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Functional Genomics in Strawberry Fruit through RNAi-mediated Silencing

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ABSTRACT

Down-regulation of gene expression by RNA interference (RNAi) has become a powerful tool to investigate gene functions *in vivo*. In this review we examine how RNAi has been used to assess gene function in strawberry fruit, including molecular mechanisms and analysis of silenced phenotypes. The down-regulated genes include *FaCHS*, *FaOMT*, *FaGT1*, *FaDFR*, *FaANS*, and *Fra* encoding proteins functioning in the flavonoid biosynthetic pathway. A comparison with stably transformed genotypes shows how spatial silencing of gene expression in fruit may affect metabolite patterns.

Keywords: firmness, flavonoid pathway, fruit ripening, pigments, quality, RNA interference

CONTENTS

INTRODUCTION

Fruit are rich in essential nutrients and are an important part of the human diet. Besides, they contain secondary plant products that bring benefits to human health (Schreiner and Huyskens-Keil 2006). Strawberries, for example are an excellent source of vitamin C and numerous health-promoting metabolites (Agius *et al*. 2003; Szajdek and Borowska 2008). To fully exploit the nutritional value of strawberry, researchers need access to the entire genetic code (genome) of strawberries as well as research tools that will allow them to study the roles of the numerous genes and to engineer beneficial agronomic traits such as pathogen resistance, drought tolerance, and fruit quality into strawberry crops (Yonekura-Sakakibara and Saito 2006).

An international consortium of researchers has already sequenced the genome of the diploid woodland strawberry, *Fragaria vesca*, which has one of the smallest genomes of economically significant plants. The 14 chromosomes of the genome comprise a total of 206 million base pairs of DNA (Shulaev *et al*. 2008, 2011). In addition to the published *F. vesca* genome sequence, a large set of approximately 50,000 expressed sequence tags (ESTs) from *F. vesca* and several thousands from the cultivated octoploid strawberry *F. x ananassa* have been deposited in GenBank. Besides, microarray experiments monitoring gene transcription activity during fruit development is available (Aharoni and O'Connell 2002).

But completely sequencing an organism's genome is just the beginning of our understanding of that organism's biology. All of the genes still need to be identified. The function of the gene products (functional RNAs and proteins) must be elucidated and the non-coding regulatory sequences need to be understood. Determination of the gene's role presents a tremendous challenge, not only because of the large number of genes to be examined, but also because defining what constitutes a 'gene' is itself a complex problem (Alonso and Ecker 2006).

FORWARD AND REVERSE GENETICS

Gene function analysis is currently performed by two fundamentally different approaches (Tisser and Bourgeois 2001). Forward genetics seeks to identify mutations that produce a certain phenotype. Conversely, reverse genetics begins with a candidate gene and analyzes the mutant

Received: 22 December, 2009. Accepted: 19 April, 2011.

Invited Review

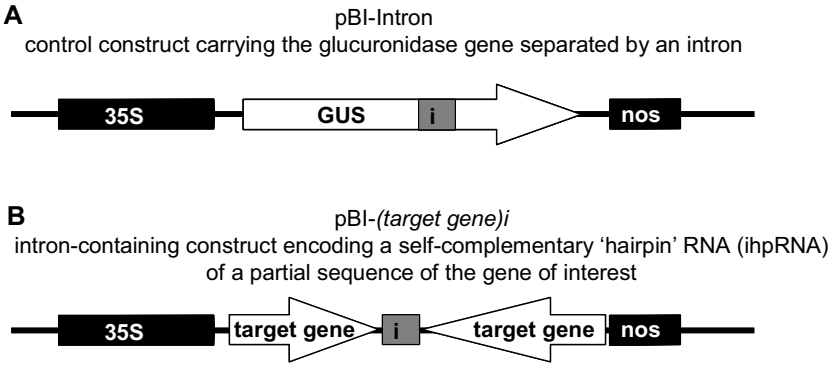

Fig. 1 Constructs used for RNAi-mediated transient gene silencing in ripening strawberry fruit. Control construct (**A**) and ihpRNA constructs (**B**).

phenotype that results upon its disruption. The advent of whole genome sequencing has led to an increased interest in reverse genetic methodologies (Alonso and Ecker 2006). Using reverse genetics, it is possible to investigate the function of all genes in a gene family, something not easily done with forward genetics. Further, one can study the function of a gene found to be involved in a process of interest in another organism, but for which no forward genetic mutants have yet been identified. Finally, the vast majority of genes have not yet been mutated in most organisms and reverse genetics allows their study. The availability of complete genome sequences combined with reverse genetics can allow every gene to be studied.

RNAi

A key to the characterization of gene function by reverse genetics is down-regulation of endogenous genes via post-transcriptional gene silencing (PTGS). Among different types of PTGS, RNA interference (RNAi) denotes a sequence-specific gene-silencing mechanism that is initiated by the introduction of double-stranded RNA (dsRNA), homologous in sequence to the silenced gene, which triggers degradation of mRNA (Filipowicz *et al*. 2005). RNAi utilizes the endonuclease Dicer to generate small interfering RNAs (siRNAs) from dsRNA. The RNAi induced silencing (RISC) complex then destroys specific target mRNAs based on sequence complementarities with the siRNA. RNAi-based silencing is an excellent strategy for reverse genetics in plants (Small 2007). It has become a powerful tool to silence the expression of target genes and study their loss-of-function phenotype, allowing analysis of gene function when mutant alleles are not available.

TRANSIENT ASSAYS

Commonly, reverse genetics is carried out by generation and evaluation of stable transgenic plants that show higher or lower transcript levels for the gene of interest (Folta and Dhingra 2006). This process is labor-intensive, time-consuming, and usually takes several months depending on the plant species used. In addition, transgene expression in transgenic plants often varies significantly due to insert position and other effects, thus complicating data analysis. Transient assays such as biolistic transient transformation, polyethylene glycol mediated transformation and electroporation provide a convenient alternative to stable transformation. In plants, the most widely used method, commonly named agroinfiltration, makes use of *Agrobacterium tumefaciens* to deliver transgenes into cells (Hellens *et al*. 2005). Compared to the generation of stably transformed plants, agroinfiltration is more rapid, and samples can be analyzed a few days after inoculation. Agroinfiltration has been used successfully in many different applications, including the analysis of disease resistance genes, plant promoters and transcription factors (Yang *et al*. 2000; Santos-Rosa *et al*. 2008).

GENE FUNCTION ANALYSIS IN STRAWBERRY

A collection of characterized protein-coding genes of *Fragaria*, including genes involved in fruit softening, pigment and aroma formation shows that functional genomics in strawberry is mainly confined to *in silico* prediction and gene expression analysis (**Table 1**). Although informative, these types of data alone are typically not sufficient to define the function of a gene, as by its very nature this information is largely correlative. Only in a few cases more reliable methods such as the testing of recombinant proteins for catalytic activity and reverse genetics approaches were

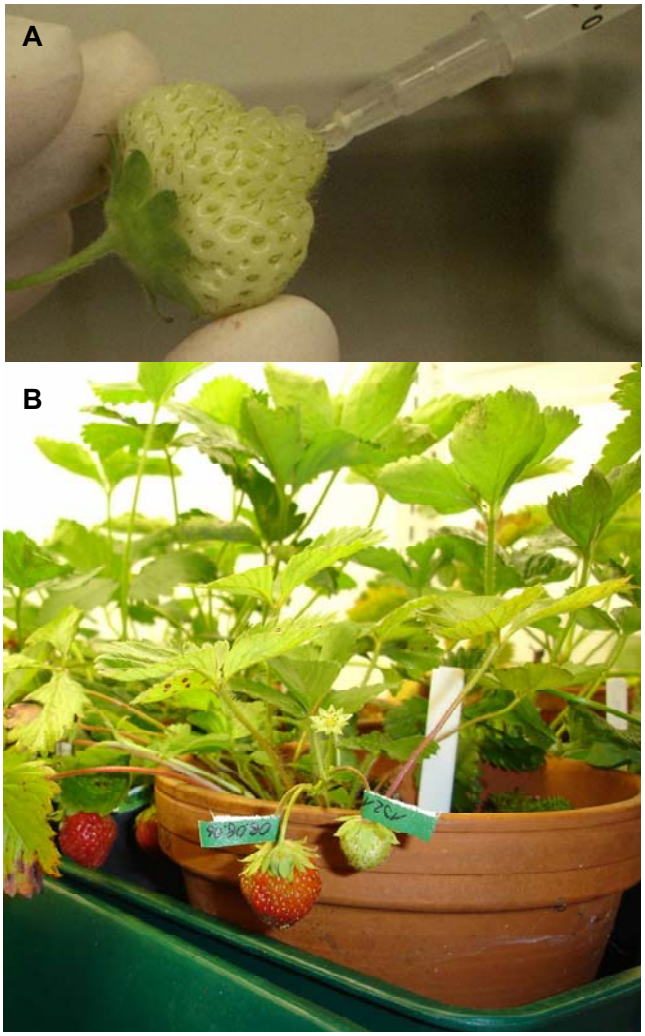

Fig. 2 Agroinfiltration of strawberry fruit and ripening of agroinfiltrated fruits. Fruits are infiltrated with a suspension of *Agrobacterium tumefaciens* harboring ihpRNA-encoding constructs (**A**). After agroinfiltration the fruits remain attached to the plant until full maturity (**B**).

Table 1 Functionally characterized structural genes in *Fragaria*.

Gene	Enzyme	Putative function	Method	Reference
		Enzyme coding genes		
FaEG3	endo-β-(1,4)-glucanase	softening, cellulose degradation	antisense	Mercado *et al.* 2010
Facel1/2	endo-β-(1,4)-glucanase	no effect on firmness	antisense	Palomer *et al.* 2006
Facel1/2	endo-β-(1,4)-glucanase	softening, cellulose degradation	antisense	Woolley *et al.* 2001
Facel1/2	endo-β-(1,4)-glucanase	softening, cellulose degradation	expression analysis	Harpster *et al*. 1998; Llop-Tous *et al.* 1999
FaEG1/3	endo-β-(1,4)-glucanase	softening, cellulose degradation	expression analysis	Trainotti *et al*. 1999
FaBG2-1/2/3	β-1,3-glucanase	pathogenesis-related	expression analysis	Khan *et al*. 2003; Shi *et al*. 2006
FaXyl1	β-xylosidase	softening, hemicellulose degradation	expression analysis	Martinez *et al*. 2004; Bustamante *et al.* 2009
FaXyl1	β-xylosidase	softening, hemicellulose degradation	expression analysis; recombinant protein testing	Bustamante *et al*. 2006
FaAra1/2/3	α-L-arabinofuranosidase	softening, hemicellulose degradation	expression analysis	Rosli *et al*. 2009
FaPE1 to 4	pectin methyl esterase	softening, pectin degradation	expression analysis	Castillejo *et al*. 2004
FaPE1 to 4	pectin methyl esterase	elicitation of defense responses	overexpression	Osorio *et al*. 2008
FaplA/B	pectate lyase	softening, pectin degradation	expression analysis	Medina-Escobar *et al.* 1997; Benitez-Burraco *et al*. 2003
Fapl	pectate lyase	softening, pectin degradation	antisense	Jimenez-Bermudez *et al*. 2002; Sesmero *et al*. 2007; Santiago-Domenech *et al*. 2008; Sesmero *et al*. 2009
FaplC	pectate lyase	softening, pectin degradation	cosuppression	Youssef *et al*. 2009
FcPG1, FcPL1	polygalacturonase, pectate lyase	softening, pectin degradation	expression analysis	Figueroa *et al*. 2008
FaPG	polygalacturonase	softening, pectin degradation	antisense	Garcia-Gago *et al*. 2009, Quesada *et al*. 2009
SpG, FaPG	polygalacturonase	softening, pectin degradation	expression analysis	Redondo-Nevado *et al.* 2001; Villarreal *et al*. 2008; Villarreal *et al.* 2009
FaChi2-1/2-2	chitinase	pathogenesis-related	expression analysis	Khan and Shih 2004
Faßgal1/2/3	β-galactosidase	softening	expression analysis, recombinant protein testing	Trainotti *et al.* 2001
FaCCR	cinnamoyl CoA reductase	firmness	expression analysis	Salentijn *et al.* 2003
FaCAD	cinnamyl alcohol dehydrogenase	firmness	expression analysis	Salentijn *et al*. 2003
FaCAD1/2	cinnamyl alcohol dehydrogenase	firmness	expression analysis, recombinant protein testing	Blanco-Portales *et al*. 2002
GalUR	D-galacturonic acid reductase	L-ascorbate biosynthesis	overexpression	Agius *et al*. 2003; Hemavathi *et al*. 2009
FaGLDH	L-galactono-1,4-lactone	L-ascorbate biosynthesis	expression analysis	Oliveira do Nascimento *et al*. 2005
SAAT	alcohol acyl-CoA transferase	aroma, fruit ester formation	expression analysis, recombinant protein testing	Aharoni *et al*. 2000
FcAAT1	alcohol acyl-CoA transferase	aroma, fruit ester formation	expression analysis	Gonzalez *et al*. 2009
FvAAT	alcohol acyl-CoA transferase	aroma, fruit ester formation	recombinant protein testing, overexpression	Beekwilder *et al*. 2004
FaAAT	alcohol acyl-CoA transferase	aroma, fruit ester formation	expression analysis	Carbone *et al*. 2006
FaNES1	S-nerolidol/S-linalool synthase	aroma, mono- and sesquiterpene formation	recombinant protein testing	Aharoni *et al*. 2004
FaNES1	S-nerolidol/S-linalool synthase	aroma, mono- and sesquiterpene formation	overexpression	Aharoni *et al.* 2004; Yang *et al*. 2008
FaPIN	pinene synthase	aroma, monoterpene formation	recombinant protein testing	Aharoni *et al.* 2004
FaPHy	pinene hydroxylase	aroma, monoterpene formation	recombinant protein testing	Aharoni *et al.* 2004
Fapdc1/3	pyruvate decarboxylase	aroma	expression analysis	Moyano *et al*. 2004
FaADH	alcohol dehydrogenase	aroma	sequence similarity	Wolyn and Jelenkovic 1990
FaOMT	*O*-methyltransferase	aroma, furanone formation	antisense	Lunkenbein *et al*. 2006c
FaOMT	*O*-methyltransferase	aroma, furanone formation	expression analysis, recombinant protein testing	Wein *et al.* 2002
FaQR	quinone (enone) oxidoreductase	aroma, furanone formation	expression analysis, recombinant protein testing	Raab *et al.* 2006; Klein *et al*. 2007
FaSDH	sorbitol dehydrogenase	sugar metabolism	expression analysis	Sutsawat *et al*. 2008; Duangsrisai *et al*. 2007
FaS6PDH	sorbitol-6-phosphate dehydrogenase	sugar metabolism	expression analysis	Duangsrisai *et al*. 2007
FagpS, FagpL1/2	ADP-glucose pyrophosphorylase	starch biosynthesis	expression analysis	Park and Kim 2007
FagpS	ADP-glucose pyrophosphorylase	starch biosynthesis	antisense	Park *et al*. 2006
FaCHS	chalcone synthase	pigment formation	antisense	Lunkenbein *et al*. 2006b
FaCHS	chalcone synthase	pigment formation	transient RNAi	Hoffmann *et al*. 2006
FaCHS, FaDFR	chalcone synthase, dihydroflavonol 4- reductase	pigment formation	expression analysis	Li *et al*. 2003
FaDFR	dihydroflavonol 4-reductase	pigment formation	expression analysis	Moyano *et al*. 1998
FaDFR	dihydroflavonol 4-reductase	pigment formation	expression analysis, recombinant protein testing	Almeida *et al*. 2007

Table 1 (Cont.)

Gene	Enzyme	Putative function	Method	Reference
		Enzyme coding genes		
FaF3h	flavanone 3-hydroxylase	pigment formation	candidate gene approach	Deng and Davis 2001
FaFHT	flavanone 3-hydroxylase	pigment formation	expression analysis, recombinant protein testing	Almeida *et al*. 2007
FaFLS	flavonol synthase	pigment formation	expression analysis, recombinant protein testing	Almeida *et al*. 2007
FaANS	anthocyanidine synthase	pigment formation	expression analysis, recombinant protein testing	Almeida *et al*. 2007
FaLAR	leucoanthocyanidin reductase	pigment formation	expression analysis, recombinant protein testing	Almeida *et al*. 2007
FaANR	anthocyanidin reductase	pigment formation	expression analysis, recombinant protein testing	Almeida *et al*. 2007
FaFGT	anthocyanidin glucosyltransferase	pigment formation	expression analysis, recombinant protein testing	Almeida *et al*. 2007
FaGT1	anthocyanidin glucosyltransferase	pigment formation	expression analysis, recombinant protein testing, transient RNAi	Griesser *et al*. 2008a
FaGT2	UDP-glucose:cinnamate glucosyltransferase	phenlypropanoid metabolism	expression analysis, recombinant protein testing, antisense	Lunkenbein *et al*. 2006a
FaGT6/7	flavonol glucosyltransferases	flavonoid metabolism	expression analysis, recombinant protein testing	Griesser *et al*. 2008b
FaCDPK1	calcium-dependent protein kinase	fruit development	expression analysis	Llop-Tous *et al*. 2002
FaACO1/2	1-aminocyclopropane-1-carboxylic acid oxidase	ethylene biosynthesis	expression study	Trainotti *et al*. 2005
FaCGS	cystathionine γ-synthase	methionine biosynthesis	expression analysis	Marty *et al*. 2000
Fapmsr	methionine sulfoxide reductase	repair of proteins and peptides	expression analysis, recombinant protein testing	Lopez *et al*. 2006
FaCCD1	carotenoid cleavage dioxygenase	lutein degradation	expression analysis, recombinant protein testing	Garcia-Limones *et al*. 2008
APxSC	cyctosolic ascorbate peroxidase	gluthathione-ascorbate cycle	expression analysis	Kim and Chung 1998a, 1998b; Kim *et al*. 2001
FacpFBP	chloroplastic fructose-1,6-diphosphate	photosynthesis	complementation assay	Serrato *et al*. 2009
FaPLD	phospholipase D alpha	membrane deterioration	recombinant protein testing	Yuan *et al.* 2005
FaSTK	serine-threonine kinases	protein modification	sequence similarity	Martinez Zamora *et al*. 2008
		Other protein coding genes		
FaCyf1	phytocystatin	cystein protease inhibitor, antifungal	recombinant protein testing	Martinez *et al.* 2005
FaEtr1/2, FaErs1	ethylene resistant, ethylene response sensor	ethylene receptor	expression analysis	Trainotti *et al.* 2005
FaTCTP	translationally controlled tumor protein	fruit ripening	expression analysis	Lopez and Franco, 2006
FaPGIP	polygalacturonase-inhibiting protein	defense	expression analysis	Mehli *et al.* 2004
FaWRKY1	transcription factor	regulator of defense	overexpression	Encinas-Villarejo *et al.* 2009
FaOLP	osmotin-like protein	pathogenesis-related, stress	expression analysis	Wu *et al.* 2001; Zhang and Shih 2007
Fxaltp	non-specfic lipid transfer protein	stress	expression analysis	Yubero-Serrano *et al.* 2003
FaNBS	nucleotide binding site protein	resistance	sequence similarity	Xu *et al.* 2007
FaRB7	tonoplast intrinsic protein	resistance	expression analysis	Vaughan *et al.* 2006
FaCBF1	cold-induced transcription factor	cold acclimation response	overexpression	Owens *et al.* 2002
STAG1	MADS box, AGAMOUS homolog	vegetative, floral, fruit development	expression analysis	Rosin *et al.* 2003
FaAP1, FaLFY	APETALA1, LEAFY	floral identity, floral integrator	expression analysis	Mouhu *et al*. 2009
FaH4	histone H4	flowering process	expression analysis	Kurokura *et al.* 2006
Fanjjs4	low molecular weight heat shock protein	seed maturation, fruit ripening	expression analysis	Medina-Escobar *et al*. 1998
FaExp1 to 6	expansin cell wall proteins	softening, cell wall disassembly	expression analysis	Civello *et al.* 1999; Dotto *et al*. 2006; Figueroa *et al.* 2009
Fcor1/2/3	cold-regulated protein	cold acclimation response	expression analysis	Ndong *et al*. 1997; Zalunskaite *et al*. 2008
FABP1	auxin-binding protein	auxin perception	expression analysis	Lazarus *et al*. 1996
FapPCM1	plant calmodulin	tuberization process, signal transduction	expression analysis	Jena *et al.* 1989
FaPIP1	plasma membrane intrinsic protein	aquaporin, water channel	expression analysis, overexpression	Mut *et al*. 2008
FaGAST	small protein with 12 cysteine residues	arresting cell elongation	expression analysis, overexpression	de la Fuente 2006
FaZIP	Zn- and Fe-regulated transporter	mineral uptake	expression analysis	Shi and Shih 2006

Table 1 (Cont.)

Gene	Enzyme	Putative function	Method	Reference
		Other protein coding genes		
FaMET, FaDRM	DNA methyltransferases	methylation of cytosine residues in DNA	expression analysis	Chang *et al*. 2009
Fra	pathogenesis-related protein, allergen	flavonoid biosynthesis	expression analysis, transient RNAi	Muñoz *et al*. 2010
FaMYB1	transcription factor	regulation of pigment biosynthesis	overexpression	Aharoni *et al*. 2001
FaHyprp	hybrid proline-rich protein	polyphenol anchoring	expression analysis	Blanco-Portales *et al*. 2004

used to assign gene functions. Only recently, a fast and easy to perform RNAi-based approach has been presented to test hypotheses about specific gene functions in strawberry (Hoffmann *et al*. 2006). In this transient assay, an *Agrobacterium* strain carrying a T-DNA expressing an intron-containing construct encoding a self-complementary 'hairpin' RNA (ihpRNA) transgene (pBI-*(target gene)i*) is injected with a syringe into receptacles of growing strawberry fruits still attached to the plant (**Figs. 1, 2**). The RNA-mediated regulation process generally results in sequence-specific inhibition of gene expression such as the degradation of complementary endogenous mRNAs. A vector carrying the glucuronidase gene (GUS) separated by an intron (pBI-Intron) serves as a control (**Fig. 1**). The general applicability of the transient RNAi method has been demonstrated by the downregulation of flavonoid biosynthesis genes (Griesser *et al*. 2008a; Muñoz *et al*. 2010).

FLAVONOID PATHWAY

Flavonoids are a major class of plant secondary metabolites that serves a multitude of functions. They have key roles in signaling between plants and microbes, in male fertility of some species, in defense as antimicrobial agents and as feeding deterrents, and in UV protection (Winkel-Shirley 2001). Flavonoids are synthesized from phenylpropanoid derivatives by condensation with malonyl-CoA (**Fig. 3**). The reaction is catalyzed by chalcone synthase (CHS) and yields naringenin chalcone. In higher plants six major subgroups are derived from this first intermediate: the chalcones, flavones, flavonols, flavandiols, anthocyanins, and condensed tannins (or proanthocyanidins). A seventh group, the aurones, is widespread, but not ubiquitous. Much effort has been directed at elucidating the flavonoid biosynthetic pathway from a molecular genetic point of view and thus it has been one of the most intensively studied metabolic systems in plants. The majority of the enzymes of flavonoid biosynthesis are members of three classes of enzymes found in all organisms: the oxoglutarate-dependent dioxygenases, NADPH-dependent reductases, and cytochrome P450 hydroxylases. CHS and chalcone isomerase appear to have a more limited ancestry. In addition to its flavor, much of the popularity of strawberry is due to the attractive red color caused by anthocyanin pigments. The major pigment is pelargonidin 3-*O*-glucoside followed by cyanidin 3-*O*-glucoside whereas quercetin and kaempferol 3-*O*-glucosides are the major flavonols (Griesser *et al*. 2008a). The chemical composition of the strawberry flavonoids has been studied in detail, but genetic and biochemical information about the numerous steps in anthocyanin biosynthesis and its regulation is still limited (Almeida *et al*. 2007).

FaCHS

CHS, the first gene in the flavonoid pathway, was chosen as a reporter gene to test the RNAi-induced silencing of gene expression in strawberry fruit by agroinfiltration. Reduction of the *CHS* function using antisense technology has led immediately to the loss of pigmentation in strawberry fruit and is thus easily detectable (Lunkenbein *et al*. 2006b). To find an efficient method for transfection of strawberry fruit different procedures were studied using the pBI-Intron vector with the GUS gene. Agroinfiltration of pBI-Intron gave the best results when the injection occurred at least 10 days post-pollination. Even after 2 days GUS activity was detectable (Hoffmann *et al*. 2006). Reproducible and efficient silencing of the *Fragaria x ananassa CHS* (FaCHS) gene was achieved with the vector pBI-*CHSi* that was generated by inserting a 303-bp fragment of *FaCHS* in the sense and antisense orientation interrupted by an intron in the pBI-Intron to replace GUS (**Fig. 1**). Agroinfiltration of pBI-*CHSi* resulted in strawberry fruit with white regions, a clear sign of impaired anthocyanin accumulation (**Fig. 4**). Fruits infiltrated with *Agrobacterium* carrying the pBI-Intron control vector turned completely red like the untreated fruit. Suppression of *FaCHS* was confirmed by semiquantitative RT-PCR, enzyme activity assays and metabolite analyses (**Fig. 5**; Hoffmann *et al*. 2006). *FaCHS*-silenced receptacles produced statistically lower levels of downstream (pelargonidin derivatives) but higher levels of upstream metabolites (phenylpropanoid derivatives) than the receptacles agroinfiltrated with the pBI-Intron control vector. Thus, *FaCHS* was successfully downregulated by agroinfitration of pBI-*FaCHSi* and led to a redirection of the intermediates of the flavonoid to the phenylpropanoid pathway.

FaOMT

In an study to clarify the biosynthesis of the aroma compound 2,5-dimethyl-4-methoxy-3(2*H*)-furanone (DMMF) from 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF) an O-methyltransferase (*FaOMT*) cDNA was obtained by screening a strawberry cDNA library, cloned, and heterologously expressed in *Escherichia coli*. The FaOMT protein catalysed the transfer of the methyl group from S-adenosyl-L-methionine (SAM), not only to HDMF but also to caffeic acid, thereby forming the corresponding *O*-methyl ethers DMMF and ferulic acid (Wein *et al*. 2002). Stable transformation of strawberry with the *FaOMT* sequence in sense and antisense orientation, under the control of the cauliflower mosaic virus 35S promoter, resulted in a near total loss of DMMF, whereas the levels of the other volatiles remained unchanged. *FaOMT* repression also affected the ratio of feruloyl 1-*O*-glucose and caffeoyl 1-*O*-glucose, indicating a dual function of the enzyme *in planta* (Lunkenbein *et al*. 2006c). To confirm the results obtained with the stably transformed strawberry plants and to validate the transient RNAi method an ihpRNA-encoding construct of *FaOMT* (pBI-*FaOMTi*) was agroinfiltrated into ripening strawberry fruits. Although, the phenotypes of the pBI-*FaOMTi* and pBI-Intron infiltrated fruits were indistinguishable (**Fig. 4**), metabolite analysis showed that the levels of DMMF in relation to HDMF was significantly reduced in the pBI-*FaOMTi* injected fruits when compared with the fruits injected with the control vector (**Fig. 5**). Since wounding of the fruit by agroinfiltration resulted in an upregulation of phenylpropanoyl glucose esters (data not shown), changes in the levels of feruloyl 1-*O*-glucose in relation to caffeoyl 1-*O*-glucose were not observed after silencing of *FaOMT* by the transient RNAi approach.

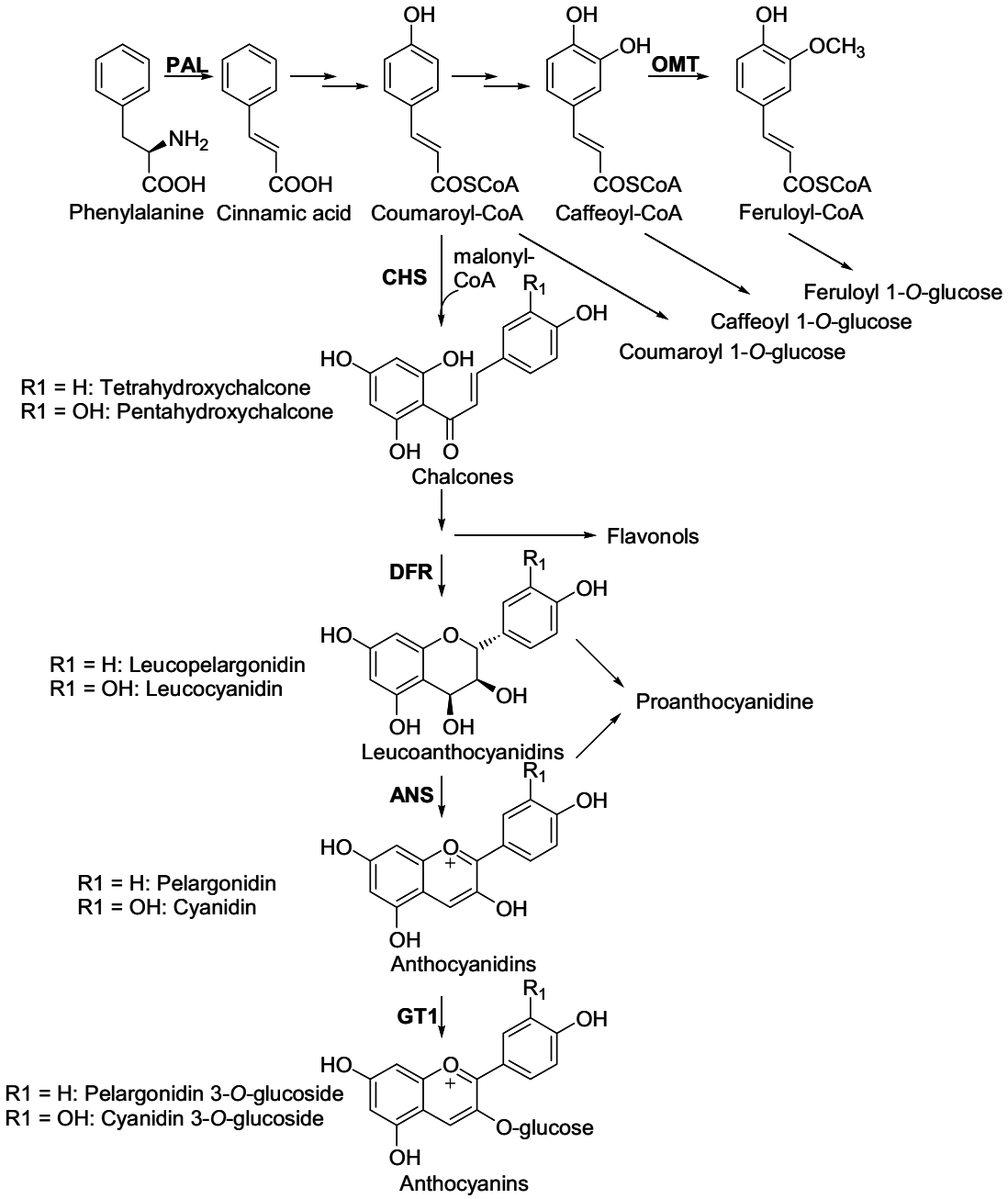

Fig. 3 Schematic view of the phenylpropanoid, flavonoid and anthocyanidin pathway. ANS anthocyanidin synthase, CHS chalcone synthase, DFR dihydroflavonol 4-reductase, GT1 anthocyanidin glucosyltransferase 1, PAL phenylalanine ammonia lyase.

FaGT1

Anthocyanidin 3-*O*-glucosyltransferase (GT1) is the enzyme that catalyzes the formation of the first stable intermediate in the anthocyanin pathway (**Fig. 3**). A putative glycosyltransferase sequence (*FaGT1*) was recently cloned from strawberry fruit cDNA (Griesser *et al*. 2008a). *In vitro* assays showed that the recombinant FaGT1 transferred glucose from the donor UDP-glucose to anthocyanidins and, to a lesser extent, to flavonols, generating 3-*O*-glucosides. To elucidate the *in planta* function of *FaGT1*, *A. tumefaciens* cells carrying an ihpRNA-encoding construct of a partial *FaGT1* sequence (pBI-*FaGT1i*) were injected into ripening strawberry fruits. This led to significant downregulation of *FaGT1* mRNA levels that correspond to reduced concentrations of pelargonidin-derived pigments in ripe fruits (**Fig. 5**). The color of the pBI-*FaGT1i* injected fruit was generally less intense and of a different hue compared to the bright red fruits of the controls (**Fig. 4**). Fruits with white regions as detected after the infiltration of pBI-*FaCHSi* were not obtained. Significant levels of epiafzelechin, formed by anthocyanidin reductase (ANR) from pelargonidin, were identified in *FaGT1*-silenced fruits (Griesser *et al*. 2008a). The result indicates competition of FaGT1 and FaANR for the common anthocyanidin substrate and shows that FaGT1 represents an important branching-point enzyme because it is channeling the flavonoid pathway to anthocyanins.

FaDFR

Dihydroflavonol 4-reductase (DFR) catalyzes the last common step in the flavonoid biosynthesis pathway leading to anthocyanins and proanthocyanidins (**Fig. 3**; Almeida *et al*. 2007). A putative *FaDFR* gene was cloned from strawberry cDNA and studied by combining biochemical and molecular approaches (Moyano *et al*. 1998; Almeida *et al*. 2007). To test the *in vivo* function of *FaDFR* by reverse genetics a pBI-*FaDFRi* construct was agroinfiltrated into strawberry fruits. The pBI-*FaDFRi* vector consisted of an ihpRNA encoding construct of a partial *FaDFR* sequence. Fruits infiltrated with pBI-*FaDFRi* showed a less intense color than the color fruits and produced significantly lower levels of

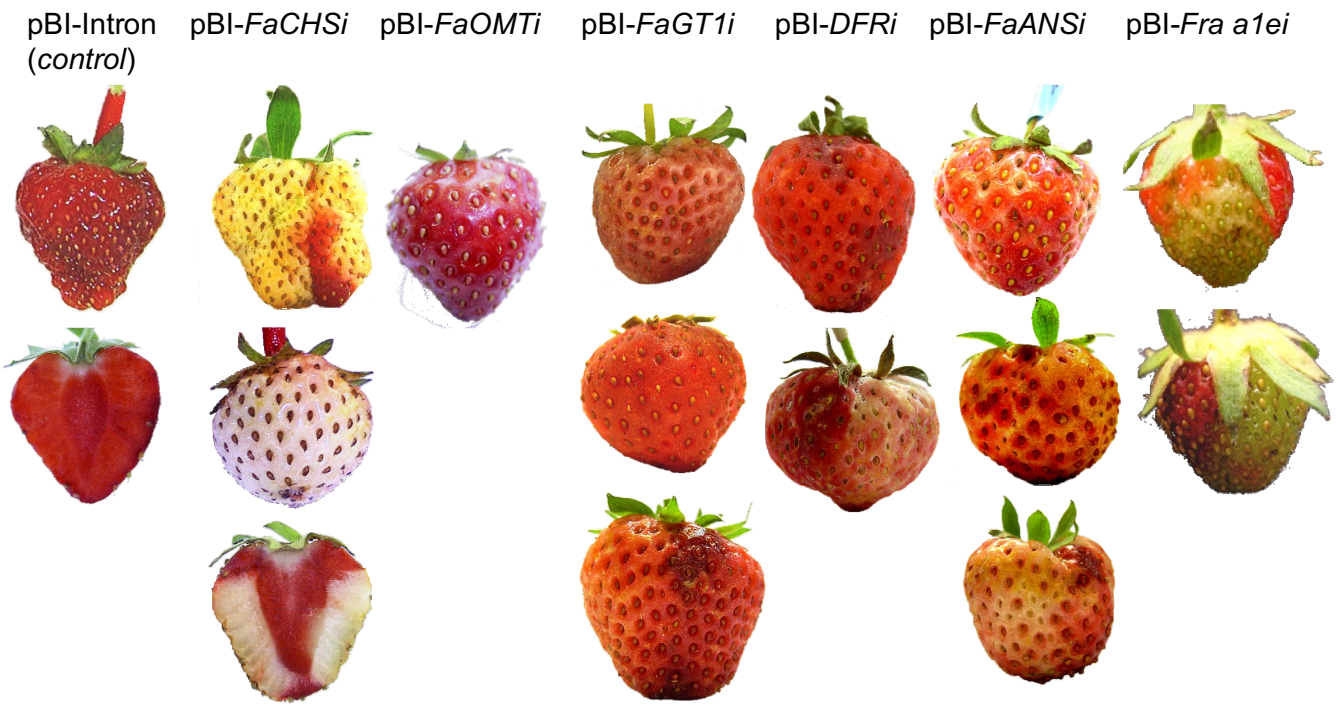

Fig. 4 Collection of phenotypes. Fruits were agroinfiltrated with the constructs referred to in the first line and remained attached to the plants until harvest. The color change in comparison to the fruits injected with the control vector pBI-Intron indicate impaired pigment formation, except for pBI-*FaOMTi*.

pelargonidin derivatives as determined by metabolite analysis (**Figs. 4, 5**). Surprisingly, the concentrations of cyanidin derivatives were not affected. Chimeric fruits with white regions were not detected.

FaANS

Anthocyanidin synthase (ANS) catalyzes the 2-oxoglutarate dependent oxidation of leucoanthocyanidins to the colored anthocyanidins (**Fig. 3**; Nakajima *et al*. 2001). A *FaANS* gene was cloned from red fruit cDNA with gene specific primers designed from published ESTs (Almeida *et al*. 2007). Functional characterization of the recombinant FaANS protein showed a preference for the substrate leucopelargonidin. The *in vivo* preference was studied after transient downregulation of the *FaANS* transcripts by agroinfiltration of a *pBI-FaANSi* vector into ripening strawberry fruits. The pBI-*FaANSi* fruits showed a similar phenotype as the pBI-*FaDFRi* injected fruits (**Fig. 4**). Metabolite analysis confirmed the significant downregulation of the levels of pelargonidin and cyanidin derivatives (**Fig. 5**). Thus, FaANS catalyzes the formation of both anthocanidins *in planta*.

Fra

The strawberry fruit allergen Fra a is a member of the Bet v1 superfamily named after the major birch pollen allergen and is ranked among a subfamily of pathogenesis-related proteins (PR-10, Muñoz *et al*. 2010). Although the allergenic properties of Bet v1 and related PR-10 proteins have been extensively studied, their biological role in plants remained elusive. In a search for strawberry genotypes with low Fra a allergen levels it was found that total allergen content was always lower in colorless (white) strawberry varieties than in red ones (Hjernø *et al*. 2006). The ripe colorless fruits that were tolerated by individuals affected by allergy were found to be virtually free from the strawberry allergen. Interestingly, several catalytically active proteins of the flavonoid pathway like FaCHS, flavanone 3-hydroxylase (F3H), and DFR were also reduced. To study whether there is a direct link between *Fra a* expression and flavonoid and anthocyanin formation *Fra a* gene expression in strawberry fruit was downregulated by RNAi targeted to *Fra a*. Fruits infiltrated with pBI-*Fraa1ei* and pBI-*Fraa3i,* representing two isoforms of *Fra a*, consistently showed white regions similar to the chimeric pBI-*FaCHSi* phenotype (**Fig. 4**). However, metabolite analysis revealed that in fruits infiltrated with pBI-*Fraa1ei* and pBI-*Fraa3i* constructs, all metabolites of the anthocyanin biosynthesis pathway showed lower levels than the controls (Muñoz *et al*. 2010). Quantitative PCR (qPCR) analysis confirmed the efficient downregulation of *Fra a* isoforms (**Fig. 6**). Besides, Fra a seems to have a regulatory function because the *FaCHS* and phenylalanine ammonia lyase (*FaPAL*) expression levels were significantly reduced in the pBI-*Fraa1ei* and pBI-*Fraa3i*-injected fruits. The results demonstrate a clear link between *Fra a* expression and flavonoid formation. The study clearly shows that the Fra a allergen has a functional role in the flavonoid pathway.

CONCLUSION

The large amount of sequence information that has been generated for *Fragaria*, and the implementation of high-throughput gene expression analyses have resulted in an increased interest in reverse genetic methodologies. One approach to generate an impaired phenotype is the down-regulation of target genes and even gene families by RNAi which is triggered by dsRNA. The dsRNA can be delivered to plants either transiently or stable by integrating dsRNA-producing transgenes. The transient RNAi approach permits rapid and highly efficient gene function discovery and validation and can be carried out in a high-throughput mode with thousands of individual plants. The recently developed transient RNAi method in *Fragaria* is suitable for analyzing genes that are highly expressed during strawberry fruit development as was demonstrated for *FaCHS*, *FaOMT*, *FaGT1*, *FaDFR*, *FaANS* and *Fra*.

ACKNOWLEDGEMENTS

This work has been supported by funds from DFG SCHW 634/10-2 and DAAD D/07/13484.

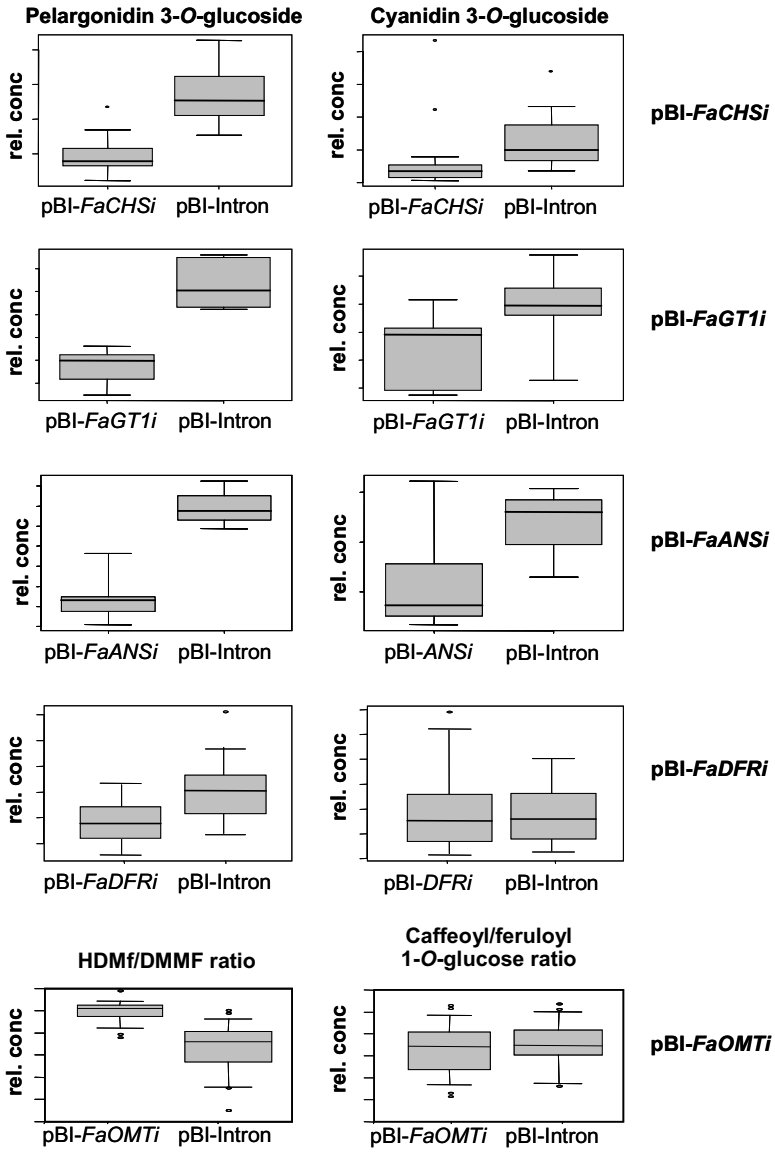

Fig. 5 Effect of gene silencing. Effects caused by various ihpRNA encoding constructs (pBI-*FaCHSi*, pBI-*FaGT1i*, pBI-*FaANSi*, pBI-*FaDFRi*, and pBI-*FaOMTi*) on selected metabolite levels (pelargonidin 3-*O*-glucoside, cyanidin-3-*O*-glucoside, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone/2,5-dimethyl-4-methoxy-3(2*H*)-furanone (HDMF/DMMF ratio) and caffeoyl 1-*O*-glucose/feruloyl 1-*O*-glucose ratio). Box plot graphs were designed for the peak areas of selected compounds. A horizontal line in the boxes indicates the medians and boxes the interquartile range. Whiskers extend to 10th and 90th percentiles. Outliers are displayed by black dots. Wilcoxon–Mann–Whitney U-test was used for non-parametric analysis of intergroup comparison.

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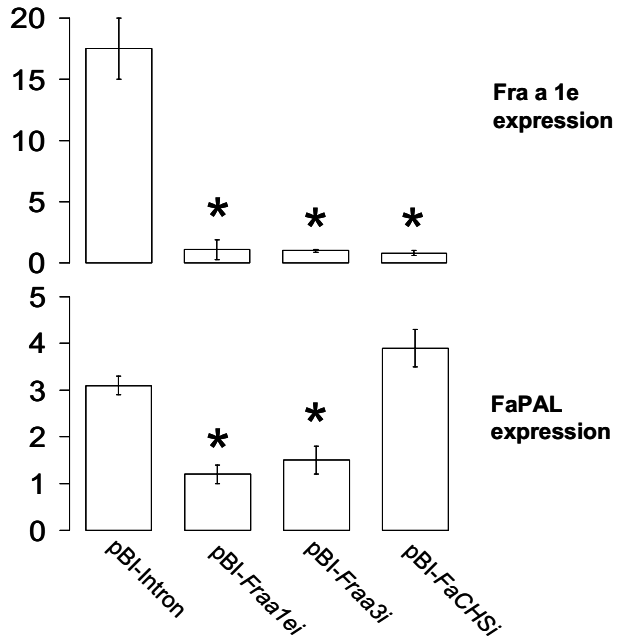

Fig. 6 Relative gene expression levels determined by qPCR. Relative transcription levels of *Fra a 1e* and *FaPAL* normalized to the interspacer gene were determined in fruits agroinfiltrated with pBI–Intron, pBI–*Fraa1ei*, pBI–*Fraa3i*, and pBI–*CHSi* (adapted from Muñoz *et al*. 2010). Statistically significant values in comparison with the values for pBI–Introns are marked with an asterisk (P = 0.05). The statistical analysis was performed by paired *t*-test function.

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