

The mannose-binding lectin gene *FaMBL1* is involved in the resistance of unripe strawberry fruits to *Colletotrichum acutatum*

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SUMMARY

The fungal pathogen *Colletotrichum acutatum* is the causal agent of strawberry (*Fragaria × ananassa*) anthracnose. Although the fungus can infect strawberry fruits at both unripe and ripe stages, the symptoms appear only on red ripe fruits. On white unripe fruits, the pathogen becomes quiescent as melanized appressoria after 24 h of interaction. Previous transcriptome analysis has indicated that a mannose-binding lectin (MBL) gene is the most up-regulated gene in 24-h-infected white strawberries, suggesting a role for this gene in the low susceptibility of unripe stages. A time course analysis of the expression of this MBL gene, named *FaMBL1* (*Fragaria × ananassa* MBL 1a), was undertaken to monitor its expression profile in white and red fruits at early interaction times: *FaMBL1* was expressed exclusively in white fruit after 24 h, when the pathogen was quiescent. *Agrobacterium*-mediated transient transformation was used to silence and overexpress the *FaMBL1* gene in 24-h-infected white and red strawberries, respectively. *FaMBL1*-silenced unripe fruits showed an increase in susceptibility to *C. acutatum*. These 24-h-infected tissues contained subcuticular hyphae, indicating pathogen penetration and active growth. In contrast, overexpression of *FaMBL1* in ripe fruits decreased susceptibility; here, 24-h-infected tissues showed a high percentage of ungerminated appressoria, suggesting that the growth of the pathogen had slowed. These data suggest that *FaMBL1* plays a crucial role in the resistance of unripe strawberry fruits to *C. acutatum*.

Keywords: *Colletotrichum acutatum*, fungal quiescence, mannose binding lectin, strawberry ripening, unripe fruit resistance.

INTRODUCTION

Fruits undergo dramatic metabolic and structural changes during ripening, which often lead to a totally different susceptibility of unripe and ripe fruit stages to economically important diseases. Generally, unripe fruits are less susceptible to fungal pathogen

infections because of the metabolic or mechanical composition of the external cell layers. For this reason, although interacting with unripe fruits, several fungal pathogens become quiescent, arresting their growth until fruits ripen and become susceptible to pathogen colonization. Here, fungi restore their growth, deeply invading the tissues and causing disease symptoms (Prusky, 1996).

Colletotrichum acutatum Simmonds is the primary causal agent of anthracnose disease of strawberry (*Fragaria × ananassa*). It infects most parts of the plant; however, as for the majority of *Colletotrichum* species, the highest losses occur on fruits (Bailey and Jeger, 1992). This pathogen attacks fruits at both immature and mature stages; however, the disease symptoms develop only on red ripe fruits, as, on white fruits, *C. acutatum* becomes quiescent as melanized appressoria after 24 h of interaction (Guidarelli *et al.*, 2011). Only when fruit ripens to the red stage does the pathogen restore its growth, and symptoms become manifest within 3 days.

In an attempt to elucidate the molecular basis of the different susceptibility to *C. acutatum*, we recently used transcriptome microarray analysis to highlight the differences in gene expression of white unripe and red ripe strawberry fruits after 24 h of interaction with this fungus. Among several genes found to be differentially regulated, a *curculin-like* (mannose-binding) lectin family protein gene was the most up-regulated gene only in white unripe inoculated fruits (Guidarelli *et al.*, 2011). This gene encodes for a B-lectin protein of the GNA (*Galanthus nivalis* agglutinin)-related mannose-binding family.

First identified as plant proteins that agglutinate human red blood cells (Van Damme *et al.*, 1998), lectins are ubiquitous proteins in the plant kingdom, capable of recognition and reversible non-catalytic binding to specific carbohydrates (Peumans and Van Damme, 1995). Although the biological role of plant lectins is still debated, their ability to bind to carbohydrates indicated a role for lectins as plant defence proteins, recognizing and binding glycoconjugates on the surfaces of microorganisms, such as bacteria and fungi (Chrispeels and Raikhel, 1991; De Hoff *et al.*, 2009; Peumans and Van Damme, 1995). Indeed, chitin-binding lectins from both wheatgerm (*Triticum* spp.) and stinging nettle (*Urtica dioica*) have been shown to display inhibitory action against different fungal pathogens on binding to fungal chitin (Broekaert *et al.*, 1989; Mirelman *et al.*, 1975). Similarly, the soybean (*Glycine*

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max) lectin β -glucan-binding protein recognizes and binds to the β -glucans, a pathogen-associated molecular pattern (PAMP) molecule present on the surface of the oomycete *Phytophthora sojae* (Fliegmann *et al.*, 2004; Mithöfer *et al.*, 2000). In addition, several lectins have been shown to accumulate in plant cells under different biotic and abiotic stresses or displaying antibacterial, antifungal or anti-insect activity, reinforcing the hypothesis of a role for these proteins in plant defence (Chrispeels and Raikhel, 1991; Peumans and Van Damme, 1995; Van Damme *et al.*, 2004).

Recently, lectins have been classified into 12 families on the basis of their domain structures and phylogenetic analysis (Jiang *et al.*, 2010). Among these, members of the mannose-binding B-lectin family, previously known as GNA-related lectins, or 'monocot mannose-binding lectins' (Van Damme *et al.*, 1995, 2008), have attracted particular attention in plant defence studies, because of their specific ability to interact with oligomannosides and mannose-type glycans, present on the surface of microbial pathogens (Barre *et al.*, 2002; Van Damme *et al.*, 1998, 2007). Mannose-binding lectins (MBLs) contain one or two GNA domains, originally characterized from the MBL of snowdrop (*Galanthus nivalis*) bulbs (Van Damme *et al.*, 1987); each of these contains three similar mannose-binding sites per subunit (Van Damme *et al.*, 2008).

The role of MBLs in plant defence has been confirmed for a number of plant species: the MBL CaMBL1 was isolated from *Capsicum annuum* leaves infected with *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*). Its GNA domain is capable of specific binding to high-mannose *N*-glycans, present on the *Xcv* surface. CaMBL1 localizes to plasma membranes and is necessary for the activation of defence-related genes, for salicylic acid accumulation and for cell death induction in infected leaves. The overexpression of the *CaMBL1* gene in *Arabidopsis* confers enhanced resistance to bacterial and fungal pathogens (Hwang and Hwang, 2011). Furthermore, a number of MBLs have been shown to display direct antifungal activity. For example, the monomeric mannose/glucose-binding lectin, isolated from the seed of red cluster pepper, *Capsicum frutescens*, is capable of inhibiting conidia germination and hyphal growth of the two fungi *Aspergillus flavus* and *Fusarium moniliforme* (Ngai and Ng, 2007); the mannose/glucose-binding lectin Dgui, extracted from the leguminous plant *Dioclea guianensis*, inhibits the conidia germination of *C. gloeosporioides*, suggesting that this protein recognizes a specific target on the surface of this fungus (Araujo-Filho *et al.*, 2010); similarly, the orchid mannose-binding proteins, gastrodianins, are active against the fungal pathogen *Valsa ambiens* (Wang *et al.*, 2001), and also against *Botrytis cinerea*, *Gibberella zeae*, *Ganoderma lucidum*, *Rhizoctonia solani* and *Phytophthora nicotianae* (Cox *et al.*, 2006; Xu *et al.*, 1998); finally, the mycelial growth of *Alternaria alternata* is inhibited by an MBL from the orchid *Dendrobium findleyanum* (Sudmoon *et al.*, 2008).

Despite the large number of reports on the defence role of plant MBLs, their mode of action and molecular mechanisms are still

poorly understood. In addition, there is no evidence of the possible role of these proteins in determining the low susceptibility of unripe fruits to microbial pathogens (Guidarelli *et al.*, 2011).

The previously reported data on the up-regulation of a gene encoding for an MBL only in white strawberries interacting with quiescent *C. acutatum* suggests that the encoded lectin could possibly have a role in the resistance of unripe strawberries to anthracnose.

Here, to investigate this hypothesis, we first performed a time course analysis of the expression of this lectin gene in both white and red fruits, to score possible differences in the regulation of its transcription, before 24 h of interaction with the fungus. Subsequently, *Agrobacterium tumefaciens*-mediated transient transformation was used to silence the expression of the lectin gene in white 24-h-infected fruits and, in parallel, to overexpress it in red ripe fruits, similarly infected. The observation of anthracnose symptoms of transfected white and red strawberries inoculated with *C. acutatum*, and histological analysis of the infected tissues, indicated that this gene strongly influences the susceptibility of ripening strawberry fruits, possibly playing an important role in the different response of these fruits to the pathogen.

RESULTS

FaMBL1 gene encodes for a B-lectin protein

In our previous study, we found that the *mannose-binding lectin* (MBL) gene (TA10594_57918) was the most up-regulated gene after 24 h of *C. acutatum* infection only in white unripe strawberry fruits, where the pathogen becomes quiescent (Guidarelli *et al.*, 2011). No difference in the expression of this gene was found in inoculated red fruits. The full-length cDNA of this gene, named *FaMBL1* (GenBank accession number: KF962716), was isolated from a 24-h-infected white fruit cDNA library (*Fragaria × ananassa* MBL): it is 1329 bp long and encodes for a protein of 442 amino acids.

The *FaMBL1* cDNA sequence was analysed using the BLASTX program (<http://blast.ncbi.nlm.nih.gov>); the encoded protein contains an N-terminus signal peptide (SP) necessary for protein secretion into the extracellular environment, a B-lectin domain containing the three-fold internal repeat QXDXNXVXY consensus sequence motif, involved in α -D-mannose recognition, and a C-terminal PAN-Apple domain, widespread in plant lectins (Van Damme *et al.*, 2008), mediating protein–protein and protein–carbohydrate interactions (Fig. 1). These features allow us to ascribe *FaMBL1* to the plant B-lectin family (Jiang *et al.*, 2010).

FaMBL1 shares 54% sequence identity with the *Arabidopsis thaliana* curculin-like (mannose-binding) lectin family protein (accession number NP_178007), 57% with the carrot (*Daucus carota*) cell attachment protein (BAD24818), 63% with the sugar beet (*Beta vulgaris*) SIEP1L protein (CAA61158), 67% with a

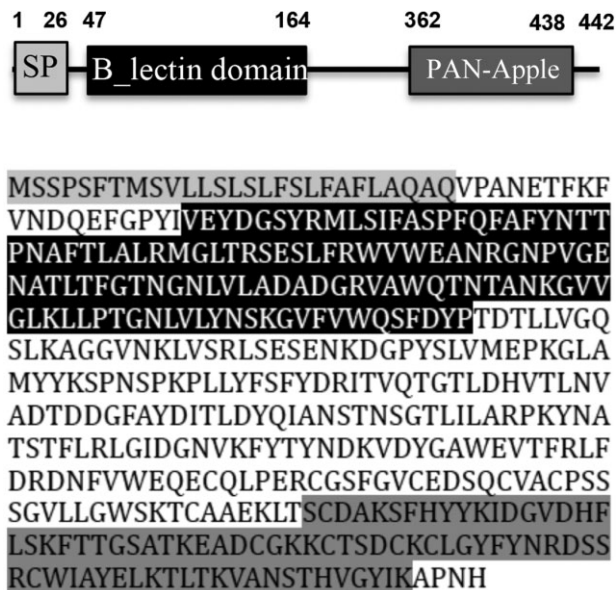


Fig. 1 FaMBL1 primary structure. Amino acid sequence of FaMBL1 and BLASTX domain prediction analysis. The signal peptide, B-lectin domain and PAN-Apple domain are highlighted.

Theobroma cacao D-mannose-binding lectin protein (EOY13258) and 100% with the woodland strawberry (*Fragaria vesca*) epidermis-specific secreted glycoprotein (XP_004294119). However, the recently characterized pepper (*Capsicum annuum*) CaMBL1 (ADG04234) shares similarity with FaMBL1, but is a shorter protein, lacking 144 amino acids at its C-terminus (Fig. S1, see Supporting Information).

Time course of *FaMBL1* gene expression in strawberry fruits infected with *C. acutatum*

In our previous study, we showed that, depending on the fruit ripening stage, at 24 h post-inoculation (hpi), *C. acutatum* infecting strawberries changes its colonization strategy: on white unripe fruits, it arrests its growth and becomes quiescent as melanized appressoria, whereas, in red ripe fruits, it is actively growing as subcuticular intercellular hyphae. In order to establish whether the up-regulation of the *FaMBL1* gene, unique to white strawberries at 24 hpi, was a specific response of unripe fruits interacting with a quiescent pathogen, or whether it also occurred in red ripe strawberries at earlier times of infection, a time course analysis of *FaMBL1* expression was performed. In particular, the *FaMBL1* transcript level was quantified by quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) in white and red fruits at earlier stages of the interaction, namely at 8 h (when conidia are still ungerminated on the fruit surface), at 16 h (when germinative tubules are produced from most conidia), at 20 h (when appressoria become apparent) and at 24 h (when *C. acutatum* proceeds with its infection only in red ripe fruits with

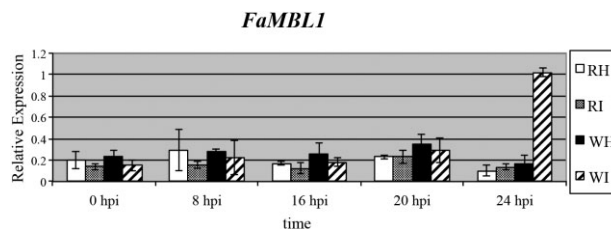


Fig. 2 Time course of gene expression analysis of *FaMBL1* in white and red strawberry fruits on *Colletotrichum acutatum* inoculation. The expression level of *FaMBL1* was quantified in white and red fruits inoculated with *C. acutatum* (WI and RI, respectively) or mock inoculated (WH and RH), and scored at 0, 8, 16, 20 and 24 h post-inoculation (hpi). Data were normalized to the transcript level of the housekeeping *elongation factor 1 α* gene. Values are the means of three biological replicates.

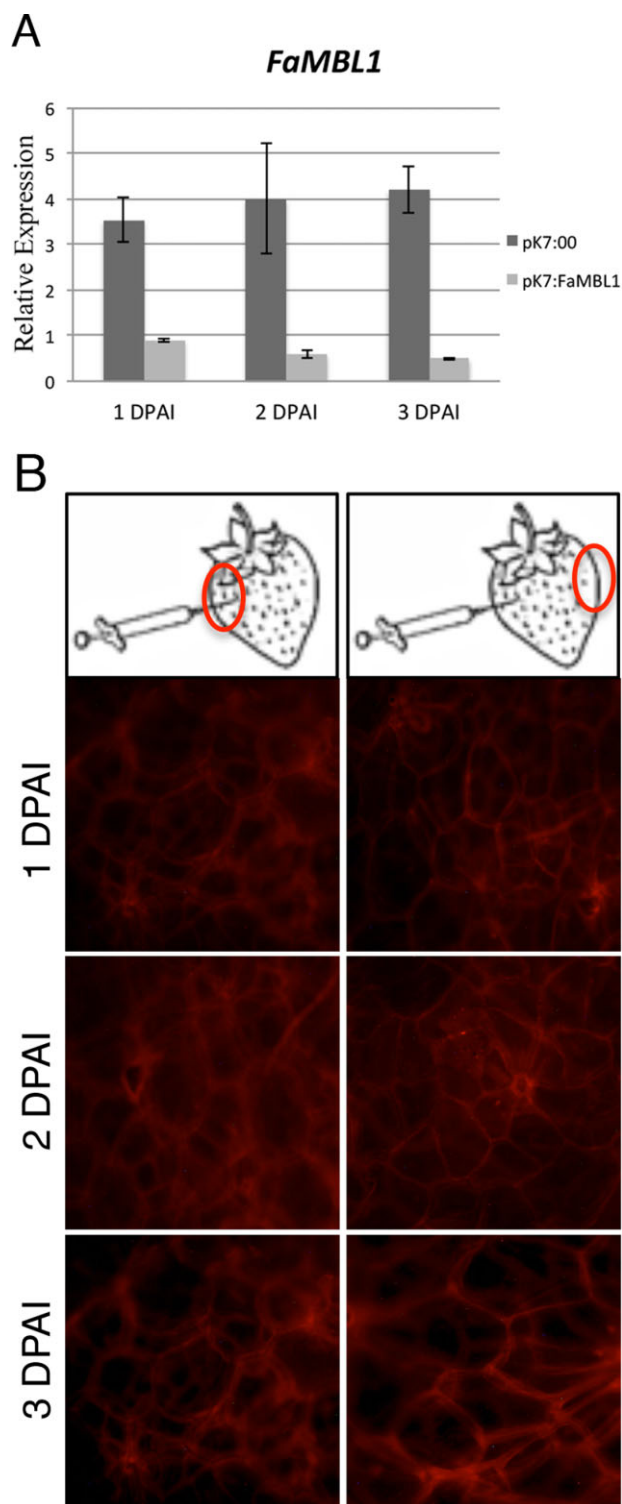
subcuticular hyphae, whereas, in white fruits, it becomes quiescent as melanized appressoria) (Guidarelli *et al.*, 2011).

By comparing inoculated fruits with mock-inoculated ones, the expression of the *FaMBL1a* gene varied significantly exclusively in white strawberry fruits at 24 hpi: here, in accordance with our previous microarray data, the *FaMBL1a* transcript level increased by about five-fold with respect to mock-inoculated fruits. The expression level of *FaMBL1* did not vary on infection in white fruits at earlier time points (0, 8, 16 and 20 hpi) (Fig. 2). In red inoculated fruits, the expression level of *FaMBL1a* did not undergo any variation with respect to mock-inoculated fruits at any of the examined time points. This indicates that the increase in the expression level of this gene is indeed specific to white unripe strawberry fruits interacting with quiescent melanized appressoria of *C. acutatum*, and could suggest that *FaMBL1* has a specific role for the resistance of white unripe strawberries to this pathogen.

FaMBL1 silencing and T-DNA integration are effectively accomplished in white and red strawberry fruits 3 days after injection with *A. tumefaciens*

To investigate the possible role of *FaMBL1* in the difference in susceptibility to *C. acutatum* of unripe and ripe strawberry fruits, we used *Agrobacterium* transient transformation to silence the increase in expression of this gene typical of white fruits after 24 hpi with *C. acutatum* and, in parallel, to induce its overexpression in red infected fruits.

To set up the best timing of *Agrobacterium*-mediated gene silencing, white strawberries were transfected for 1, 2 or 3 days with *A. tumefaciens* carrying *FaMBL1* silencing vectors, harvested and inoculated with *C. acutatum*. The *FaMBL1* expression level was scored by qRT-PCR at 24 hpi. The silencing vector (pk7:*FaMBL1*) consisted of an intron-containing hairpin (ihp) construct generated by inserting a partial fragment of the *FaMBL1* gene in the sense and antisense orientation interrupted by an intron, which determined the formation of self-complementary hairpin RNA (ihp-RNA), leading to an RNAi-induced degradation



of the specific gene target mRNAs. As a negative control, to check that agroinfiltration did not interfere with *FaMBL1* gene expression independent of silencing, white strawberries were transformed with *Agrobacterium* carrying the empty vector (pk7:00) for

Fig. 3 Timing of T-DNA integration in strawberry fruits. (A) Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis of *FaMBL1* expression in silenced white strawberry fruits infiltrated with *Agrobacterium* carrying the construct pk7:*FaMBL1* (pk7:*FaMBL1*) or the empty vector control (pk7:00). *FaMBL1* expression was scored from 1 to 3 days post-agroinjection (DPAI). Data were normalized to the expression level of the housekeeping *elongation factor 1 α* gene. Values are the means of three biological replicates. (B) Fluorescence microscope analysis of strawberry tissues at 1–3 DPAI carrying the plasmid pk7GWIWG2 II-RedRoot. Transfected strawberries were observed both proximal (left) and distal (right) to the *Agrobacterium* infiltration point.

1, 2 or 3 days, harvested and assayed for *FaMBL1* gene expression after 24 h of *C. acutatum* infection. The increase in gene expression, normally induced by the 24-h *C. acutatum* inoculation in wild-type strawberries, was drastically decreased in strawberry transformed with pk7:*FaMBL1* even after 1 day of agroinfiltration. Silencing was still effective after 3 days (Fig. 3A). In the white control strawberries, transfected with empty vector and infected with *C. acutatum*, *FaMBL1* gene expression increased, indicating that 1, 2 or 3 days of agroinfiltration did not alter significantly the *FaMBL1* transcriptional response.

Similarly, the T-DNA integration and overexpression timing in strawberry fruit on agroinfiltration was scored by transfecting fruits with the plasmid pk7GWIWG2 II-RedRoot, allowing the overexpression of the DsRED fluorescent protein. RedRoot fluorescence was monitored after 1, 2 and 3 days of agroinfiltration. Fluorescence became apparent even after 1 day of agroinfection and remained clearly visible up to 3 days after infiltration (Fig. 3B), indicating that the overexpression of the transfected gene is maintained very efficiently during this time. Furthermore, tissues both close and far from the injection point became fluorescent, indicating that the transfection occurred in the whole fruit (Fig. 3B).

Taking into account these data, 3 days was chosen as the agroinfiltration time for the subsequent experiments.

Silencing of *FaMBL1* in white unripe strawberries affects fruit resistance to *C. acutatum*

The induction of *FaMBL1* gene expression at 24 hpi of *C. acutatum* was evaluated by qRT-PCR in pk7:*FaMBL1* [3 days post-agroinfiltration (DPAI) with the *FaMBL1* silencing vector], pk7:00 (3 DPAI with empty silencing vector, as control) and in wild-type white strawberries. After 24 h of infection, *FaMBL1* expression was strongly induced by *C. acutatum* in both wild-type and control infiltrated strawberries (pk7:00), where its transcript level increased by about 10- and eight-fold, respectively. In contrast, in the pk7:*FaMBL1* transfected strawberries, the induction of *FaMBL1* gene expression was almost abolished (Fig. 4A).

With respect to disease susceptibility, 5 days after *C. acutatum* inoculation, the wild-type and pk7:00 control agroinfiltrated

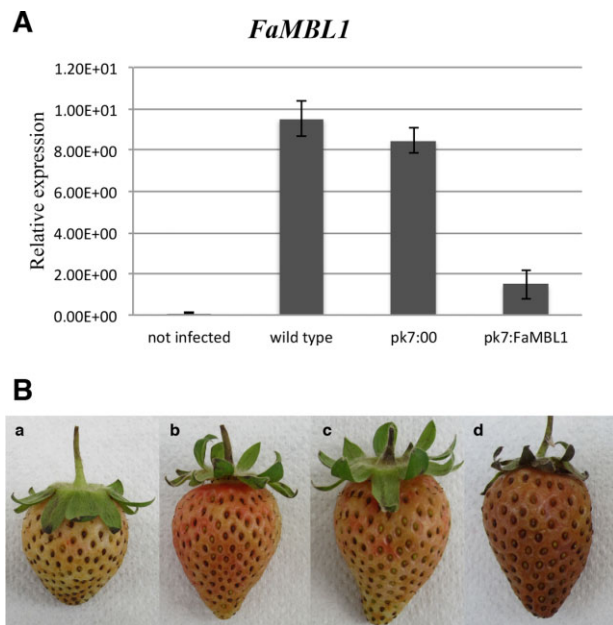


Fig. 4 Infection of *Colletotrichum acutatum* in *FaMBL1*-silenced white strawberry fruits. (A) Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis of *FaMBL1* expression in white wild-type or transfected strawberries 24 h after inoculation with *C. acutatum*. The transcript levels of mock-inoculated wild-type fruits (not infected) and 24-h *C. acutatum*-inoculated fruits (wild-type) were compared with those of mock-silenced fruits (pk7:00) and *FaMBL1*-silenced fruits (pk7:FaMBL1), both 24 h after inoculation with *C. acutatum*. Data were normalized to the expression level of the housekeeping *elongation factor 1 α* gene. Values are the means of three biological replicates. (B) Disease symptom analysis in mock-infected white fruits (not infected) (a), wild-type white fruits (b), mock-silenced white fruits (pk7:00) (c) and *FaMBL1*-silenced white fruits (pk7:FaMBL1) (d), all (b–d) infected for 5 days with *C. acutatum*.

whitestrawberries did not show anthracnose symptoms. This is consistent with our previously published results, showing that *C. acutatum* infecting white unripe strawberries becomes quiescent (Guidarelli *et al.*, 2011), and indicates that agroinfiltration does not alter the resistance of unripe fruits to this pathogen. However, at this time (5 days post-infection), the white pk7:*FaMBL1*-silenced fruits showed typical anthracnose symptoms with brown necrosis in the whole fruit (Fig. 4B), suggesting that *FaMBL1* gene silencing strongly affects the resistance of white fruits to *C. acutatum*.

The histological analysis of the 24-hpi-infected tissues highlighted the presence of melanized appressoria in wild-type and pk7:00 control strawberries, whereas penetration events with hyphae internal to the epidermal layers were distinguishable in pk7:*FaMBL1*-silenced fruits (Fig. 5). This suggests that, in these fruits, *C. acutatum* does not become quiescent, but proceeds in its colonization process, whereas, in wild-type and pk7:00 control strawberries, the pathogen becomes quiescent.

Overexpression of *FaMBL1* in red ripe strawberries decreases the fruit susceptibility to *C. acutatum*

The overexpression of *FaMBL1* was induced in red ripe strawberries by infiltration for 3 days with *Agrobacterium* carrying the plasmid 35S:*FaMBL1*. Overexpression in these strawberries was evaluated by qRT-PCR at 24 hpi of *C. acutatum*; here, the expression of the *FaMBL1* gene increased more than 400-fold with respect to the control red fruits (non-infected, infected wild-type or infected 35S:00) (Fig. 6A), indicating that overexpression was very efficient and that agroinfiltration itself did not alter *FaMBL1* expression.

Five days after *C. acutatum* inoculation, wild-type red fruits and 35S:00 agroinfiltrated control fruits showed the typical anthracnose symptoms with dark and sunken lesions on the fruit surface (Fig. 6B). Symptom development was drastically slowed in 35S:*FaMBL1* red fruits, supporting the hypothesis that this gene plays a role in the susceptibility of strawberries to *C. acutatum*.

Sections of 24-hpi-infected red fruits were analysed microscopically; penetrated hyphae underneath the fruit surface were detected in 35S:00 control fruits and in wild-type ripe strawberry fruits (Fig. 7); in contrast, in 35S:*FaMBL1* fruits, a higher percentage of melanized appressoria of *C. acutatum* without penetration events was clearly visible in the tissue, suggesting that here the pathogen slowed its growth.

DISCUSSION

The majority of post-harvest losses in fruit production are caused by fungal pathogen infections occurring at the pre-harvest immature stage. Here fungi become quiescent until the fruits ripen, provoking symptoms only in mature fruits, when the commercial value is at its highest. For this reason, knowledge of the molecular basis of the low susceptibility of unripe fruits is of great importance, in particular for the development of new control strategies.

In this study, we isolated and functionally characterized the strawberry *FaMBL1* gene, previously found to be up-regulated in unripe strawberry fruits interacting with quiescent melanized appressoria of *C. acutatum* (Guidarelli *et al.*, 2011).

MBLs are B-lectin proteins believed to play a role in the innate immune response through their ability to bind to carbohydrates on the surface of microbial pathogens (Barre *et al.*, 2001; Peumans and Van Damme, 1995; Vijayan and Chandra, 1999;). The molecular basis of their mode of action is still uncharacterized; furthermore, their structure is very variable in terms of peptide length and domain composition, as indicated by Van Damme *et al.* (2008).

Following the description reported by Van Damme *et al.* (2008), *FaMBL1* encodes a protein similar to the GNA-related lectins identified in the Brassicaceae family; it includes an N-terminus signal peptide necessary for protein secretion into the extracellular

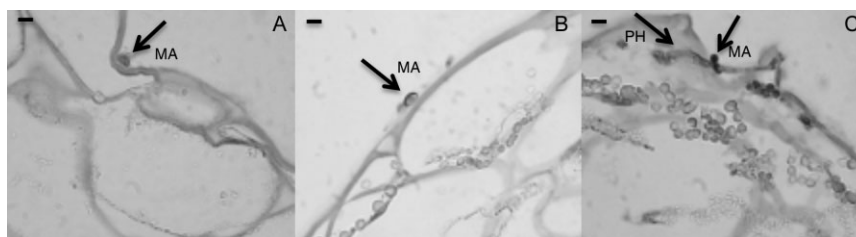


Fig. 5 Histological analysis of 24-h *Colletotrichum acutatum*-infected *FaMBL1*-silenced white strawberry fruits. Optical microscopy of wild-type white fruits (A), mock-silenced white fruits (pk7:00) (B) and silenced white fruits (pk7:*FaMBL1*) (C), all inoculated for 24 h with *C. acutatum*. Tissue slices were stained with haematoxylin and eosin. Melanized appressoria (MA) and intercellular hyphae (PH) are indicated. Bar, 10 μ m.

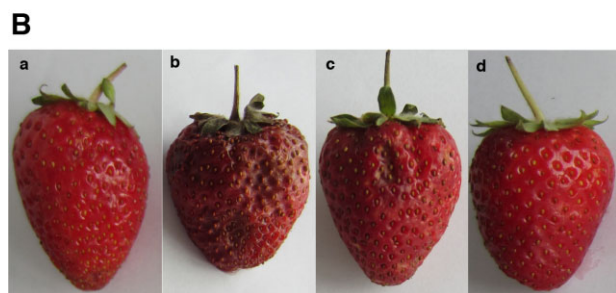
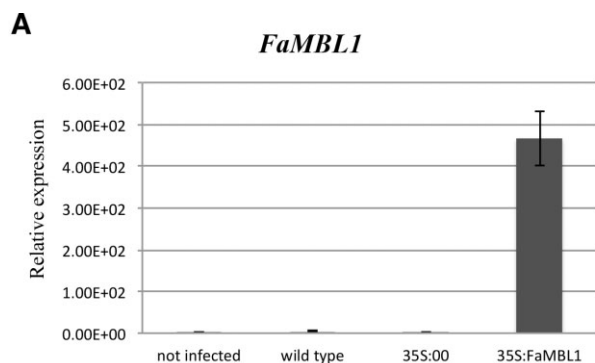


Fig. 6 Infection of *Colletotrichum acutatum* in *FaMBL1*-overexpressing red strawberry fruits. (A) Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis of *FaMBL1* expression in red wild-type or transfected strawberries 24 h after inoculation with *C. acutatum*. The transcript levels of wild-type mock-inoculated fruits (not infected) and 24-h *C. acutatum*-inoculated fruits (wild-type) were compared with those of mock-overexpressing fruits (35S:00) and *FaMBL1*-overexpressing fruits (35S:FaMBL1), both at 24 hpi with *C. acutatum*. Data were normalized to the expression level of the housekeeping *elongation factor 1 α* gene. Values are the means of three biological replicates. (B) Disease symptom analysis in red wild-type mock-infected fruits (a), red wild-type fruits (wild-type) (b), mock-overexpressing red fruits (35S:00) (c) and *FaMBL1*-overexpressing red fruits (35S:FaMBL1) (d), all (b–d) infected for 5 days with *C. acutatum*.

environment, followed by three mannose-binding domains, with the QxDxNxVxYx consensus motif for α -D-mannose recognition and a C-terminal PAN-Apple domain (Fig. 1). *FaMBL1* lacks the S-locus glycoprotein domain (SLP), playing a role in *Brassica* self-incompatibility, which is anyway poorly conserved among GNA-related lectins. For several MBL proteins, the ability to bind *in vitro* to mannose residues has been established: many bind with a weak

affinity to mannose, but strongly interact with oligomannosides and high-mannose *N*-glycans (Van Damme *et al.*, 2008).

For most plant lectins, the ability to recognize and bind to glycoconjugates on the surface of microorganisms through GNA-related domains is strictly related to their defence role (Peumans and Van Damme, 1995). On establishment of contacts with a host surface, pathogens release extracellular matrix (ECM) material containing glycoproteins with mannose residues, which are recognized by and bind to the mannose-binding domains of MBLs (Barre *et al.*, 2001; Sugui *et al.*, 1998). In particular, for most *Colletotrichum* species, it was found that the production and secretion of ECM material are associated with the developing appressorium process and are highest when melanized appressoria formation is accomplished (Bailey *et al.*, 1992). Using lectin cytochemistry, the specific sugar composition and abundance on the surface of *Colletotrichum* spp. appressoria were monitored during appressoria formation: α -D-mannose residues, in the form of mannans or mannoproteins, and galactose, were detected as the most abundant sugars in ECM material at the stage of melanized appressoria (O'Connell *et al.*, 1992; Pain *et al.*, 1996).

The time course analysis of *FaMBL1* gene expression revealed that this gene is up-regulated exclusively in white unripe strawberry fruits after 24 h of infection with *C. acutatum*, at the same time as the formation of quiescent melanized appressoria. No change in the expression of this gene was detected in white fruits at earlier time points. Furthermore, *FaMBL1* gene expression was not altered in red fruits interacting with the pathogen, either during the early infection stages, such as conidia germination and appressoria formation, or after 24 h of infection, when penetration had occurred and subcuticular hyphae were visible (Guidarelli *et al.*, 2011) (Fig. 2). Such a specific up-regulation of the *MBL* gene in unripe fruits at 24 hpi with *C. acutatum* could suggest that, similar to other GNA-related lectins, *FaMBL1* is capable of the recognition of and binding to carbohydrates present on the surface of quiescent melanized appressoria. To test this hypothesis, the binding ability of the *FaMBL1* GNA domain to mannans and mannoproteins of *C. acutatum* appressoria should be tested. Similarly, it would be interesting to characterize the downstream effects of this interaction on both fungal pathogen growth and strawberry fruit susceptibility.

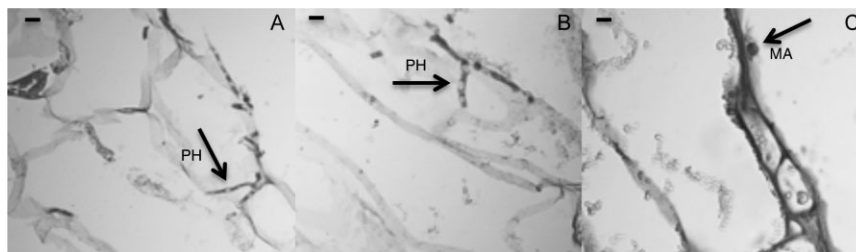


Fig. 7 Histological analysis of 24-h *Colletotrichum acutatum*-infected *FaMBL1*-overexpressing red strawberry fruits. Optical microscopy of wild-type red fruits (A), mock-overexpressing red fruits (35S:00) (B) and overexpressing red fruits (35S:*FaMBL1*) (C), all inoculated for 24 h with *C. acutatum*. Tissue slices were stained with haematoxylin and eosin. Melanized appressoria (MA) and intercellular hyphae (PH) are indicated. Bar, 10 μ m.

We have shown that silencing of *FaMBL1* expression in 24-h *C. acutatum*-inoculated white fruits leads to enhanced disease susceptibility and the appearance of early disease symptoms at unripe stages (Fig. 4). The histological analysis of infected tissues showed that this phenotype is associated with pathogen penetration events, characterized by the presence of subcuticular hyphae, a clear sign of active pathogen colonization (Fig. 5), normally absent in white unripe fruits. However, 24-h *C. acutatum*-inoculated red ripe fruits overexpressing the *FaMBL1* gene were shown to have delayed anthracnose symptoms, which normally appear 3 days after infection. A high percentage of melanized appressoria was observed in these fruits (Figs 6 and 7).

The altered susceptibility of transfected strawberry fruits supports the hypothesis that *FaMBL1* plays a crucial role in the differential susceptibility of fruits to *C. acutatum*. Consistently, silencing of the homologous *CaMBL1* gene in pepper leaves infected with *Xcv* enhanced disease susceptibility, decreased the accumulation of reactive oxygen species (ROS) and reduced the expression of *pathogenesis-related* (*PR*) genes. By contrast, its overexpression in Arabidopsis conferred resistance to *Alternaria brassicicola* (Hwang and Hwang, 2011). The GNA-related domain of *CaMBL1* is capable of binding to mannose and *N*-acetylgalactosamine residues, and is responsible for cell death induction; it is believed that its binding activity is related to its defence role (Hwang and Hwang, 2011). Similarly, on recognition and binding to specific mannose residues on *C. acutatum* appressoria, *FaMBL1* could induce downstream signals leading to the expression and activation of defence signals in strawberry fruits. However, *FaMBL1* could also act directly against the fungus, inhibiting its growth on binding to fungal oligomannoside residues.

Furthermore, the expression of the *FaMBL1* gene only in white infected strawberry fruits indicates that the ripening process dramatically changes the regulation of transcription of this specific lectin gene.

Interestingly, our past microarray study revealed that, together with the *FaMBL1* gene, several genes encoding hormone biosynthesis enzymes were expressed exclusively in 24-h-infected white fruits, and not in red ones: among these were, for example, *aminocyclopropane carboxylate oxidase* (*ACO*), and *allene oxide*

cyclase (*AOC*) genes, involved in synthesis of ethylene (ET) and jasmonic acid (JA), respectively, and the *abscisic acid* (*ABA*)-*responsive element* (*ARE*) and *phosphatase 2C* (*PP2C*) genes, involved in the ABA biosynthesis (Guidarelli *et al.*, 2011). These data suggest that ET, JA and ABA could play roles in regulating *FaMBL1* transcription during ripening. In support of this, *AtLEC*, a lectin gene in Arabidopsis, was shown to be induced by fungal cell wall oligosaccharides, as well as JA and ET hormones (Lyou *et al.*, 2009), and jasmonate-inducible lectins were identified from *Nicotiana* spp. (Lannoo *et al.*, 2006). In addition, a recent transcriptome analysis of ripening tomato interacting with *Botrytis* highlighted a role for ET, JA and ABA hormones in the resistance of unripe fruits to fungal pathogens (Blanco-Ulate *et al.*, 2013).

Our results indicate that an MBL protein plays an important role in the resistance of unripe stages of fruits to fungal pathogens. Future investigations should be addressed to clarify the molecular basis of the function of the *FaMBL1* protein in unripe fruit resistance, to elucidate the upstream events regulating its transcription and to reveal the downstream events leading to the inhibition of fungal growth.

EXPERIMENTAL PROCEDURES

Fungal and plant material

Colletotrichum acutatum was isolated from strawberry fruits showing severe anthracnose symptoms and identified by morphological analysis and sequencing of ribosomal DNA internal transcribed spacer (ITS) regions. The isolate was grown on potato dextrose agar (Sigma, St. Louis, MO, USA) at 20 °C for 10 days. For fruit infections, stock solutions ($\geq 100\times$) of conidial suspensions ($\geq 10^8$ conidia/mL) were prepared by washing the colonies with 5 mL of sterile distilled water containing 0.05% (v/v) Tween-80 and quantifying the conidia concentration with a haemocytometer.

Fragaria \times *ananassa* cv. 'Alba' plants cultivated in pots in a glasshouse were used for all experiments. Standard growing conditions were maintained at 20 °C with a 16-h photoperiod.

Time course of gene expression analysis

For the time course of *FaMBL1* gene expression analysis, white unripe (about 20 days after anthesis, daa) and red ripe (about 30 daa) strawberry

fruits were harvested and inoculated with *C. acutatum* by dipping for 1 min in a solution containing 10^6 conidia/mL suspension (diluted in water from the prepared stock) or with distilled water (mock control), and stored at 20 °C and 70% relative humidity for 8, 16, 20 and 24 h. After that time, the fruit surface was excised with a clean scalpel and immediately frozen in liquid nitrogen and transferred to -80 °C until use. Total RNA was prepared as described by Lopez-Gomez and Gomez-Lim (1992) with minor modifications. For qRT-PCR experiments, first-strand cDNA was synthesized from 1 µg of total RNA in a volume of 20 µL with oligo-d(T)17 and Superscript III (Invitrogen Life Technology, Carlsbad, CA, USA), following the manufacturer's instructions. The cDNA concentration in the RT mix was quantified using a ND-1000 UV spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), and 1 µg of cDNA was used for qRT-PCR experiments, employing an MX3000 thermal cycler (Stratagene, La Jolla, CA, USA) and Platinum Sybr-Green Kit (Invitrogen Life Technology), according to the manufacturer's instructions.

The *elongation factor 1 α* gene, having constitutive expression, was used to normalize raw data and to calculate relative transcript levels. Experiments were performed in three independent biological replicates of cDNA, each run in three technical replicates. Means were subjected to analysis of variance (ANOVA).

Plasmid construction, *Agrobacterium* transformation and plant transfection

The pK7GWIWG2(II), pK7GWIWG2 II-RedRoot and pK7WG2 vectors, described in Karimi *et al.* (2002), were obtained from the VIB Department of Plant Systems Biology, Ghent University, Belgium (<http://gateway.psb.ugent.be>).

For *FaMBL1* silencing, the partial sequence of the *FaMBL1* gene was amplified from the white inoculated strawberry cDNA pool using the *FaMBL1*-silencing forward primer (5'-CACCATGCAAGTCCCGGCAACGAAAC-3') and *FaMBL1*-silencing reverse primer (5'-TTTGCCATGCAACGCGGCCGTCGGCGT-3'). The PCR product was directionally cloned into entry vector pENTR/D-TOPO (Invitrogen Life Technology). The *FaMBL1* gene fragment was subsequently cloned into GATEWAY ready pK7GWIWG2(II) RNAi vector in sense and antisense orientation spaced by an intron, using the LR reaction of GATEWAY recombination-based cloning (Invitrogen Life Technology). To confirm the correct orientation, the resulting RNAi construct was checked using PCR [two reactions: one with the CaMV35S promoter forward primer (5'-ATTACAATTTACTATTCTAGTCG-3') and *FaMBL1*-silencing reverse primer; a second with the CaMV35S terminator forward primer (5'-TTTTGCGGACTCTAGCATGGCCG-3') and the *FaMBL1*-silencing reverse primer] and digested using *Xba*I and *Hind*III restriction enzymes. Sequencing of PCR products was performed to ensure the specificity of the gene target.

In order to overexpress *FaMBL1* in red fruits, the full-length sequence of *FaMBL1* was isolated from the cDNA library of white unripe strawberry fruits infected with *C. acutatum* and directionally cloned into entry vector pENTR/D-TOPO using the primers *FaMBL1*Over forward (5'-CACCATGCTCTCTTCATTACAAT-3') and *FaMBL1*Over reverse (5'-TATACTAGTGATTAGGTGCTTGTATG-3'). The *FaMBL1* gene was, in turn, cloned into pK7WG2 using GATEWAY cloning technology, as described above. The plasmid constructs were checked by PCR [CaMV35S promoter forward primer and attB2 reverse primer (5'-ACCATTTGTACAAGAAA-3')],

by digestion (*Xba*I and *Hind*III restriction enzymes) and by DNA sequencing.

The resulting plasmids (pK7:*FaMBL1* for silencing, 35S:*FaMBL1* for overexpression) were introduced into *A. tumefaciens* strain EHA105 using the freeze-thaw shock method (Holsters *et al.*, 1978).

The *A. tumefaciens* strains EHA105, containing pK7:*FaMBL1*, and 35S:*FaMBL1* were grown at 28 °C in Luria-Bertani (LB) medium with appropriate antibiotics. When the culture reached an optical density at 600 nm (OD_{600}) of about 0.8, *Agrobacterium* cells were harvested and resuspended in a modified MacConkey agar (MMA) medium [Murashige and Skoog salts, 10 mM 2-(*N*-morpholino)ethanesulphonic acid (MES), pH 5.6, 20 g/L sucrose and 200 µM acetosyringone], according to Spalore *et al.* (2001). After 1 h of incubation at 22 °C, the *Agrobacterium* suspension was injected into fruits still attached to the plant.

The silencing time was scored by agroinfiltrating white fruits with the plasmid pK7:*FaMBL1*. After 1, 2 or 3 days, fruits (10 fruits for each transfection time) were harvested and inoculated with *C. acutatum* conidia for 24 h, as described previously. Tissues from the surface of the whole fruit were collected and RNA was isolated, as described previously. qRT-PCR was used to evaluate the *FaMBL1* transcript level, as described above. Similarly, for the determination of the integration timing of T-DNA on the strawberry genome, nine white fruits were agroinfiltrated with a plasmid pK7GWIWG2 II-RedRoot containing a red fluorescent protein gene. Strawberry fruits were harvested after 1, 2 or 3 days (three fruits per condition), and tissues were analysed using a Nikon Eclipse TE2000-E fluorescence microscope (Nikon, Melville, NY, USA).

The *A. tumefaciens* strains EHA105, containing pK7:*FaMBL1*, and 35S:*FaMBL1* were grown at 28 °C in LB medium with appropriate antibiotics. When the culture reached an OD_{600} of about 0.8, *Agrobacterium* cells were harvested and resuspended in MMA medium. After 1 h of incubation at 22 °C, the *Agrobacterium* suspension was evenly injected into fruits still attached to the plant using a sterile 5-mL syringe.

FaMBL1 silencing and overexpression

For silencing, 50 white strawberry fruits at about 17 daa (3 days before the 'white' stage) were agroinfiltrated with pK7:*FaMBL1* for 3 days. They were harvested and inoculated with *C. acutatum*, as described above. Twenty of these fruits were collected 24 h after inoculation: 15 fruits were used for *FaMBL1* gene expression analysis to confirm gene silencing. For this, RNA was isolated and qRT-PCR was performed, as described above. Five fruits were used for histological analysis of the fungal infection, as described by Guidarelli *et al.* (2011). The other 30 fruits were phenotypically observed after 5 days of inoculation for anthracnose symptom evaluation.

Similarly, for overexpression, 50 red fruits at about 27 daa (3 days before the 'red' stage) were agroinfiltrated with plasmid 35S:*FaMBL1* and treated as for silencing, except that, for better phenotype evaluation, 30 fruits were observed 3 days instead of 5 days after *C. acutatum* inoculation.

As controls for silencing and overexpression, the same number (50) of white and red fruits were agroinfiltrated with pK7:00 (the empty silencing vector) and 35S:00 (the empty overexpression vector) plasmids, or left in the wild-type condition. Phenotype observation, gene expression and histological analysis were performed as described above.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Multiple sequence alignment of FaMBL1 and mannose-binding lectins (MBLs) from other species. FaMBL1 protein sequence (KF962716) was aligned with woodland strawberry (*Fragaria vesca*) epidermis-specific secreted glycoprotein (XP_004294119), cacao (*Theobroma cacao*) MBL (EOY13258), sugar beet (*Beta vulgaris*) SIEP1L protein (CAA61158), pepper (*Capsicum annuum*) CaMBL1 (ADG04234), carrot (*Daucus carota*) cell attachment protein (BAD24818) and Arabidopsis curculin-like (mannose-binding) lectin protein (NP_178007). The mannose-binding domain is boxed.