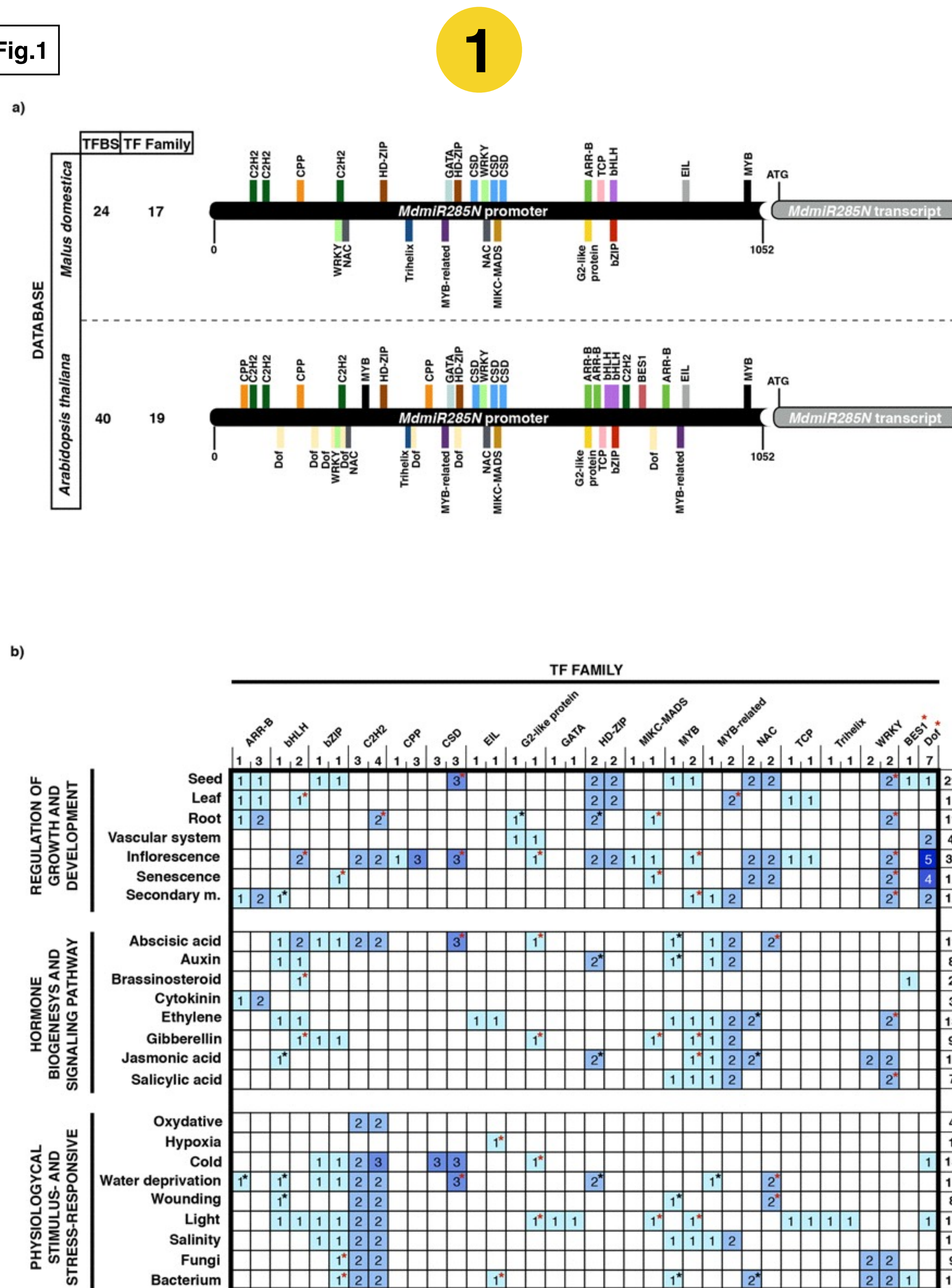
INTRODUCTION AND AIM OF THE WORK

Malus x domestica microRNA MdmiR285N is a potential key regulator of plant immunity, as it has been predicted to target 35 RNA transcripts coding for different disease resistance proteins (Toll Interleukin 1 Receptor-Nucleotide Binding Site-Leucine Rich Repeat, SUPPRESSOR of NPR-1 CONSTITUTIVE, Calcium-Dependent Protein Kinase) involved in plant defense to pathogens. In this study, as part of a long-term goal to identify a promising miRNA for potential genetic improvement of apple, 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 gene driven by the promoter of *MdmiR285N* was examined in transgenic apple, demonstrating that *MdmiR285N* was expressed during the vegetative growth phase. Similarly, in transgenic *Arabidopsis thaliana*, spatial and temporal patterns of *GUS* expression revealed that *MdmiR285N* was differentially regulated during seed germination, vegetative phase change, and reproductive development. 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 host-pathogen infections. Overall, these data suggest that MdmiR285N is involved in the biotic stress response, plant growth, and reproductive development.

EXPERIMENTAL PROCEDURES AND RESULTS

1

Fig.1



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. In both plant species examined (apple and *Arabidopsis thaliana*), a considerable series of putative TFBSs and corresponding TFs was identified regulating the *MdmiR285N* gene promoter (Fig. 1a) during different stages of plant growth and development, especially seed formation, vegetative (leaf and root) and reproductive (inflorescence) development, organs senescence and secondary metabolism (Fig. 1b). In addition, a putative functional profile was associated with phytohormones biogenesis and signaling pathways, particularly to those of abscisic acid, ethylene and jasmonic acid (Fig. 1b). Finally, potential responses to multiple physiological stimulus and stress conditions, especially light intensity, water availability, temperature conditions and bacterial infections, were also predicted (Fig. 1b).

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 *Arabidopsis thaliana*, thus suggesting also that the regulation of genes involved in defense responses may be similar between the two plant species examined.

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Fig.2

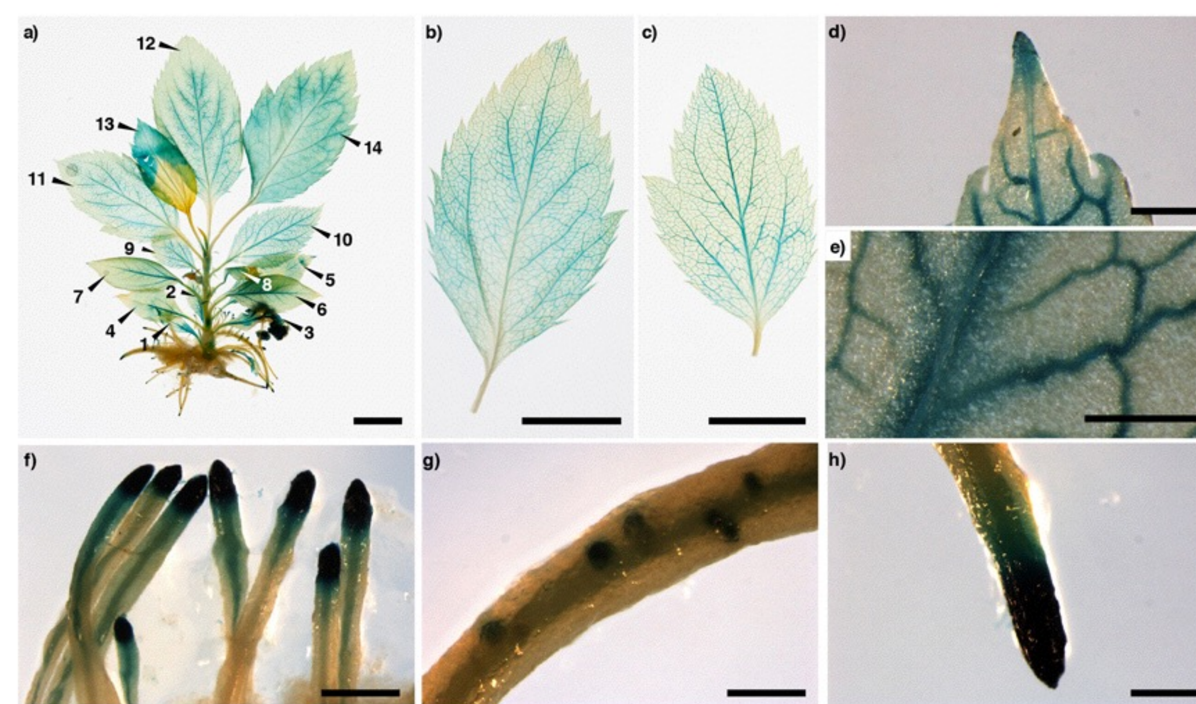


Fig.3

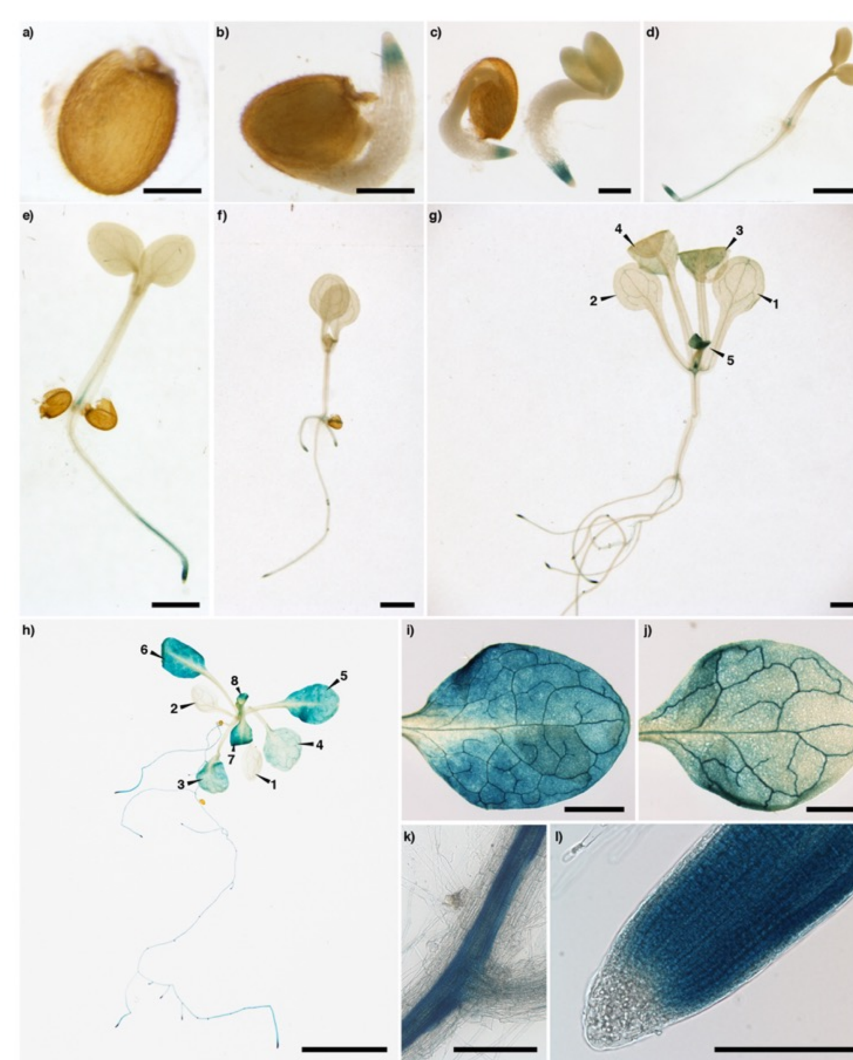
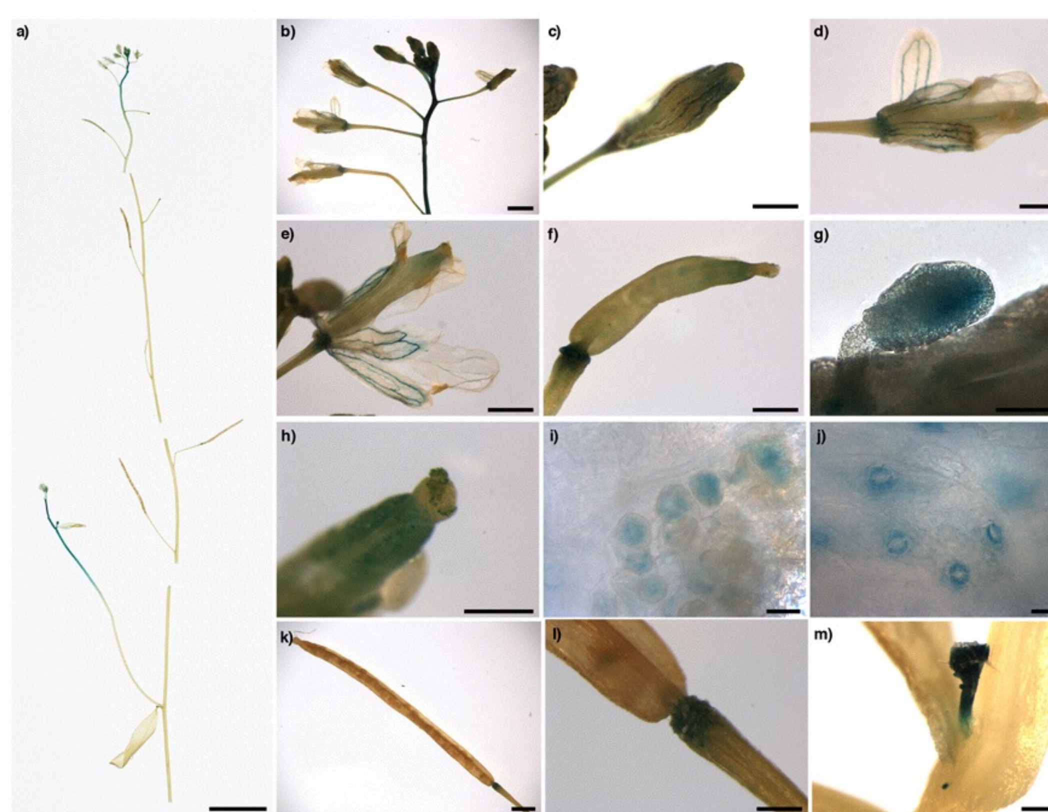


Fig.4



According to the *in silico* analysis, in apple the histochemical GUS staining revealed that *MdmiR285N* is expressed during the plant vegetative development (Fig. 2). GUS expression was detected in the shoot apical meristem, in the stem and in the leaf vascular tissue (Fig. 2a-e). In roots, strong GUS expression was detected in the tip of primary and secondary roots and in the meristems of emerging lateral roots, in the root vascular system, and in the root elongation zone up to the root maturation region (Fig. 2f-h).

In *Arabidopsis 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 (Fig. 3a). In the later stages of seed germination, GUS staining was evident in the root apical meristem (RAM), and elongation and maturation zones of emerging, fully germinated and elongating seedlings (Fig. 3b-g). In the later phases of vegetative growth (Fig. 3g,h), in multiple organs of the seedling a gradual increase of GUS signal was observed. Roots (Fig. 3g,h,k,l) were strongly stained according to the pattern previously described, however strong GUS staining was also visible in the SAM (Fig. 3g,h), in the parenchyma cells of leaves (Fig. 3g-j), and in the leaf vascular tissue (Fig. 3g-j).

In *Arabidopsis thaliana*, *MdmiR285N* expression was regulated also during the reproductive development (Fig. 4). Strong GUS expression was observed in the stalk and flowers (Fig. 4a,e) in the silique (Fig. 4f,k,l), the ovule (Fig. 4g), the stigma apex (Fig. 4h), the pollen grains on stigma (Fig. 4i), and the guard cells of stigma cover (Fig. 4j). Finally, strong GUS signal was also observed in axillary buds (Fig. 4m).

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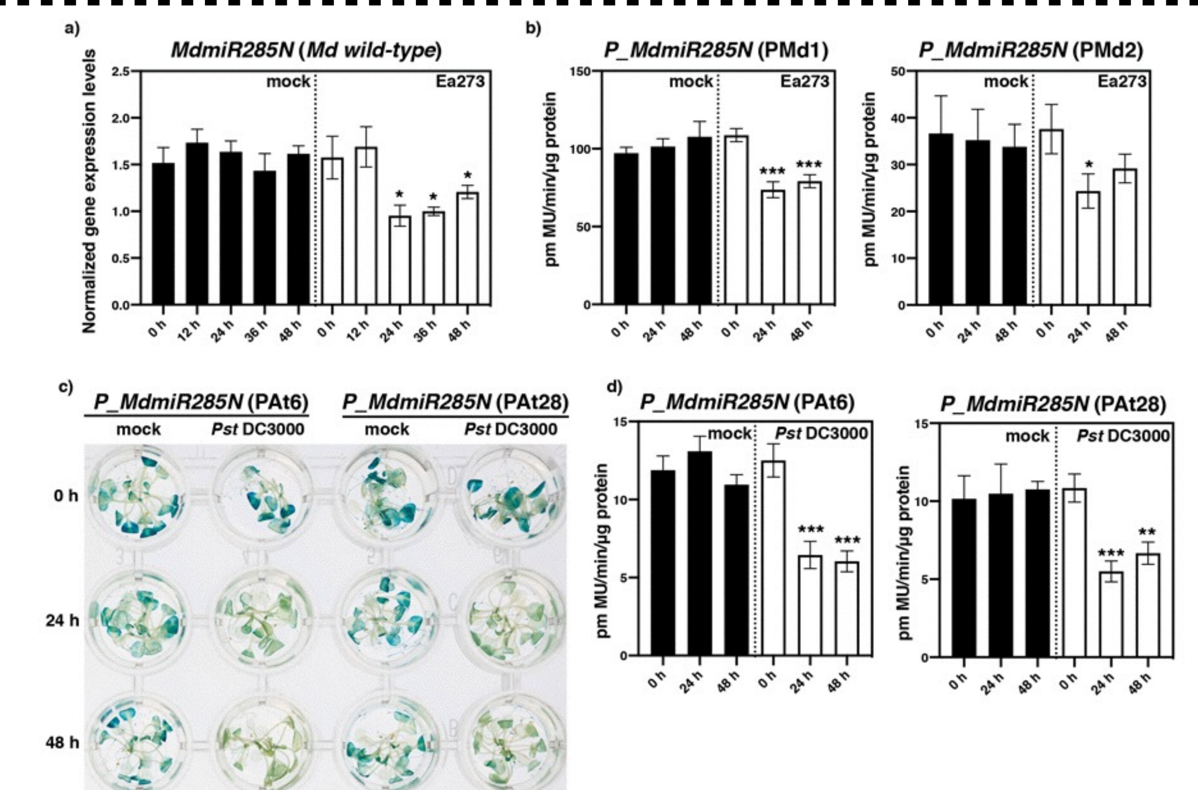
Fig.5

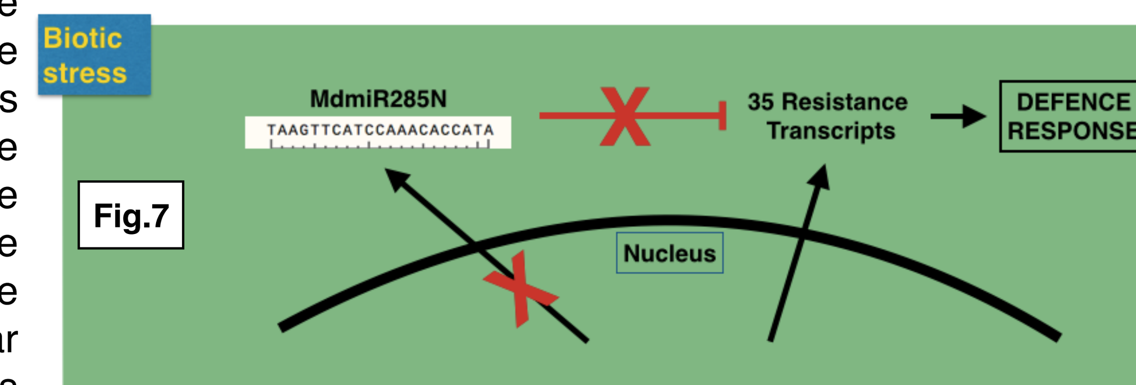


The investigation of putative changes in the expression profile of *MdmiR285N* in response to environmental stimuli, such as bacterial infections, may provide insights into the biological roles of this novel and uncharacterized apple miRNA.

In apple, when soil-acclimated *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 (Fig. 5a). Differently, if plants experienced the bacterium (*Erwinia amylovora*), the abundance of mature *MdmiR285N* transcripts decreased significantly and specifically 24, 36 and 48 hours after the application of the stress (Fig. 5a). Consistent results were obtained when the stimulatory effect of *Erwinia amylovora* on *MdmiR285N* expression was investigated in transgenic apples (Fig. 5b). A decrease of GUS activity was confirmed 24 and 48 hours after infection. In *Arabidopsis thaliana*, a similar pattern of expression of the *MdmiR285N* promoter was observed in the transgenic lines throughout *Pseudomonas syringae* pv. *Tomato* DC3000 infection (Fig. 5c,d).

MAIN CONCLUSIONS

Overall, *MdmiR285N* appeared down-regulated in both plant species examined thus suggesting an increase of its targeted disease resistance transcripts during pathogen infection. A fine regulation of disease resistance proteins is mandatory for a correct plant growth and development. Disease resistance proteins were indeed shown to have a cost to plants because if unregulated they can trigger autoimmunity in the absence of pathogen infection and inhibit plant growth. 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 (Fig. 6), and to ensure rapid induction of disease resistance proteins during pathogen invasion (Fig. 7). 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 (Fig. 6,7).



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