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RESEARCH PAPER

Demethylation of oligogalacturonides by *FaPE1* in the fruits of the wild strawberry *Fragaria vesca* triggers metabolic and transcriptional changes associated with defence and development of the fruit

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Abstract

Ectopic expression of the strawberry (*Fragaria*×*ananassa*) gene *FaPE1* encoding pectin methyl esterase produced in the wild species *Fragaria vesca* partially demethylated oligogalacturonides (OGAs), which conferred partial resistance of ripe fruits to the fungus *Botrytis cinerea*. Analyses of metabolic and transcriptional changes in the receptacle of the transgenic fruits revealed channelling of metabolites to aspartate and aromatic amino acids as well as phenolics, flavanones, and sesquiterpenoids, which was in parallel with the increased expression of some genes related to plant defence. The results illustrate the changes associated with resistance to *B. cinerea* in the transgenic *F. vesca*. These changes were accompanied by a significant decrease in the auxin content of the receptacle of the ripe fruits of transgenic *F. vesca*, and enhanced expression of some auxin-repressed genes. The role of these OGAs in fruit development was revealed by the larger size of the ripe fruits in transgenic *F. vesca*. When taken together these results show that in cultivated *F. ananassa* FaPE1 participates in the de-esterification of pectins and the generation of partially demethylated OGAs, which might reinforce the plant defence system and play an active role in fruit development.

Key words: Defence signalling, fruit, oligogalacturonides, pectin methyl esterase, strawberry.

Introduction

Fruit growth and ripening are complex developmental processes that involve many events contributing to the textural and constitutional changes in the fruits and determining their final composition. Cell wall disassembly is one of the main processes occurring at the end of the ripening period and its rate and extent are crucial for the maintenance of fruit quality and integrity (Matas *et al.*, 2009). For this reason, maintenance of firmness has long been the target for breeders in many crops to minimize post-harvest decay. Since strawberry is particularly sensitive to such textural changes, this characteristic currently limits the breadth of its commercialization (Perkins-Veazie, 1995). Polysaccharide solubilization and depolymerization have previously been reported in ripening strawberry fruits (Rosli *et al.*, 2004). They are the combined result of activity by enzymes such as polygalacturonases, pectate lyases, and pectin methyl esterases (PMEs) acting on pectins (Nogata *et al.*, 1993; Medina-Escobar *et al.*, 1997; Castillejo *et al.*, 2004; Quesada *et al.*, 2009); cellulases degrading cellulose (Abeles and Takeda, 1990); and endo β-1,4-glucanases and β-xylosidases acting on hemicelluloses (Harpster *et al.*, 1998; Trainotti *et al.*, 1999; Bustamante *et al.*, 2006; Mercado *et al.*, 2010). In addition, β-galactosidase and α-L-arabinofuranosidase are involved in the cleavage of the side

Abbreviations: IAA, indole-3-acetic acid; OGA, oligogalacturonide; PME, pectin methyl esterase; PR proteins, pathogenesis-related proteins; RLK, receptor-like kinase.

chains of the cell wall polymers (Trainotti *et al.*, 2001; Rosli *et al.*, 2009). The sequential action of this suite of enzymes results in changes in cell wall properties leading to fruit softening.

In parallel with these changes in the cell wall in many fruits a dramatic increase in susceptibility to necrotrophic pathogens has been reported (Prusky, 1996). It is now accepted that cell wall disassembly can be a key component of this susceptibility (Flors *et al.*, 2007; Cantu *et al.*, 2008). However, there is increasing evidence that molecules that are either components of the cell wall or the result of their partial degradation, such as the oligogalacturonides (OGAs), contribute to disease resistance (Hématy *et al.*, 2009).

Pectin is a cell wall polymer that suffers major changes during fruit ripening since it is not only the target of degrading enzymes (Medina-Escobar *et al.*, 1997; Castillejo *et al.*, 2004), but also the source of OGAs, which have been shown to display elicitor activity (Ridley *et al.*, 2001). OGAs elicit responses ranging from fortification of the cell wall to the synthesis of defence molecules, such as phytoalexins, pathogenesis-related (PR) proteins, and reactive oxygen species (De Lorenzo and Ferrari, 2002; Field *et al.*, 2006; Van Loon *et al.*, 2006). The effectiveness of OGAs as eliciting molecules rests on specific structural requirements, such as their size (Aziz *et al.*, 2004) and degree of methyl esterification (Wiethölter *et al.*, 2003; Osorio *et al.*, 2008). In addition to their eliciting capacity, it is known that the OGAs also affect several aspects of plant growth and development (Côté and Hahn, 1994; Melotto *et al.*, 1994). On this point the antagonism found between OGAs and auxin actions is relevant (Ferrari *et al.*, 2008).

Pectin methyl esterase (PME) is a key enzyme of pectin degradation catalysing the demethylation of galacturonyl residues of high-molecular-weight pectin. Four *PME* genes in strawberry (*FaPE1–4*) have been reported previously (Castillejo *et al.*, 2004) and isoelectric focusing of cell wall extracts from ripe fruits has revealed up to six basic isoforms (Draye and Van Cutsem, 2008). In a previous study the fruit-specific *FaPE1* was ectopically expressed in *Fragaria vesca*; two major effects were found (Osorio *et al.*, 2008). First, the pectin of the red fruits in the transgenic *F. vesca* displayed a lower degree of methyl esterification in a block-wise pattern. This is important in fruit softening since this esterification pattern has a positive effect on Ca^{2+} binding to the pectin, and eventually in cell wall integrity (Willats *et al.*, 2001). In tomato this role has been assigned to the PME Pmeu1 since silencing of the corresponding gene resulted in enhanced fruit softening during ripening (Phan *et al.*, 2007). The second effect was the appearance of partially demethylated OGAs in parallel with increased resistance of the ripe fruits to *Botrytis cinerea*.

Here the results of transcriptomic and metabolomic analyses of the ripe receptacle of *F. vesca* overexpressing *FaPE1* are presented. The objective was to shed light on the role played by the strawberry enzyme FaPE1 in the generation of partially demethylated OGAs and the subsequent role played by these compounds in plant defence and/or development.

Materials and methods

Plant material and sample collection

F. vesca and two independent transgenic lines with the PME (*FaPE1*) cDNA in the sense orientation under control of a single constitutive (CaMV35S) promoter (line 4 and line 15; described in Osorio *et al.*, 2008), and *Fragaria×ananassa* Duch. cv. Camarosa plants were grown in a greenhouse under natural light conditions. Transgenic *F. vesca* plants used in this work correspond to the second, third, and fourth vegetative generation.

The *F. ananassa* fruits harvested in four different developmental stages corresponded to: green, G; white, W; turning (at least 25% surface red), T; red, R. Pools of fruits from a single plant have been considered single biological replicates (eight plants were used in total). Analyses of both primary and secondary metabolites in transgenic *F. vesca* were performed in six separate pools of red fruits of ~30 fruits each. Each pool was from one individual plant. All fruits were frozen immediately in liquid nitrogen and achenes were removed using a scalpel on frozen fruits. For microarray analysis, one biological replicate was considered as a pool of red fruits collected from *F.vesca* wild type and line 4 from eight individual plants each. The two biological replicates are pools from different seasons. For the qRT-PCR analysis, red fruits were harvested as pools of two individual plants for each replicate (eight plants divided into four groups). Red fruits of *F. vesca* at identical ripening stage were used for auxin content measurement.

Protein extraction, immunoblot analysis, and PME activity

Protein extraction, immunoblot analyses, and PME activity from *F.ananassa* fruits were performed according to Osorio *et al.* (2008).

Degree of pectin esterification and chemical de-esterification of OGAs

Degree of esterification and chemical de-esterification of OGAs from *F. ananassa* red fruits were analysed according to Osorio *et al.* (2008).

RNA isolation and gene expression analysis

Total RNA was isolated from *F. vesca* fruits without achenes according to the method described by Manning (1991). Integrity of the extracted RNA was checked by electrophoresis under denaturing conditions after treating the RNA with RNase-free DNaseI (Roche). First-strand cDNA synthesis of 2 μg of RNA in a final volume of 20 ml was performed with Moloney murine leukaemia virus reverse transcriptase, Point Mutant RNase H Minus (Promega), according to the supplier's protocol using oligo(dT) T19 primer.

Expression of *PR5* by real-time qRT-PCR was as previously described (Osorio *et al.*, 2008). Expression of *LRR1* (Acc.No. DY671714), *LRR2* (Acc.No. DY668749), *LRR3* (Acc.No. AJ871783), *WRKY1* (Acc.No. DY667265), *WRKY2* (Acc.No. AJ871772), β-xylosidase (Acc.No. AY486104), (1,4)-β-mannan endohydrolase (Acc.No. GT149809), and *PR5* (Acc.No. EU289405) was analysed by real-time qRT-PCR using the fluorescent intercalating dye SYBR Green in an iCycler detection system (Bio-Rad; http://www.bio-rad.com/). Relative quantification of the target expression level was performed using the comparative Ct method. The list of primers used is provided as supporting information (Supplementary Table S4 at *JXB* online). Expression data were normalized to reference 18S–26S ribosomal gene (interspacer) (Casado-Díaz *et al.*, 2006).

Microarray analysis

Oligo (60-mer length) design for expression analysis was performed by NimbleGen Systems Inc. after receiving >14 500 sequences from *F. vesca* (GenBank), *F. ananassa* (GenBank and the authors'

ad hoc generated strawberry database), and *Prunus persica* (GenBank) in a file in FASTA format. A minimum of three oligos was printed per probe and three blocks were printed per dataset.

RNA was extracted from the receptacle of ripe red fruits from two biological replicates of the wild type and L4 as described above. These two replicates comprised eight different plants each, and were harvested in two different seasons. The RNA was then used for the synthesis of double-stranded cDNA according to the protocol described in the Invitrogen SuperScript™ Double-Stranded cDNA Synthesis Kit. Sample labelling and hybridization with three probes per target, were performed by NimbleGen Systems Inc. The data were loaded into R, and expression estimates were generated using RMA (Bolstad *et al.*, 2003) as suggested by the expression analysis section of the NimbleGen users' manual (www.nimblegen.com). The Bioconductor package limma (Smyth, 2004) was used to identify differentially expressed genes using a moderated *t* test and false discovery rate control (Benjamini and Hochberg, 1995).

Extraction, derivatization, and analysis of polar metabolites using GC-MS

Metabolite analysis by GC-MS was carried out essentially as described by Lisec *et al.* (2006) and Fernie *et al.* (2004). The mass spectra were cross-referenced with those in the Golm Metabolome database (Kopka *et al.*, 2005).

Extraction and analysis of semi-polar metabolites and UPLC-FT-ICR-MS measurements

Metabolite extraction and UPLC-FT-ICR-MS were performed as described previously (Giavalisco *et al.*, 2008). In brief, 250 mg of frozen red fruits without achenes was extracted in 1 ml of chloroform/metanol/water (1:2.5:1). The homogenized tissue was incubated for 20 min at 30 °C on an orbital shaker followed by a 10-min sonication. The sample was then centrifuged at 4000 *g* for 5 min and the supernatant was transferred to a fresh Eppendorf tube before concentrating it to dryness in a Speed-vac (Centrivac, Heraeus, Hanau, Germany).

Chromatographic separation, mass spectrometric measurements, and data analysis were performed using a Waters Acquity UPLC system (Waters, Milford, MA, USA) using an HSS T3 C18 reverse phase column (100×2.1 mm i.d., 1.8-µm particle size; Waters) and a LTQ FT-ICR-Ultra mass spectrometer (Thermo-Fisher, Bremen, Germany), as described by Giavalisco *et al.* (2009). Mass peaks from these spectra were picked and aligned (SIEVE; Thermo-Fisher) leading to peak lists containing accurate mass, retention time, and intensity of each detected peak from each sample. The accurate masses of each detected peak were searched against a compiled version of three biological databases [KNApSAcK (http://kanaya.naist.jp/KNApSAcK/), Metabolome.Jp (http://www.metabolome.jp/), and KEGG (http://www.genome.jp/kegg/)] leading to the assignment of an elemental composition and a compound name to the masses that found a match in the database. The annotations for all the peaks that showed significant differences between wild-type and transgenic plants were validated by manual inspection of the correct assignment of the assigned adducts and search for masses derived from in-source fragmentation. The mass error for the database searches and the fragment assignments was always <2 parts per million (average value 0.8 ppm) resulting in confident elemental composition assignments. The MS/MS fragmentation of the metabolites was compared with candidate molecules found in databases, and verified with earlier literature on similar compounds, especially when the presence of the metabolite was reported in strawberry.

Determination of indole-3-acetic acid

Determination of indole-3-acetic acid (IAA) was performed essentially as described by Peng *et al.* (1999). In brief, frozen red fruits without achenes (6 g) were homogenized and extracted overnight in 20 ml of 80% methanol. After extraction, each sample was reduced *in vacuo* and diluted with 20 ml of water. The aqueous phase was adjusted to pH 2.8 with 1 M HCl and partitioned four times with equal volumes of ethyl acetate. The ethyl acetate extracts were combined and evaporated to dryness. The residue was dissolved in 1 ml of 10% methanol and applied to a pre-equilibrated C_{18} cartridge (Waters; http://www.waters.com). The column was washed with aqueous acetic acid (pH 3.0), and then IAA was eluted with 80% methanol. After evaporation to dryness, the samples were derivatized as described (Lisec *et al.*, 2006) and analysed by comparison with authentic standard using GC-MS.

Results

FaPE1 participates in pectin de-esterification during strawberry fruit ripening

Polyclonal antibodies raised against two antigenic peptides derived from the conserved C-terminal portion of the FaPE1–4 proteins (Osorio *et al.*, 2008) detected a single band of 67 kDa (Fig. 1A). Western blot showed that the PME protein content of the strawberry fruits increased

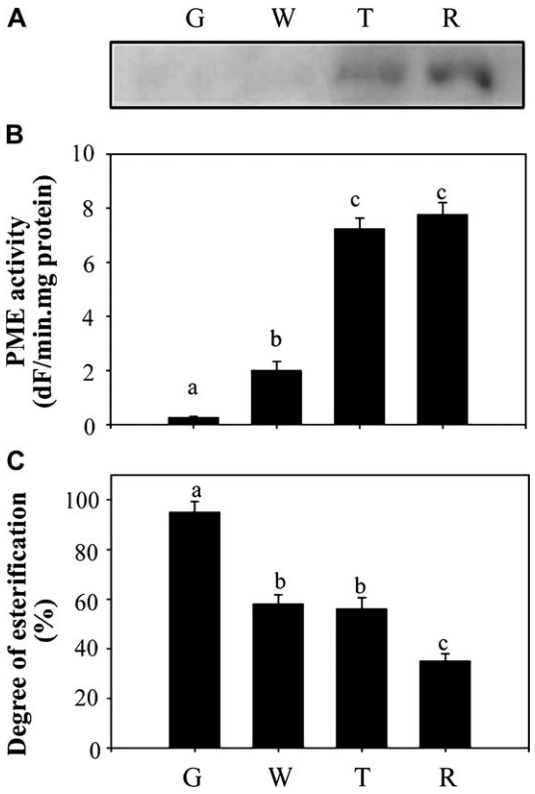

Fig. 1. Analysis of *F. ananassa* fruits. (A) Immunoblot analysis of proteins from *F. ananassa* fruits. Equal amounts of proteins from fruits at different ripening stages were loaded. Molecular mass is indicated on the right. (B) PME activity of extract from *F.ananassa* fruits at different stages of ripening. (C) Percentage of pectin methyl esterification from *F.ananassa* fruits at different stages of ripening. Results are the mean values of four individual plants. Values marked with a different letter differ significantly from each other ($P<0.05$). Stages of fruits: G, green; W, white; T, turning; R, red.

during ripening, reaching its maximum abundance at the (red) ripe stage (Fig. 1A). This pattern correlated with measurements of total PME activity (Fig. 1B), which showed an increase in protein after the white stage, and reached and maintained a maximum value at the turning and red stages. In the opposite direction, the degree of pectin methyl esterification decreased from green to red fruits (Fig. 1C). However, this inverse relationship was not perfect since the high increase in activity between the white and turning stages was not paralleled by a decrease in the degree of methylation. Our best explanation is that changes in total PME activity measured *in vitro* are the result of changes in isoenzymes, whose contribution to demethylation of pectin *in vivo* might be different for each. Effectively, it is known that the action of these enzymes *in muro* within the cell wall is influenced by a wide range of factors, such as pH and the presence of inhibitors, and their effects are specific for each isoenzyme (Pelloux *et al.*, 2007). This means that not all the isoenzymes have the same demethylation activity *in vivo*. Therefore, changes in the expression of isoenzymes would be revealed by western blot (Fig. 1A) and total PME activity *in vitro* (Fig.1B), but not necessarily by demethylation *in planta* (Fig. 1C).

Ripe fruits of strawberry F. ananassa *produce eliciting OGAs*

Transgenic *F. vesca* lines exhibiting ectopic expression of the fruit-specific *FaPE1* of *F. ananassa* have been previously generated. These transgenic lines had modified oligosaccharides (OGAs) (Osorio *et al.*, 2008). Here the size of OGAs from ripe fruits of *F. ananassa*, which naturally expresses *FaPE1*, were analysed and compared with the size of OGAs extracted from ripe *F. vesca* fruits of wild type and one transgenic line (L4) (Fig. 2A). The gel filtration profile of the *F. vesca* OGAs has been previously published (Osorio *et al.*, 2008), but they are also included in Fig. 2A to facilitate comparison with the *F. ananassa* OGAs. It is clear that OGAs from the *F. ananassa* fruits showed the same size as the OGAs from the transgenic line, but were smaller than the OGAs from wild-type *F. vesca*. When OGAs from *F. vesca* fruits of wild-type and transgenic (L4) plants, and from *F. ananassa* were de-esterified they eluted in the same fraction (Fig. 2A). From these results it might be concluded that OGAs from *F. ananassa* and from *F. vesca* have the same degree of polymerization but differ in their degree of esterification.

It was known that OGAs from transgenic *F. vesca* fruits overexpressing *FaPE1* had eliciting capacity, reflected by the induction of *PR5* gene expression when ripe fruits of *F. vesca* were inoculated with them, but this increased *PR5* expression did not occur when the OGAs were de-esterified (Osorio *et al.*, 2008). *PR5* expression in ripe fruits of both *F. vesca* and *F. ananassa* was then analysed after injection of 500 µg of OGAs extracted from *F. vesca* or from *F. ananassa*, before and after de-esterification (Fig. 2B). Expression of *PR5* was clearly up-regulated in fruits of both species injected with OGAs from *F. ananassa* and from transgenic *F. vesca* compared with fruits injected with OGAs from wild-type

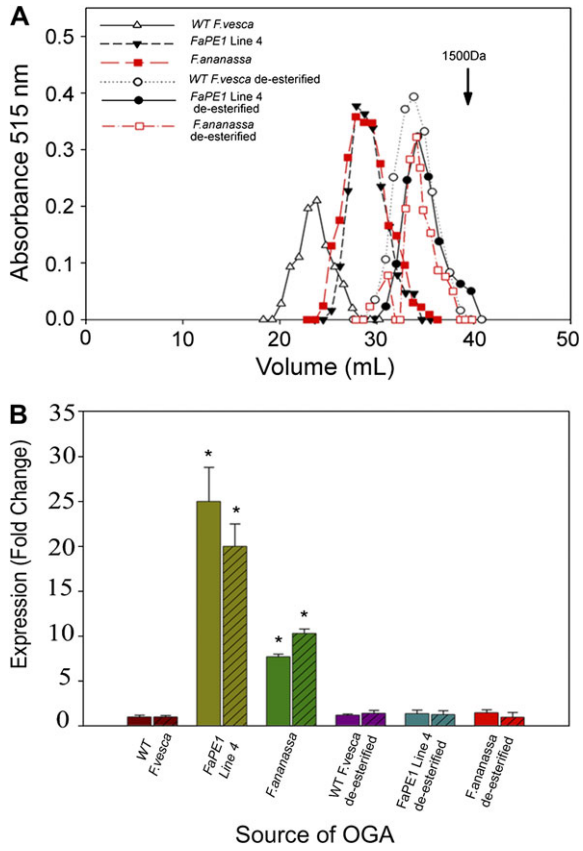

Fig. 2. Analysis of OGAs from *F. ananassa* fruit, and their effect on *PR5*. (A) Gel-filtration profiles of OGAs extracted from ripe fruits and after de-esterification of wild type and *FaPE1* line 4 (black symbols) and *F. ananassa* (red symbols). A Bio-Gel P-4 column was used. Fractions (0.8 ml) were assayed for uronic acid content. (B) Quantitative RT-PCR of *PR5* in ripe fruits of *F. vesca* (solid bars) and *F. ananassa* (lined bars) 48 h after injection of 500 µg of the specific OGAs shown in (A). An asterisk denotes values that are significantly different as determined by *t* test ($P<0.01$). Results are the means of independent assays ($n=4$). Data from wild type and *FaPE1* line 4 have been published previously in Osorio *et al.* (2008).

F. vesca. This increased expression did not occur with chemically de-esterified OGAs from different sources (Fig. 2B). Taken together these results associate partial esterification of OGAs with their capacity to increase *PR5* expression, as shown previously (Osorio *et al.*, 2008). Whether or not this is a defence-specific response should be studied. To this purpose the focus in this work was on the metabolic and transcriptional changes associated with the presence of partially demethylated OGAs. *F. vesca* was selected as a model since comparison between the wild-type and transgenic lines was direct because they represent near-isogenic lines, the only difference being the *FaPE1* gene.

Metabolites in ripe fruits of wild-type and transgenic F. vesca *overexpressing* FaPE1

The changes in metabolites in two independent transgenic lines (L4 and L15) overexpressing *FaPE1*, which showed

a similar phenotype of resistance to *B. cinerea* (Osorio *et al.*, 2008), were analysed and compared with the wild type. The receptacle of ripe fruits was analysed using an established GC-MS protocol (Fernie *et al.*, 2004; Lisec *et al.*, 2006) (Table 1). The two transgenic lines exhibited significantly higher content of aromatic amino acids phenylalanine, tryptophan, and tyrosine (Phe, Trp, Tyr), and aspartate (Asp). Simultaneously, a significant decrease was found in the proteinogenic amino acids alanine (Ala) and proline (Pro), and the non-proteinogenic β-alanine and 4-amino-butyrate (GABA). *O*-acetylserine, which also decreased in the transgenic lines, is an intermediate in the incorporation of inorganic sulphur into the amino acid cysteine (Wirtz *et al.*, 2001) and probably reflects changes in this amino acid (which was itself undetected using the authors' own protocol). Of particular interest is the significant decrease in spermidine, one of the polyamines known to play a major signalling role in plants (Kusano *et al.*, 2008).

Our data displayed significant decreases in the main hexoses glucose and fructose (Glc and Fru), their corresponding 6-phosphate derivatives as well as in glucose oxidation products such as saccharic acid and the disaccharide sucrose and the trisaccharide raffinose. Among these, changes in Glc, Fru, and sucrose appear to be more relevant since they are by far the main components of the sugar content in the receptacle of the ripe cultivated strawberry (Olsson *et al.*, 2004) continuously increasing during ripening (Fait *et al.*, 2008). Regarding the organic acids there were no significant changes in tricarboxylic acid cycle intermediates, but a decrease was found in glyceric and tartaric acids, and an increase in D-galacturonic acid. This latter is probably a consequence of the higher PME activity, which in turn will result in higher demethylation of the pectins (Osorio *et al.*, 2008). When summed together these changes suggest a dramatic shift in the primary metabolism of the transgenic fruits when the metabolites were quantified absolutely (Supplementary Table S1, at *JXB* online).

Given that Phe is the obligate precursor of the phenylpropanoid pathway and presented a 5-fold increase (Table 1), altered composition of intermediates of this pathway in the transgenic fruits was predicted. Therefore an exhaustive analysis of secondary metabolite profiling was conducted, using a non-targeted approach, of extracts derived from the receptacle of both the *F. vesca* transgenic lines (L4 and L15), and wild type. Methanol-soluble constituents were analysed using UPLC-FT-ICR-MS in positive and negative ionization mode. A total of 1524 chromatographic peaks were analysed of which 57 showed alterations between the two transgenic lines and wild type. Following the identification process these 57 secondary metabolites were tentatively assigned to specific compound classes (Supplementary Table S2, at *JXB* online).

The results, summarized in a heat map (Fig. 3), show a significant decrease in most of the galloyl derivatives and ellagitannin, which are derived directly from the shikimate pathway. Many of the hydroxylated derivatives of cinnamic and benzoic acids, precursors of benzoates, salicylates, coumarins, lignin, and flavonoids, increased in both

Table 1. Primary metabolite levels in the receptacle of red fruits of *F. vesca* plants overexpressing *FaPE1*

Metabolites were determined in the receptacle from red fruits. Data are normalized to the mean response calculated for the wild type. Values presented are mean±standard error of six replicates. Values in bold denote significant differences as determined by ANOVA analysis ($P<0.01$).

Amino acids	Wild type	Line 4	Line 15
Alanine	1.00±0.04	**0.59±0.04**	**0.62±0.05**
β-Alanine	1.00±0.02	**0.60±0.04**	**0.73±0.04**
Asparagine	1.00±0.07	0.88±0.05	0.89±0.05
Aspartate	1.00±0.03	**1.60±0.05**	**1.45±0.04**
GABA	1.00±0.02	**0.43±0.02**	**0.90±0.01**
Glutamate	1.00±0.06	1.06±0.04	1.11±0.03
Glycine	1.00±0.07	0.72±0.06	0.69±0.08
Methionine	1.00±0.04	1.15±0.05	0.98±0.02
Phenylalanine	1.00±0.02	**5.15±0.18**	**4.32±0.08**
Proline	1.00±0.03	**0.41±0.01**	**0.55±0.02**
Serine	1.00±0.03	**0.80±0.02**	**0.87±0.05**
Threonine	1.00±0.03	1.09±0.04	0.96±0.05
Tryptophan	1.00±0.05	**1.65±0.07**	**1.86±0.04**
Tyrosine	1.00±0.05	**1.91±0.05**	**1.63±0.05**
Valine	1.00±0.04	1.29±0.13	1.10 ±0.12
Organic acids			
2-Oxoglutaric acid	1.00±0.08	1.09±0.06	1.15±0.05
Dehydroascorbic acid	1.00±0.04	**1.40±0.04**	**1.69±0.07**
Fumaric acid	1.00±0.07	1.20±0.06	0.86±0.06
Galacturonic acid	1.00±0.06	**8.10±0.11**	**6.32±0.23**
Glucuronic acid	1.00±0.08	1.30±0.07	0.96±0.05
Glyceric acid	1.00±0.03	**0.71±0.02**	**0.70±0.04**
Malic acid	1.00±0.04	1.20±0.07	0.89±0.04
Quinic acid	1.00±0.02	1.00±0.04	0.93±0.06
Saccharic acid	1.00±0.05	**0.42±0.04**	**0.82±0.01**
Succinic acid	1.00±0.09	0.93±0.04	1.33±0.02
Tartaric acid	1.00±0.03	**0.52±0.02**	**0.79±0.02**
Sugars and sugar alcohols			
Erythritol	1.00±0.03	1.14±0.02	0.93±0.03
Fucose	1.00±0.06	0.89±0.05	0.81±0.08
Fructose	1.00±0.02	**0.50±0.03**	**0.80±0.04**
Fructose-6-P	1.00±0.05	**0.82±0.02**	**0.59±0.02**
Galactinol	1.00±0.07	0.71±0.02	1.12±0.04
Glucose	1.00±0.05	**0.46±0.03**	**0.76±0.04**
Glucose-6-P	1.00±0.07	0.75±0.05	0.93±0.03
1-*O*-Methylglucopyranoside	1.00±0.04	1.12±0.03	0.96±0.07
Maltose	1.00±0.07	0.74±0.05	0.97±0.04
Maltitol	1.00±0.10	1.34±0.05	0.88±0.04
Maltotriose	1.00±0.08	1.20±0.05	1.05±0.07
Raffinose	1.00±0.06	**0.51±0.02**	**0.45±0.04**
Sucrose	1.00±0.05	**0.53±0.02**	**0.74±0.02**
Trehalose α α'	1.00±0.04	1.12±0.05	0.84±0.08
Xylose	1.00±0.08	0.72±0.09	1.35±0.07
Miscellaneous			
Glycerol	1.00±0.08	0.67±0.10	0.80±0.07
myo-Inositol	1.00±0.04	**0.61±0.03**	**0.80±0.02**
Inositol-1-P	1.00±0.08	1.32±0.07	0.71±0.09
Phosphoric acid	1.00±0.06	1.14±0.08	0.81±0.03
O-Acetylserine	1.00±0.03	**0.51±0.03**	**0.69±0.02**
Spermidine	1.00±0.05	**0.62±0.02**	**0.81±0.02**

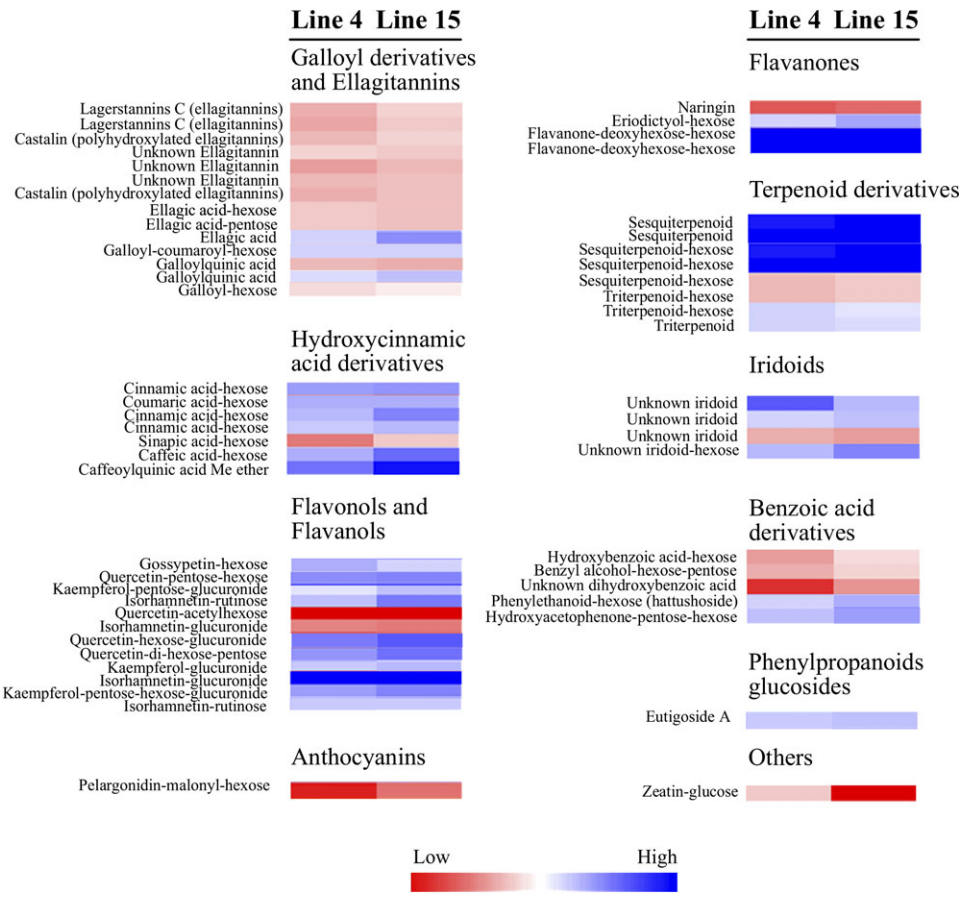

Fig. 3. Distribution of tentatively identified secondary metabolites in *F. vesca* red fruits overexpressing *FaPE1*. The colours indicate the proportional content of each putatively identified metabolite among the samples as determined by the intensity of each detected peak using UPLC-FT-ICR-MS. The lowest intensity is depicted as red and the highest intensity blue (see colour bar at the bottom). The exact values of each metabolites peak are provided in Supplementary Table S3 at *JXB* online.

transgenic lines. Thirteen products of the flavonoid pathway, flavonols, flavanols, and flavanones, exhibited a dramatic increase in both transgenic lines and only three were down-regulated. Apart from metabolites derived from the shikimate and phenylpropanoid pathways, significantly differing terpenoid derivative metabolites were detected. In the class monoterpene iridoids, three and one metabolites, respectively, were up- and down-regulated in both transgenic lines and in the triterpenoid class two were up-regulated and one down-regulated. Dramatic accumulation of four sesquiterpenoids was also observed, and only one metabolite in this class was down-regulated.

Transcript changes in transgenic F. vesca *overexpressing* FaPE1

A custom array was prepared from 14 785 non-redundant DNA sequences from *F. vesca*, *F. ananassa*, and *P. persica*. A total of 7697 sequences from *F. vesca*, which were freely available in public databases, were selected. Since global comparison between homologous sequences from *F. vesca* and *F. ananassa* revealed an average identity of >93%, 5957 non-redundant sequences from *F. ananassa* were selected, which were added for the preparation of the oligonucleotide microarray. Finally, the opportunity of using a considerable number of sequences from *P. persica*, a species of the same Rosaceae family as the *Fragaria* species (Shulaev *et al.*, 2008) was assessed. The average identity between homologous *F. vesca* and *P. persica* sequences was >87%. This high level of homology was sufficient to consider some of the *P. persica* sequences as a potential source of new probes to be used in the expression studies. A selection of 1131 sequences of this species that were annotated for metabolic pathways relevant in fruit ripening and not represented in the annotated *F. vesca* and *F. ananassa* sequences were selected. In total these three sequence sets accounted for the 14 785 probes used in the expression studies. Taking account of the number of genes for a closely related species like *P. persica* (http://www.rosaceae.org/peach/genome) it is estimated that around one-third of the *F. vesca* transcriptome is represented in the microarray.

The microarray was used to compare transcript levels in the receptacle of red fruits from the wild type and the transgenic line L4 (Supplementary Table S3, at *JXB* online). Stringency conditions used in the assay allowed a distinction to be made between the non-homologous probes selected for the design of the microarray. Extracting significantly changed genes after Benjamini Hochberg correction

($P \leqslant 0.05$) gave a total of 729 non-redundant sequences that showed higher expression in the transgenic line and 667 with lower expression.

To make tables within the article not too exhaustive, a conservative *P*-value cut-off of 0.01 was used. The genes showing significant changes ($P \leqslant 0.01$) are reproduced in Tables 2 and 3 (for a more comprehensive list see Supplementary Table S2 at *JXB* online). The overexpression of *FaPE1* produces a general activation of biosynthetic processes of the carbon metabolism, including genes involved in photosynthetic electron transport. Genes with highest values for fold change are classified in the arbitrary categories of signalling, defence, hormone action, and cell wall metabolism (Table 2). Of special interest are the genes related to auxin action due to the antagonism proposed between the action of auxin and oligosaccharides (OGAs) (Ferrari *et al.*, 2008). Moreover, interplay between auxin and pathogen responses has been proposed to take place at multiple levels, such as biosynthesis, transport, signalling, and response (Kazan and Manners, 2009). The auxin content of the receptacle of transgenic red fruits of *F. vesca* was then measured, and found to be significantly lower than the wild type (Fig. 4A). The diminished content of auxin also occurred in transgenic L15, which was not used in the microarray experiments but presented the same resistance phenotype as L4. Interestingly, red fruits of these two transgenic lines were larger in size than fruits of *F. vesca* wild type (Fig. 4B)

Analysis of genes with lower expression in the transgenic line showed distribution associated with carbohydrate metabolism at different steps, as occurs with the hydrolysis of linear glucans by β-amylase and at the final step of glycosylation represented by the UDP-glucose glucosyltransferase (Table 3). Other metabolic processes affected are lignin biosynthesis represented by cinnamoyl CoA reductase, laccase, and peroxidase, and cysteine biosynthesis

Table 2. Sequences up-regulated in the receptacle of red fruits of transgenic line L4 of *F. vesca* overexpressing *FaPE1*

The expression study was performed in a microarray system as described in the Materials and methods. Sequences were selected after establishing a $P<0.01$. The Bioconductor package limma (Smyth, 2004) was used to identify differentially expressed genes using a moderated *t* test and false discovery rate control (Benjamini and Hochberg, 1995).

GenBank Acc. No.	Sequence description	Fold change L4/wild type
Metabolism		
CO816702	Chlorophyll binding (*LHCA1*)	8.4
GT151146	Chlorophyll a/b binding protein	7.9
CO817454	Chlorophyll a/b binding	7.7
GT149794	Chloroplast chlorophyll a/b binding protein	7.0
CO817033	Photosystem II light harvesting complex gene 1.5	6.9
DY667514	RuBisCO activase	6.7
CX661120	Chlorophyll a/b binding protein CP29	6.5
CX661807	Chlorophyll binding (*LHCA1*)	6.4
DY672111	Fructose-bisphosphatase precursor	6.2
DY672688	Chlorophyll a/b binding protein	6.0
DY671922	Photosystem II subunit Q-2	5.7
DY674768	Chlorophyll binding (*LHCA1*)	5.4
DY669570	Chloroplast ribulose–bisphosphate carboxylase oxygenase activase large protein isoform	5.2
CX661883	Photosystem II light harvesting complex gene 1.4	5.0
DV438556	Photosystem II subunit Q-2	5.0
DY674975	23 kDa polypeptide of oxygen-evolving complex	4.9
DY671983	Photosystem II oxygen-evolving complex protein 3-like	4.7
CO816905	Probable photosystem I chain XI precursor	4.6
DY675836	Photosystem II subunit Q-2	4.6
DY673544	Light harvesting complex of photosystem II 5	4.2
GT150608	RuBisCO activase	4.1
DY668266	Sucrose-phosphate synthase-like protein	4.1
DY673647	Phosphoenolpyruvate carboxykinase	3.9
GT149612	Glyceraldehyde-3-phosphate dehydrogenase a, chloroplast precursor	3.7
GT149992	Glyceraldehyde-3-phosphate dehydrogenase a, chloroplast precursor	3.7
CO817015	Fructose-bisphosphate aldolase	3.5
CO817284	Glycosyl hydrolase family 1 protein	3.1
DY670922	Chlorophyll a/b binding protein 1	3.0
CO817839	Photosystem II subunit Q-2	3.0
CX309712	Fructose-bisphosphate aldolase	2.9
DY672627	Cytochrome P450	2.5
DY673749	Pyruvate orthophosphate dikinase	2.2

Table 2. *Continued*

GenBank Acc. No.	Sequence description	Fold change L4/wild type
GT151838	Succinic semialdehyde dehydrogenase	6.2
DY667672	Cytochrome p450 monooxygenase	6.1
CX661274	Acyltransferase	3.0
GT151410	*Oleo1* (Oleosin1)	2.2
Signalling		
DY671714	Receptor-like kinase	23.3
GT150375	Mitogen activated protein kinase	17.5
GT151311	Phosphoinositide-specific phospholipase C family protein	2.9
DY668719	ATP binding/kinase/protein serine/threonine kinase	2.5
AY429427	MADS domain-containing transcription factor	2.4
DV439963	ATP binding/kinase/protein serine/threonine kinase	1.7
Defence		
DY667796	Metallothionein-like protein	26.7
DY667992	DNAJ heat shock N-terminal domain-containing protein	15.6
DY670442	DNAJ-like protein	11.8
CO381839	NADPH oxidase	7.4
GT148982	Allergen Pru2	4.7
Auxin		
GT149402	Auxin-repressed kDa protein	23.3
DY676028	Auxin-repressed protein	15.2
DV440644	Auxin-repressed 12.5 kDa protein	14.9
GT149171	Auxin-repressed protein	8.9
CO817753	IAA-induced protein 4	4.6
Ethylene		
AJ851829	1-Aminocyclopropane-1-carboxylate oxidase	3.1
Cell wall		
DY674083	β-Xylosidase	25.5
AY486104	β-Xylosidase	20.5
GT150504	Pectinesterase family protein	12.4
AY324809	Pectinesterase family protein (*FaPE1*)	8.4
CX661881	Pectinesterase family protein	8.1
DY672512	Xyloglucan endotransglucosylase/hydrolase 9	3.4
DY675164	Xyloglucan endotransglucosylase/hydrolase 9	3.0
DY673321	β-Glucosidase	2.4
GT150520	Glucan endo-1,3-β-glucosidase	2.2
DY666944	UDP-D-glucuronate 4-epimerase 6	2.2
DY668670	Glucan endo-1,3-β-glucosidase	1.9
Others		
DY674021	Potassium channel	21.0
CO379585	NA	17.0
DY671017	Amino acid permease-like protein	15.7
DV438739	NA	13.7
DY670699	NA	11.6
DY667933	Ankyrin repeat family protein	9.5
DY668571	Peptide transporter	8.5
DY675427	NA	7.9
DY674019	Amino acid transporter family protein	7.8
DY675639	Unknown protein	6.3
DY670803	Serine carboxypeptidase II	6.3
DY669100	Cyclic nucleotide-regulated ion channel protein	6.1
DY671564	Asterase/lipase/thioesterase family protein	5.5
DY669965	NA	5.1
AB219569	NA	5.0

Table 2. *Continued*

GenBank Acc. No.	Sequence description	Fold change L4/wild type
GT151822	NA	4.8
GT149428	NA	4.7
CO379857	NA	4.6
CO817860	Ankyrin repeat family protein	4.6
GT149739	NA	4.2
DY671828	Metal ion binding	4.1
CO817838	DNA binding	4.0
DY669494	Cyclase family protein	3.9
DV440102	NA	3.9
CO381427	NA	3.5
DV440453	NA	3.5
GT151230	Prunin precursor	3.4
CX661321	Zinc transporter 5 precursor	3.3
GT151587	Oligopeptide transporter (yellow stripe-like 2)	3.3
DY672846	Potassium transporter 2	3.2
CX661150	NA	3.2
DY672106	NA	3.1
DV438897	NA	3.1
CO818160	NA	3.1
GT150558	Inositol transporter 4	3.0
DV438540	Cyclin-dependent protein kinase regulator/protein binding	3.0
CX661035	Protein binding/ubiquitin–protein ligase/zinc ion binding	3.0
DY672730	MATE efflux family protein	2.8
GT152272	Nutrient reservoir (Cruciferina)	2.8
DV439423	NA	2.8
GT151843	Nutrient reservoir (Cruciferina)	2.8
DY672238	NA	2.8
GT150218	Nutrient reservoir (Cruciferina)	2.7
DV438492	NA	2.6
CO381589	Nutrient reservoir (Cruciferina)	2.6
DY671641	NA	2.6
DY670874	Histone H3	2.5
DV438317	Metal ion binding	2.5
CO380103	NA	2.4
DY672929	NA	2.4
GT151774	Nutrient reservoir (Cruciferina)	2.4
CO816755	Calmodulin binding/catalytic	2.3
CO817458	NA	2.3
DY674055	Zinc finger (C2H2 type) family protein	2.3
DY672630	Binding	2.2
DY666754	Calcium-dependent cysteine-type endopeptidase/cysteine-type endopeptidase	2.1
DY667692	NA	2.1
DY674677	NA	2.1
DY672812	EDA30 (embryo sac development arrest 30)	2.1
DY668283	NA	2.0
CO816895	NA	2.0
DV438752	Xanthine/uracil permease family protein	1.9
DY672213	Male sterility MS5 family protein	1.9

Table 3. Sequences down-regulated in the receptacle of red fruits of transgenic line L4 of *F. vesca* overexpressing *FaPE1*

The expression study was performed in a microarray system as described in the Materials and methods. Sequences were selected after establishing a $P<0.01$. The Bioconductor package limma (Smyth, 2004) was used to identify differentially expressed genes using a moderated *t* test and false discovery rate control (Benjamini and Hochberg, 1995).

GenBank Acc. No.	Sequence description	Fold change wild type/L4
Metabolism		
DY668170	β-Amylase	39.1
GT151071	Glycosyl hydrolase family expressed	22.4

Table 3. *Continued*

GenBank Acc. No.	Sequence description	Fold change wild type/L4
CO816906	β-Amylase	20.2
CO817208	Glycosyl hydrolase family expressed	18.4
CO817751	*O*-acetylserine (thiol) lyase (*OAS-TL*) isoform A1	18.0
DY668554	UDP-glucose glucosyltransferase	17.5
GT152052	Cysteine synthase	16.3
CO817617	Carbonic anhydrase	13.2
GT151016	UDP-glucose glucosyltransferase	5.5
CX661291	Carbonic anhydrase 1	5.1
DY671280	UDP-glucose glucosyltransferase	4.8
DY669935	UDP-glycosyltransferase	4.4
DY671623	Cinnamyl-alcohol dehydrogenase	3.9
CO816952	Serine acetyltransferase 1	3.8
DY670430	UDP-glucose glucosyltransferase	3.8
CO381569	Alternative oxidase 1A	3.7
GT151580	Sulphate adenylyltransferase (ATP)	2.5
DY675250	2-Oxoglutarate-dependent dioxygenase	2.4
DY670982	Pyridoxine biosynthesis 1.2	2.3
DY674635	Oxidoreductase	2.1
Signalling		
DY668308	Serine threonine protein kinases	15.5
DY672721	Sterol regulatory element-binding protein site 2 protease	8.2
DY670038	Sterol regulatory element-binding protein site 2 protease	7.8
DY668034	Leucine zipper	5.1
Defence		
GT149690	Small heat shock protein	34.6
CX309745	Heat shock protein	34.3
DY671878	Heat shock protein 18	12.7
DV439408	Thioredoxin-like	5.1
CX661962	Pathogenesis-related thaumatin family protein	4.8
GT151254	Lipid transfer protein	4.7
GT150102	Heat shock protein 18.2	3.2
GT150184	Heat shock protein 18.2	2.6
CO817046	Pollen Ole e 1 allergen and extensin family protein	2.5
DY673431	Disease resistance protein (NBS-LRR class)	2.5
CX662137	Heat shock protein 18.2	2.5
CO817988	Heat shock protein 18.2	2.4
GT148962	Heat shock protein 17.4	2.4
DY667157	Heat shock protein 17.6 kDa class II	2.3
GT150281	Heat shock protein 17.6 kDa class II	2.1
DY670885	Heat shock protein 18.2	2.1
CX661280	Heat shock N-terminal domain-containing protein	2.1
CX309670	Heat shock protein 70	2.0
CX662201	Mitochondrion-localized small heat shock protein 23.6	1.9
Hormone		
DY676220	ABA- and ripening-induced protein	4.3
DV439312	Abscisic stress ripening-like protein	4.1
Cell wall		
DY675690	Pectinesterase inhibitor	10.7
DV439771	Peroxidase	7.6
DY635780	Peroxidase	6.0
Others		
DY667655	NA	134.1
CO381782	NA	105.2
CX662074	Protein	73.3
GT150988	Protein	44.0
DY676171	NA	32.1

Table 3. *Continued*

GenBank Acc. No.	Sequence description	Fold change wild type/L4
CX661434	NA	31.4
GT149209	NA	30.0
CX661917	Hypothetical protein	27.2
GT151111	NA	24.8
CX661399	Hypothetical protein	21.9
GT149504	NA	20.1
GT149218	NA	19.3
DY671058	Polynucleotide phosphorylase polyadenylase	19.0
CO378910	NA	9.5
DY671369	Hypothetical protein	9.3
CO381802	NA	9.2
DY675861	Glutamate receptor 2.3	8.8
GT151351	Unknown protein	7.7
DY666709	Hypothetical protein	6.7
GT151547	Hypothetical protein	6.4
DY673480	Multidrug efflux family protein	6.2
CO816985	Hypothetical protein	6.1
DY669410	Hypothetical protein	6.1
DY670906	Porin-like protein	5.6
DV440632	NA	4.9
CO381788	NA	4.9
DV439808	NA	4.8
DV438560	Unknown protein	4.8
GT150021	Cinnamoyl CoA reductase-like protein	4.8
DY671815	NA	4.7
DV439862	NA	4.6
GT150217	NA	4.6
DY671827	NA	4.6
DY670432	NA	4.4
DY672652	NA	4.2
DY668601	NA	4.1
DY667680	RPT2 (root phototropism 2) protein binding	4.0
DY669461	NA	3.9
DV439643	NA	3.8
CX661927	NA	3.8
DY674943	NA	3.6
CO380657	NA	3.6
CO380695	NA	3.6
CO381358	NA	3.4
CX661524	Yellow-leaf-specific gene 8	3.3
CX661866	UVB-resistance protein-related/regulator of chromosome condensation (*RCC1*) family protein	3.3
CO378645	NA	3.1
DY674097	NA	3.1
CO379715	NA	3.1
DY673421	Vacuolar sorting protein 9 domain-containing protein	3.0
DY671602	DNA-binding family protein	3.0
DY668516	*Phytoclock 1*; DANN binding	2.9
DY668737	Low PSII accumulation 1	2.8
GT149149	NA	2.8
CX661552	Fibrillin	2.8
DY667414	Mitochondrial substrate carrier family protein	2.8
DY671736	Lipocalin	2.7
DV439266	Sugar transport protein 13	2.7
CO381215	NA	2.7
CO380557	NA	2.6
DY675350	GTP binding/GTPase	2.6
GT151004	NA	2.5
DY675275	NA	2.5
DV440067	NA	2.5

Table 3. *Continued*

GenBank Acc. No.	Sequence description	Fold change wild type/L4
DY669887	Zinc finger (B-box type) family protein	2.5
CO380577	NA	2.5
CO380259	NA	2.5
CO816724	NA	2.5
DY673668	Potassium:hydrogen antiporter	2.4
DY675052	*SOUL-1*; binding	2.4
DY669425	Camphor resistance CrcB family protein	2.3
CO381340	NA	2.3
CO380041	NA	2.3
DY671656	NA	2.2
DY675816	NA	2.2
DY670526	DNA binding/calmodulin binding/transcription factor (*TGA1*)	2.2
GT151076	Temperature-induced lipocalin	2.1
CX661704	NA	2.0
CO381906	NA	2.0
DV439251	NA	2.0
DY673901	Hydrolase, α/β-fold family protein	2.0
GT151835	Identical protein binding/serine-type endopeptidase	1.9
DY672131	WD-40 repeat family protein	1.8

as reflected by the changes in cysteine synthase, *O*-acetylserine lyase, and serine acetyltransferase (Table 3).

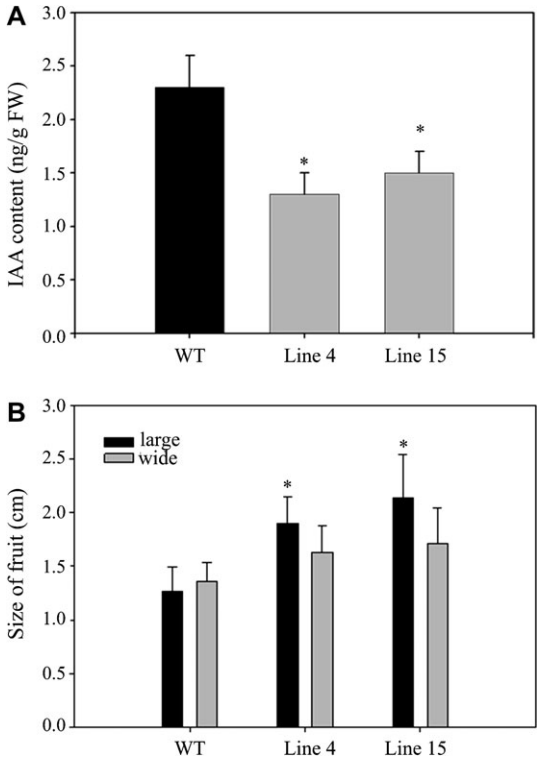

Fig. 4. Analysis of auxin content and size of red fruits of *F. vesca* overexpressing *FaPE1*. (A) Auxin (IAA) content (ng g^{-1} FW) from *F.vesca* wild-type and *FaPE1* transgenic plants (line 4 and line 15). Size, large and wide, of red fruits from *F.vesca* wild-type and *FaPE1* transgenic plants (line 4 and line 15). An asterisk denote values that are significantly different as determined by *t* test ($P<0.01$).

Also noteworthy are the changes in genes involved in signalling pathways such as the sterol regulatory element-binding protein site 2 protease, which has been described to be directly involved in the regulation of lipid metabolism (Duncan *et al.*, 1997), and a serine threonine protein kinase. However, major changes occur in genes encoding proteins of unknown function.

Fruits of two independent lines of F. vesca *overexpressing* FaPE1 *exhibit higher expression of defence-related and cell wall-modifying genes than wild-type* F. vesca

The drastic changes found in the expression of transgenic line 4 (L4) compared with wild type, were further confirmed by qRT-PCR studies. Moreover, the study was extended to another independent transgenic line (L15), which showed a similar phenotype to L4. Thus, amongst the genes that are up-regulated in transgenic *F. vesca* L4 compared with wild type, one of the largest fold changes (>23-fold) was exhibited by a sequence (Acc. No. DY671714) annotated as encoding a protein kinase belonging to the family of receptor-like kinases (RLKs) (Table 2), here named *LRR1*. Some members of this family play a central role in signalling during pathogen recognition, and subsequent activation of plant defence mechanisms (Afzal *et al.*, 2008). In the authors' microarray analysis another RLK (Acc. No. DY668749), named *LRR2*, was identified that displayed a >5-fold increase in expression in the transgenic line L4 ($P\leqslant0.05$; Supplementary Table S2 at *JXB* online). The expression of *LRR1* and *LRR2* was further evaluated by qRT-PCR in the receptacle of red fruits of wild-type *F. vesca* and two different transgenic lines (L4 and L15) overexpressing *FaPE1*. As shown in Fig. 5A, *LRR1* and *LRR2* showed increased expression in the transgenic lines

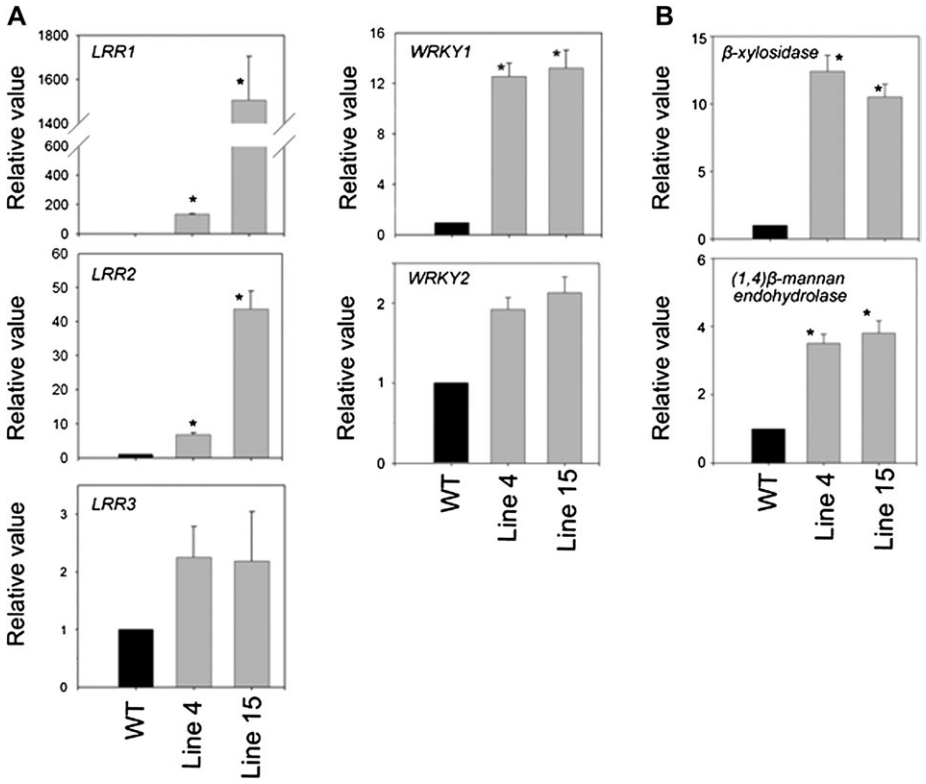

Fig. 5. Expression of defence and cell wall-related genes in *F. vesca* red fruits overexpressing *FaPE1*. (A) Expression of kinases and *WRKY* transcription factor genes. (B) Cell wall-related genes. The values represent the mean±standard error of four individual plants. An asterisk indicates values determined by *t* test to be significantly different from the wild-type value ($P<0.01$). Genes are as follows: *LRR1*, DY671714; *LRR2*, DY668749; *LRR3*, AJ871783; *WRKY1*, DY667265; *WRKY2*, AJ871772; β-xylosidase, AY486104; (1,4)β-mannan endohydrolase, GT149809.

L4 and L15 compared with the wild type, thus confirming the microarray results. Previous studies on strawberry transcriptional changes associated with infection by *Colletotrichum* identified another member of this family, *Falrrk-1* (Acc. No. AJ871783) whose expression was higher in a moderately resistant cultivar (Andana) compared with another susceptible cultivar (Camarosa) (Casado-Díaz *et al.*, 2006). The expression of this gene, here named *LRR3*, was not significantly different in the L4 and L15 transgenic lines of *F. vesca* in comparison with the wild type (Fig. 5A).

The microarray study identified a *WRKY*-type gene (*WRKY1*, Acc. No. DY667265), with higher expression in the ripe receptacle of the L4 *F. vesca* compared with the wild type (6.5-fold; $P\leqslant0.17$; Supplementary Table S2). WRKY proteins form a superfamily of transcription factors involved in the regulation of various physiological programmes, including pathogen defence (Eulgem *et al.*, 2000). Expression analysis in strawberry after *Colletotrichum* infection also identified a different *WRKY*-type gene, *Fawrky-1* (*WRKY2*, Acc. No. AJ871772), whose transcripts were highly increased after infection (Casado-Díaz *et al.*, 2006). Expression of these *WRKY*-type genes was studied in the ripe receptacle of *F. vesca* and the two transgenic lines, L4 and L15. Whereas the expression of *WRKY1* was higher in the transgenic L4 and L15 compared with the wild type of *F. vesca*, there was no difference in the expression of *WRKY2* between these lines (Fig. 5A).

It is known that changes in the cell wall might be a mechanism by which plants protect themselves from pathogenic penetration (Hückelhoven, 2007; Hématy *et al.*, 2009). Overexpression of *FaPE1* has been shown to produce significant changes in the composition of the cell wall (Osorio *et al.*, 2008). In microarray expression analysis it was found that a gene encoding a β-xylosidase (Acc. No. AY486104) showed >20-fold increased expression in transgenic *F. vesca* (L4) (Table 2). Analysis of its expression by real-time PCR in two different transgenic lines of *F. vesca* overexpressing *FaPE1* (L4 and L15) confirmed the microarrays results (Fig. 5B). Analysis of the expression by qRT-PCR of other genes involved in cell wall synthesis, modification, or degradation only gave a significant difference between wild-type and transgenic *F. vesca* in the case of the gene encoding (1,4)-β-mannan endohydrolase (Acc. No. GT149809) (2.6-fold; $P\leqslant0.02$; Supplementary Table S2, at *JXB* online), which was induced in the transgenic lines (Fig. 5B).

Discussion

Transgenic F. vesca *fruits display characteristics of active defence response*

Metabolomic and transcriptomic studies were restricted to the receptacle of the ripe fruit where resistance to

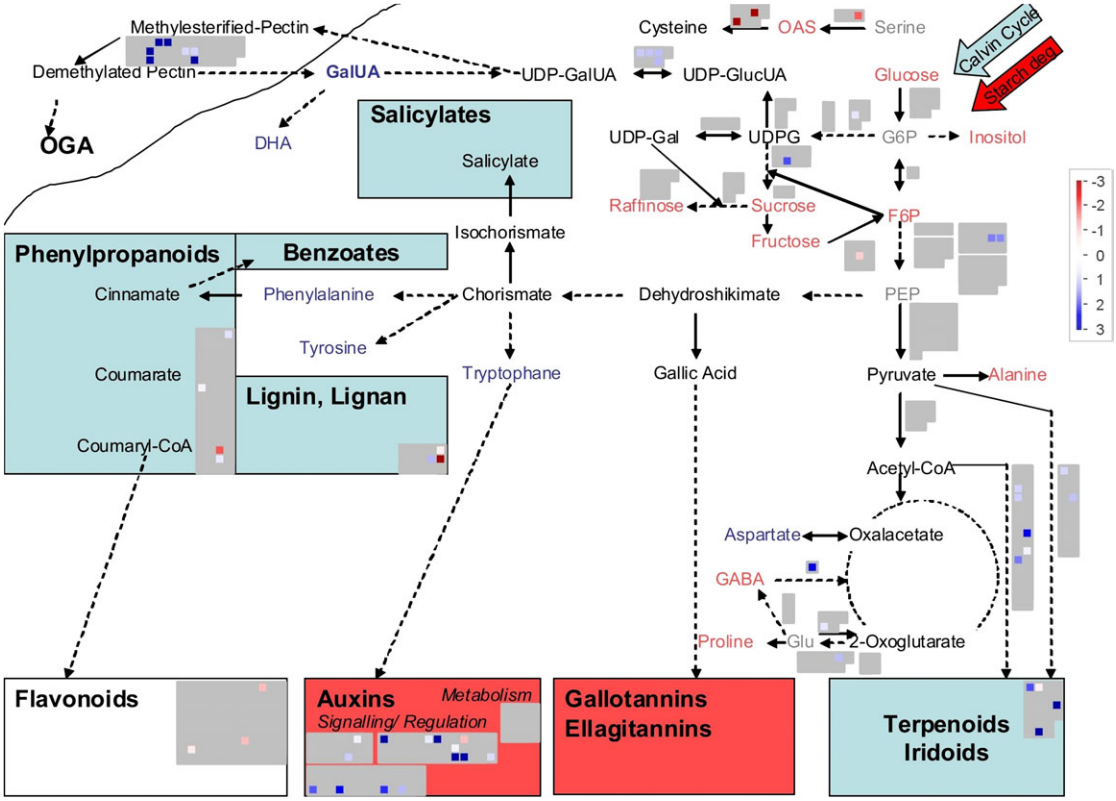

Fig. 6. Overview of changes in metabolites and transcripts upon overexpression of *FaPE1* in *F. vesca*. Transcripts and metabolites are visualized as small squares and the metabolite name, respectively. Up-regulated transcripts are depicted in shades of blue where up-regulation of 3 log2 units leads to saturation in blue and down-regulation by 3 log2 units to saturation in red. Metabolites are only shown in one shade of blue or red, if a significant change was detected to enhance the visibility. Metabolites that were not measured are depicted in black and metabolites where no significant change could be detected in either line are shown in grey. Secondary metabolite classes are depicted as coloured boxes, where the colour of the box is either red, if most metabolites are down-regulated, blue if most are up-regulated, or white if no clear response was detected. Transcripts responsible for the interconversion of metabolites in these classes are shown within the boxes. Solid arrows represent a single reaction whereas dashed arrows indicate a sequence of reactions.

inoculation by fungal spores was previously found (Osorio *et al.*, 2008). An overview of the changes in metabolites and expression of corresponding genes after overexpression of *FaPE1* in *F. vesca* is presented in Fig. 6.

In relation to metabolic changes a general decrease of the most abundant sugars in the receptacle of ripe fruits coincident with the up-regulation of many genes involved in the photosynthetic carbon assimilate processes was observed. The metabolic fate of these assimilates would appear to be aromatic amino acids, and secondary metabolites derived thereof, especially phenolic compounds, flavanones, and terpenoid derivatives. In this last group, changes are dramatic for sesquiterpenoids and their hexose derivatives. Plant secondary metabolites with antimicrobial activity, also known as phytoalexins, are low-molecular-weight compounds that are structurally diverse and often restricted in their occurrence to a limited number of plant species. It is known that in *Solanaceus* plants sesquiterpenoid phytoalexins are synthesized (Kuc, 1995). Although the final chemical structure of the sesquiterpenoids found in *F. vesca* are not known it can be hypothesized that they might play a defence role in this species. Equally, a defence role against *B. cinerea* can be attributed to the increase in some of the phenolic compounds in transgenic *F. vesca*, since in the cultivated strawberry it has been reported that lower content of these compounds led to higher susceptibility to *B. cinerea* (Hanhineva *et al.*, 2009). In the case of amino acids higher changes were in Asp and aromatic amino acids, and a genome-wide *in silico* analysis found that a group of the Asp-family catabolic genes and the highly coordinated aromatic amino acid groups were both positively associated with fungal response genes (Less and Galili, 2009).

Regarding transcript changes, in addition to those directly related to the metabolic changes (Fig. 6), changes associated with a defence response are reported here. In this category should be included genes encoding PR10 proteins, WRKY (Eulgem *et al.*, 2000), and metallothioneins (Butt *et al.*, 1998; Dauch and Jabaji-Hare, 2006). Also, the diminished expression of the glucosyltransferase genes may also represent a specific defence response. Glycosyltransferases constitute a broad family of enzymes that are able to recognize very different substrates, from hormones to secondary metabolites, using UDP-glucose as the most common sugar donor in plants (Bowles *et al.*, 2006). It has been recognized that glycosylation/deglycosylation alters the bioactivity of some defence compounds (Morrissey and

Osbourn, 1999). Moreover, the antimicrobial activity of some metabolites such as saponins is related to the maintenance of the sugar residues (Morrissey and Osbourn, 1999). Finally, induced expression of a gene encoding ACC oxidase could be associated with defence since at least in tomato ethylene responses are important for resistance of tomato to *B. cinerea* (Díaz *et al.*, 2002).

The relative contribution to *B. cinerea* resistance of cell wall modifications and concomitant production of secondary antimicrobial metabolites has often been questioned. Here, significant transcriptional changes in genes encoding β-xylosidase and (1,4)-β-mannan endohydrolase are reported. The polymer target of these two genes is hemicellulose (Martínez *et al.*, 2004; Moreira and Filho, 2008). Whilst the exact function of these two hemicellulose-degrading enzymes is not clear within the plant defence response, there are reports on the induction of endoxylanases as elicitors of defence responses in plants (Beliën *et al.*, 2006) that could potentially also hold true for necrotrophic pathogens such as *B. cinerea*. In summary, these metabolic and expression data support that defence response is activated in the receptacle of transgenic *F. vesca* overexpressing *FaPE1*.

Auxin is involved in the defence response and developmental changes in transgenic F. vesca *overexpresing* FaPE1

The presence of OGAs, with some requirements of size and degree of methylation, has been previously associated with plant defence response (De Lorenzo and Ferrari, 2002; Field *et al.*, 2006; Van Loon *et al.*, 2006). There is also a documented role for these compounds in plant development (Côté and Hahn, 1994; Melotto *et al.*, 1994). In addition to OGAs, auxin has been directly involved in this crosstalk between defence and development in plants. In addition to the largely known role played by auxin in plant development (Zhao, 2010) more recently it has been demonstrated effectively that this hormone modulates plant defence responses (Wang *et al.*, 2007; Zhang *et al.*, 2007; Kazan and Manners, 2009). More interestingly, there are also reports illustrating antagonism between auxin and the action of OGAs (Ferrari *et al.*, 2008).

It was found that transgenic plants of *F. vesca* had an altered development pattern. The greater size in two independent lines expressing *FaPE1* was clear at the vegetative stage (results not shown) and in fruits. Mediation of auxin in these developmental changes is supported by the following arguments. First, and most pertinently, auxin content in the receptacle of the transgenic fruits at the ripe stage was significantly lower, and this is accompanied by enhanced expression of several auxin-repressed genes. Second is that growth of the strawberry fruit receptacle is known to be dependent on auxin delivered from the achenes in the early stages (Nitsch, 1950), but later in development it is the decrease in auxin in the receptacle that triggers the ripening process since there are many ripening genes that are negatively regulated by auxin (Aharoni *et al.*, 2002). It is anticipated that this pivotal role played by auxin in strawberry fruit development holds true for the fruits of *F. vesca*. The question remains as to whether this reduced auxin content is also related to the defence phenotype presented by the transgenic fruits (Osorio *et al.*, 2008). At least in other species that seems to be the case; for instance in *Arabidopsis*, pathogen defence against *Pseudomonas syringae* (Chen *et al.*, 2007) and against *B. cinerea* (Llorente *et al.*, 2008) appears to be modulated by the auxin produced following pathogen challenge, since a functional auxin signalling pathway is required for an effective defence response. Furthermore, there are reports that directly link auxin changes to plant defence response (Park *et al.*, 2007; Ding *et al.*, 2008)

Ripe transgenic fruits were not only lower in auxin content when compared with wild type but also contained a new fraction of partially demethylated OGAs with eliciting capacity (Osorio *et al.*, 2008). Both compounds, OGAs and auxin, have been reported to be involved in plant development and plant defence with many reports showing antagonistic roles for OGAs and auxin in different aspects of plant development and defence (Branca *et al.*, 1988; Bellincampi *et al.*, 1993; Ferrari *et al.*, 2008). It would be important to know whether or not they act independently or if there is an interaction between their signalling pathways. For this purpose it is important to consider that the first event in this model is the demethylating activity of PME and the subsequent generation of partially demethylated OGAs. Accordingly, the molecular changes that follow, including a change in auxin content, would be downstream processes initiated by this initial change. Therefore, change in auxin content is a direct or indirect consequence of the partially demethylated OGA build-up in the transgenic fruits. Intermediate steps in this crosstalk are presently unknown. It is speculated here that the first interaction of OGAs occurs with RLK proteins, which have been reported to play a central role in pathogen recognition and downstream steps of plant defence (Afzal *et al.*, 2008). The corresponding genes are among those with highest increased expression in transgenic *F. vesca*.

FaPE1 in F. ananassa *has a role in the production of partially demethylated OGAs in ripe fruits*

The expression of three PME-encoding genes in the fruits of strawberry (*F. ananassa*) that show a different pattern during development has been reported previously (Castillejo *et al.*, 2004). Here it is shown that the global content of all PMEs increases in the fruits as they ripen, and discrepancy between enzyme level and demethylation of pectins might be indicative of the different capacity of demethylating pectins *in muro*. FaPE1 is one of these enzymes specifically expressed in fruit that displays increasing expression during the ripening process up to a maximum at the turning stage (Castillejo *et al.*, 2004). When *FaPE1* is expressed in wild-type *F. vesca* the occurrence of a new OGA fraction (Osorio *et al.*, 2008) that has a deep effect on defence and fruit development of the transgenic plants has been detected.

This OGA fraction is also present in the ripe fruits of strawberry (*F. ananassa*) that naturally expresses *FaPE1*. It is here proposed that FaPE1 plays a central role in the endogenous production of OGAs in the fruits of this species, in addition to a possible role in demethylating pectins.

OGAs are α-1,4-oligogalacturonides probably resulting from the action of endo-polygalacturonase on the pectin polymers of the cell wall (Ridley *et al.*, 2001). In strawberry two genes encoding endo-polygalacturonases have been reported to be expressed in fruits with different patterns as development proceeds, and it has been proposed that one (*FaPG1*) is involved in the release of OGAs (Quesada *et al.*, 2009). The presence of partially demethylated OGAs here reported will require the activity of a PME in addition to the polygalacturonase activity. Since it is known that FaPE1 is able to produce block-wise demethylation of pectins in transgenic *F. vesca* (Osorio *et al.*, 2008), and the highest expression of FaPE1 in *F. ananassa* fruits is at the turning stage (Castillejo *et al.*, 2004), prior to the peak of FaPG1 expression (Quesada *et al.*, 2009), we deduce that activity of FaPE1 might proceed to FaPG1 activity. All together, these data and those in the literature indicate that FaPE1 in cultivated *F. ananassa* plays a role not only in pectin disassembly during fruit ripening, but also in the generation of the fraction of partially demethylated OGAs.

Concluding remarks

The combined results presented here demonstrate that the eliciting OGAs produced in the diploid *F. vesca*, as a result of the expression of *FaPE1*, trigger a highly coordinated and specific response that is evident at both transcriptional and metabolite levels (MapMan; http://tinyurl.com/OsorioReviewerLink; Supporting online information; Usadel *et al.*, 2009). It is contested here that these results reflect the role played by naturally produced OGAs in plant defence. In addition, the specificity of the chemical composition and structure of these OGAs is also emphasized as it is demonstrated that the degree of demethylation is critical for the triggering of the plant response. The presence of partially demethylated OGAs is associated with a decrease in the auxin content, thus providing an example of the interaction of these two compounds in plant developmental and defence responses. Exhaustive analysis performed here of two *F. vesca* genotypes, differing in the expression of a single gene encoding PME, provides an important system for advancing the knowledge of this pathogen response–development interplay with OGAs and auxin as active compounds.

Supplementary data

The following materials are available in the online version of this article.

Supplementary Table S1. Absolute metabolites concentrations (μg g^{-1} FW) of red fruits of *F.vesca* plants overexpressing *FaPE1*. Value are presented as mean±SE of six replicates; values set in bold type were determined by ANOVA analysis to be significantly different ($P<0.05$) from the wild type.

Supplementary Table S2. Tentatively identified metabolites in the UPLC-FT-ICR-MS and their abundancy in red fruits of *Fragaria vesca* plants overexpressing *FaPE1*. Metabolites were determined in receptacle from red fruits. Data are normalized to the mean response calculated for the wild type. Values presented are mean±standard error of six replicates and are significant differences by ANOVA analysis ($P<0.01$).

Supplementary Table S3. Microarray expression data.

Supplementary Table S4. Primers.

Supporting online data. MapMan; http://mapman.gabipd.org/web/guest/mapmanweb.

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