The Flavonoid Biosynthetic Enzyme Chalcone Isomerase Modulates Terpenoid Production in Glandular Trichomes of Tomato1[C][W][OPEN]

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Flavonoids and terpenoids are derived from distinct metabolic pathways but nevertheless serve complementary roles in mediating plant interactions with the environment. Here, we show that glandular trichomes of the anthocyanin free (af) mutant of cultivated tomato (Solanum lycopersicum) fail to accumulate both flavonoids and terpenoids. This pleiotropic metabolic deficiency was associated with loss of resistance to native populations of coleopteran herbivores under field conditions. We demonstrate that Af encodes an isoform (SICH1) of the flavonoid biosynthetic enzyme chalcone isomerase (CHI), which catalyzes the conversion of naringenin chalcone to naringenin and is strictly required for flavonoid production in multiple tissues of tomato. Expression of the wild-type SICH1 gene from its native promoter complemented the anthocyanin deficiency in af. Unexpectedly, the SICH1 transgene also complemented the defect in terpenoid production in glandular trichomes. Our results establish a key role for SICH1 in flavonoid production in tomato and reveal a link between CHI and terpenoid production. Metabolic coordination of the flavonoid and terpenoid pathways may serve to optimize the function of trichome glands in dynamic environments.

Flavonoids and terpenoids (isoprenoids) comprise two of the largest groups of specialized metabolites in higher plants. They serve myriad and often complementary roles in plant acclimation to changing environmental conditions. For example, flavonoids and terpenoids directly repel attack by insect herbivores and microbial pathogens and also mediate plant communication with symbiotic bacteria, natural enemies of arthropod herbivores, and parasitic plants (Hirsch et al., 2003; Rasmann et al., 2005). Pigmented flavonoids and volatile terpenes provide visual and olfactory cues, respectively, for the attraction of insect pollinators (Dixon and Paiva, 1995; Grotewold, 2006; Vogt, 2002). Much of the scientific interest in flavonoids and terpenoids reflects their importance in food quality, nutrition, and human health (Dillard and German, 2000; Middleton et al., 2000; Dixon et al., 2005; Bohmiller and Keeling, 2008).

Flavonoids are synthesized from Phe derivatives generated via the shikimate and phenylpropanoid pathways (Tohge et al., 2013; Fig. 1). Chalcone synthase (CHS; EC 2.3.1.74) condenses 4-coumaroyl-CoA and malonyl-CoA to form the open-chain flavonoid naringenin chalcone, which is converted to naringenin by chalcone isomerase (CHI; EC 5.5.1.6). Naringenin defines a key branch point for the synthesis of several major classes of flavonoids, including flavanones, flavones, and anthocyanins. Flavonoid biosynthesis is controlled by transcription factors that coordinate the expression of multiple biosynthetic genes in response to environmental and developmental cues (Dixon and Paiva, 1995; Grotewold, 2006; Vogt, 2010). Terpenoids originate from distinct metabolic pathways in which the C5 precursors dimethyl allyl diphosphate and isopentenyl diphosphate are sequentially combined to produce the building blocks for C10 monoterpenes, C15 sesquiterpenes, and higher molecular weight derivatives. Plastid-derived terpenoids are synthesized by the methyl-erythritol phosphate (MEP) pathway (Fig. 1), whereas most extraplastidic terpenoids are produced from the cytosolic mevalonic acid pathway (Bohmiller and Keeling, 2008). Relatively little is known about the regulatory mechanisms governing terpenoid biosynthesis in higher plants (Patra et al., 2013).

The terpenoid and flavonoid biosynthetic pathways are supplied with carbon skeletons generated in primary...
Figure 1. Schematic overview of terpenoid and flavonoid biosynthetic pathways. The names of compounds and select enzymes (italicized) are as follows: ANS, anthocyanidin synthase; CDP-ME, 4-cytidine 5'-diphospho-2-C-methyl-o-erythritol; CDP-MEP, 2-phospho-4-cytidine 5'-diphospho-2-C-methyl-o-erythritol; CMK, 4-(cytidine 5'-diphospho)-2-C-methyl-o-erythritol kinase; DADP, 3-deoxy-o-arabino-heptulosonate 7-phosphate; DFR, dihydroflavonol 4-reductase; DMAIP, dimethylallyl diphosphate; DXP, 1-deoxy-o-xylulose 5-phosphate; DXPS, DXP synthase; DXR, DXP reductoisomerase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; FPP, farnesyl diphosphate; FPPS, FPP synthase; GPP, geranyl diphosphate; GPPS, GPP synthase; HDR, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase; IPP, isopentenyl diphosphate; MCT, 2-C-methyl-o-erythritol 4-phosphate cytidylyltransferase; MTS, monoterpene synthase; PAL, Phe ammonia lyase; PEP, phosphoenolpyruvate; STS, sesquiterpene synthase. Note that although some sesquiterpenes in tomato are synthesized via the MEP pathway (as shown for simplicity; Sallaud et al., 2009), most sesquiterpenes are derived from the cytosolic mevalonate pathway. [See online article for color version of this figure.]

metabolism but, otherwise, are thought to operate independently of one another in most plant tissues (Fig. 1). Recent studies, however, are beginning to uncover metabolic and regulatory connections between these two major branches of specialized metabolism. For example, dimethyl allyl diphosphate is used for the synthesis of prenylated flavonoids and other terpenophenolics in glandular trichomes of some plant species (Nagel et al., 2008; Wang et al., 2008; Shen et al., 2012; Voo et al., 2012). There is also evidence that members of the MYB family of transcription factors play a role in coordinating metabolic activity between the flavonoid and terpenoid biosynthetic pathways (Bedon et al., 2010; Ben Zvi et al., 2012).

Glandular trichomes provide an excellent model system in which to study the production of specialized metabolites in a single, experimentally accessible cell type (Wagner, 1991; Lange et al., 2000; Schilmiller et al., 2008; Tissier, 2012; Voo et al., 2012). The type VI glandular trichome of tomato (Solanum lycopersicum) is particularly well suited for the analysis of flavonoid and terpenoid synthesis, which dominates the metabolic activity of this specialized epidermal structure (Duffey and Isman, 1981; Kennedy, 2003; van Schie et al., 2007; Schilmiller et al., 2010; Kang et al., 2010b). Mutants affected in the chemical composition, morphology, and density of type VI glands have provided insight into the biological roles of glandular trichomes in resistance to insect herbivores (Li et al., 2004; Kang et al., 2010a, 2010b; Bleeker et al., 2012). Both the development and biosynthetic capacity of type VI trichomes are responsive to environmental inputs and are regulated in part by the stress hormone jasmonate (Li et al., 2004; Boughton et al., 2005; van Schie et al., 2007; Peiffer et al., 2009; Tian et al., 2012). The mechanisms that coordinate the developmental and metabolic plasticity of glandular trichomes in tomato and other species are largely unknown.

The anthocyanin free (af) mutant of tomato was identified more than a half-century ago as an x-ray-induced variant that lacks anthocyanin production in all tissues (Burdick, 1958). In contrast to other anthocyanin-deficient mutants of tomato, Rick et al. (1976) showed that af foliage exhibits a lower density of type VI glandular trichomes, reduced emission of leaf volatiles, and increased susceptibility to herbivorous flea beetles. These pleiotropic phenotypes led to the suggestion that af may define a regulatory gene that controls both flavonoid production and trichome development (De Jong et al., 2004), but this hypothesis has remained untested. Here, we demonstrate that Af encodes a member (SICH1) of the CHI family of enzymes that catalyzes the synthesis of a key branch point intermediate [2(S)-naringenin] in the flavonoid pathway. Transgenic complementation experiments showed that Af/CHI1 is required not only for the synthesis of anthocyanins and other flavonoids but also plays a role in promoting the accumulation of terpenoids in glandular trichomes. These findings suggest a role for CHI1 in coordinating the developmental plasticity and metabolic activity of an epidermal structure that shields against biotic stress.
RESULTS

The af Mutant Is Deficient in Flavonoid Accumulation in Type VI Glandular Trichomes

Under growth conditions in which wild-type plants accumulate relatively high anthocyanin levels, af mutant plants lacked visible anthocyanin pigmentation in all tissues, including leaves, stems, and hypocotyls (Fig. 2, A–D). This defect is associated with a reduced density of type VI glandular trichomes on leaves, as reported previously (Rick et al., 1976). We found that the density of type VI trichomes on the adaxial surface of af leaves (400 ± 54 cm⁻²) was approximately 30% of that on wild-type leaves (1,379 ± 129 cm⁻²). Light microscopy and scanning electron microscopy confirmed these findings and also showed that the size of type VI glands on af leaf and stem tissue is reduced in comparison with the wild type (Fig. 2, E and F).

Chlorogenic acid (caffeoylquinic) and rutin (quercetin-3-O-rutinoside), which are both derived from 4-coumaroyl-CoA (Fig. 1), are two of the most abundant phenolic compounds in tomato leaves and type VI glands, respectively (Duffey and Isman, 1981; Kang et al., 2010b). To further characterize the effect of af on phenolic metabolism, we used liquid chromatography-mass spectrometry (MS) to measure the levels of chlorogenic acid, rutin, and other nonvolatile secondary metabolites in leaf-dip extracts obtained by brief immersion of excised leaflets into acidified methanol. af leaves contained less than 10% of wild-type levels of rutin and other flavonol derivatives, including kaempferol rhamnoside, a quercetin trisaccharide, and 3-O-methylmyricetin (Fig. 3A). Wild-type and af leaves contained similar levels of chlorogenic acid and its precursor, quinic acid, as well as acyl sugars and glycoalkaloids (α-tomatine and dehydrotomatine). Metabolite analyses performed with isolated type VI trichomes confirmed that the mutant produces only trace levels of rutin in gland cells (Fig. 3B). Low levels of acyl sugars were found in type VI extracts from both the wild type and af, whereas chlorogenic acid, quinic acid, and glycoalkaloids were not detected in these extracts (Fig. 3B). These results indicate that af, in addition to blocking anthocyanin production in tissues throughout the plant, impairs the flavonoid branch of phenylpropanoid metabolism in type VI trichome glands.

The af Mutant Exhibits Increased Susceptibility to Herbivorous Beetles

The defense-related roles of glandular trichome-derived metabolites led us to test whether af plants are affected in resistance to native populations of arthropod herbivores. Greenhouse-grown wild-type and af seedlings were transplanted to the field and subsequently monitored for the presence of herbivores and associated leaf damage. Potato flea beetle (Epitrix cucumeris) was observed much more frequently on the mutant than on neighboring wild-type plants (Fig. 4A). Moreover, af foliage received more herbivore damage than wild-type leaves, as determined by counting the number of flea beetle feeding sites (Fig. 4B). We also found that af plants were visited by two additional beetle species, Colorado potato beetle (Leptinotarsa decemlineata) and blister beetles (Epicauta funebris), which were not observed on wild-type plants (Fig. 4, C and D). These results are consistent with previous field studies showing that the af mutant is compromised in resistance to native populations of the tobacco flea beetle (Epitrix hirtipennis; Rick et al., 1976).

af Is a Null Mutation in CHI

The Af locus was previously mapped to an interval on chromosome 5 that is defined by the Solanum pennellii introgression line IL5-2 ( Tanksley et al., 1992; Eshed and Zamir, 1995). Fine-mapping of the locus allowed us to position Af to an approximately 200-kb region flanked by markers SGN-U571424 and CT93 (Fig. 5A). Among the approximately 31 hypothetical genes in this region were two tandemly duplicated genes predicted to encode the flavonoid pathway enzyme CHI (Fig. 5B).
well-established role of CHI in flavonoid biosynthesis indicated that these genes, which we designated CHI1 (Solyc05g010320) and CHI2 (Solyc05g010310), were good candidates for Af. Reverse transcription (RT)-PCR analysis showed that CHI1 transcripts are expressed in both wild-type and af leaves, whereas expression of CHI2 was not detected in leaves of either genotype (Supplemental Fig. S1). This finding is consistent with publicly available gene expression data for CHI1 and CHI2 (http://solgenomics.net). Sequencing of wild-type- and af-derived genomic clones containing CHI2 did not reveal nucleotide polymorphisms at this locus. CHI1 genomic and complementary DNA (cDNA) clones isolated from af, however, contained a six-nucleotide deletion (preceded by a G-to-T transversion) within the last exon. This polymorphism generates a premature stop codon that truncates the C-terminal 18 amino acids of the protein (Fig. 5C).

Based on an x-ray crystal structure of CHI (Jez et al., 2000), the C-terminal truncation of CHI1 in af is predicted to affect an α-helix (α7) that does not participate directly in enzyme catalysis. Therefore, we performed experiments to determine whether the mutant form of SICH1 is enzymatically active. His-tagged derivatives of wild-type (CHI1WT) and mutant (CHI1af) CHI1 were expressed in Escherichia coli and purified to homogeneity. The amount of soluble CHI1af recovered from induced Escherichia coli cells was low in comparison with CHI1WT, and we found that this difference in expression resulted largely from partitioning of CHI1af to the insoluble fraction (Supplemental Fig. S2A; data not shown). In vitro assays performed with affinity-purified proteins showed that CHI1WT rapidly metabolizes the substrate naringenin chalcone. In contrast, the activity of CHI1af was not greater than that of control reactions lacking enzyme (Fig. 5D).

We used immunoblot assays to determine whether CHI1af accumulates in the af mutant. Polyclonal antibodies raised against CHI1WT reacted strongly and specifically with a single polypeptide in wild-type leaves (Fig. 5E). The apparent molecular mass of this protein, which was recovered in both supernatant and pellet fractions of crude leaf extracts, matched the predicted size of CHI1 (24.6 kD). The anti-CHI1 antibody did not specifically recognize proteins in the soluble or particulate fraction from af leaves. Control experiments performed with recombinant enzymes showed that the absence of immunoreactive CHI1 in af did not result from a failure of the antibody to recognize CHI1af (Supplemental Fig. S2B). These results indicate that the af mutation eliminates the enzymatic activity of SICH1 and also prevents accumulation of the protein in planta.

Figure 3. af leaves are deficient in the production of flavonoid-related metabolites. Levels of the indicated secondary metabolites were measured in leaf-dip extracts (A) and isolated type VI glands (B). Each data point represents the mean ± se of four biological replicates from wild-type (WT) and af plants. Asterisks represent significant differences between wild-type and af plants (unpaired Student’s t test: *P < 0.05, **P < 0.01, ***P < 0.001). nd, Not detected.
We used chemical and genetic complementation approaches to test whether the af mutant is impaired in CHI1-mediated conversion of naringenin chalcone to naringenin. Consistent with a metabolic block at this step, supplementation (through the cut stem) of seedlings with exogenous naringenin led to anthocyanin accumulation in the major veins of naringenin-treated but not mock-treated af plants (Fig. 6A). In genetic experiments, we used Agrobacterium tumefaciens-mediated transformation to introduce the wild-type CHI1 gene, expressed from the native CHI1 promoter, into the af mutant background. We recovered 16 independent primary (T0) transformants that accumulated anthocyanins in leaf and stem tissue following the transfer of explants from tissue culture medium to soil. Four representative anthocyanin-accumulating (AC+) lines (CHI1::CHI1-2, CHI1::CHI1-3, CHI1::CHI1-4, CHI1::CHI1-9) and one anthocyanin-deficient (AC−) line (CHI1::CHI1-20) were selected for further analysis (Fig. 6B and C). All five of these transgenic lines tested positive by PCR for the presence of the pCHI1::CHI1 transgene. Western-blot analysis confirmed that CHI1 protein accumulated in the AC+ lines but not the AC− line (Fig. 6C). Spectrophotometric assays showed that the level of anthocyanin in the AC+ lines was more than 5-fold that in the af parental line and ranged from 68% to 113% of that in wild-type leaves. The anthocyanin content of CHI1::CHI1-20 was not significantly different from that in leaves of the af mutant. These collective findings demonstrate that the anthocyanin deficiency of the af mutant results from a loss of function of a single CHI isoform, SlCHI1.

SlCHI1 Modulates Terpenoid Production in Type VI Glandular Trichomes

Given the identification of af as a CHI1 mutant, we next addressed the question of why af plants lack of the typical aroma of tomato foliage (Rick et al., 1976). Gas chromatography (GC)-MS was employed to analyze the volatile composition of wild-type and af leaves and type VI trichomes. Analysis of leaf-dip extracts showed that af produces less than 15% of wild-type levels of several monoterpenes, including α-pinene, 2-carene, α-phellandrene, α-terpinene, and β-phellandrene (Fig. 7A). The levels of two sesquiterpenes, δ-elemene and β-caryophyllene, were also reduced in af leaves. Analyses of extracts from isolated type VI trichome glands, which are the main source of tomato leaf
terpenoids (Kang et al., 2010a, 2010b), yielded similar results (Fig. 7B).

To investigate the genetic basis of the terpenoid deficiency in af trichomes, we measured the level of β-phellandrene (the major monoterpenene) in progeny from a cross between af and its parental line (cv Red Cherry). Analysis of the anthocyanin phenotype in both F1 and F2 (260 F2 plants) confirmed that af segregates as a monogenic recessive mutation (197 AC+ plants and 63 AC- plants; $\chi^2 = 0.04$, $P = 0.8415$) and further showed that the AC+ and AC- phenotypes within this population are unambiguous (Fig. 2). The β-phellandrene content in type VI glands of F1 plants was approximately one-third of that in glands from the wild-type parent but significantly greater than that of af plants (Supplemental Fig. S3A), suggesting that af is semidominant with respect to the terpenoid phenotype. Analysis of β-phellandrene levels in leaf-dip extracts from 56 randomly selected F2 individuals (34 AC+ plants and 22 AC- plants) showed that the

Figure 5. The Af gene encodes CHI. A, Fine genetic mapping of Af delimited the target gene to an interval between markers SGN-U571424 and CT93 on chromosome 5. Numbers in parentheses indicate the number of recombination events identified between markers and the target gene. B, Physical map of the region defined by markers SGN-U571424 and C2_At3g55120. Black boxes indicate exons within three predicted genes (arrows). The asterisk denotes the location of the mutation identified in the last exon of CHI1. C, Wild-type (WT) and af-derived nucleotide sequence (top) encoding the C terminus of CHI1 and the corresponding deduced amino acid sequence (bottom). Stop codons are indicated by boldface letters and depicted in the predicted amino acid sequence by asterisks. D, Enzymatic activity of CHI1 WT and CHI1 af. Purified recombinant CHI1 WT and CHI1 af proteins (0.125 μg) were mixed with substrate (naringenin chalcone), and the amount of the substrate was measured spectrophotometrically ($A_{340}$) at various times thereafter. Control reactions in which enzyme was omitted were performed in parallel and used to correct for nonenzymatic turnover of the substrate. E, Western-blot analysis of CHI protein levels in wild-type and af leaves. Crude leaf extract was separated into supernatant (sup.) and pellet fractions by centrifuging at 21,000g for 25 min at 4˚C. The resulting supernatant (10 μg of protein) and a proportional amount of detergent-solubilized protein in the pellet fraction were blotted and probed with a polyclonal antibody against tomato CHI1 (top). The polyvinylidene difluoride membrane was stained with Coomassie blue after western-blot analysis (bottom). Arrows denote CHI1 and the large subunit of Rubisco (RbcL).
amount of β-phellandrene in AC+ plants was consistently greater than that in AC− individuals (Supplemental Fig. S3B). The mean level of β-phellandrene in AC− (af/af) plants was 30.5% of that in AC+ (Af/−) siblings. This finding establishes a genetic linkage between the anthocyanin and terpenoid phenotypes.

The terpenoid deficiency in af trichomes could result from the loss of CHI1 function or, alternatively, a secondary mutation closely linked to the Af locus. To distinguish between these possibilities, we measured the terpenoid content in leaves of the CHI1::CHI1 transgenic lines described above. The level of the major monoterpenoid (β-phellandrene) in type VI glands isolated from four independent complemented (AC+) lines ranged between 39% and 97% of that in wild-type trichomes.
(Fig. 8A). Similarly, the level of β-caryophyllene in these lines ranged between 93% and 110% of wild-type levels (Fig. 8B). In all four complemented lines, terpenoid levels were significantly greater than those in the congenic af parent. The terpenoid content of glands isolated from the noncomplemented (AC−) control line (CHI1::CHI1-20) was comparable to that in the af mutant. Analysis of terpenoid levels in leaf-dip extracts obtained from the same genotypes gave similar results (Supplemental Fig. S4A). These findings indicate that the terpenoid deficiency of af leaves is caused by the defect in CHI1.

DISCUSSION

Af Encodes a CHI

Mutations affecting anthocyanin pigmentation have been instrumental not only as markers in classical plant genetics but also as tools to identify flavonoid biosynthetic genes and associated regulatory loci in many plant species (Grotewold, 2006). Several mutations in tomato cause complete loss of anthocyanin pigments, which, depending on environmental conditions, typically accumulate in hypocotyls, stems, abaxial leaf surfaces, and cortical cells at the base of trichomes (von Wettstein-Knowles, 1967). Here, we provide several independent lines of evidence to demonstrate that anthocyanin deficiency in the af mutant results from loss of function of an isoform (CHI1) of CHI. The small deletion identified in the CHI1 gene from af plants abolishes CHI enzymatic activity and also prevents the accumulation of CHI1 protein. Transgenic complementation experiments showing that the anthocyanin deficiency is restored by expression of the wild-type CHI1 gene from its native promoter showed that the af mutation is responsible for the defect in flavonoid production. Consistent with a biochemical role for SICH1 in the cyclization of naringenin chalcone to the corresponding flavanone (naringenin), exogenous naringenin restored anthocyanin accumulation in vascular tissues of the af leaves. A search of the tomato genome (http://solgenomics.net) identified seven genes annotated as CHI or CHI-like sequences. Phylogenetic analyses showed that only two of these, CHI1 and CHI2, are closely related to canonical CHIs that catalyze the cyclization of chalcones to tricyclic (S) flavonones (Supplemental Fig. S5). The five remaining CHI-like genes in tomato are predicted to encode polypeptides most closely related to noncatalytic CHI-fold family proteins, which bind fatty acid substrates (Ngaki et al., 2012). The apparent lack of CHI2 expression, together with the strong flavonoid deficiency of the af mutant, indicates that CHI1 is the major isoform for flavonoid production in tomato leaves. As expected, we found that af plants are deficient in the production of other naringenin-derived metabolites, including rutin and several flavonol glycosides. The af mutant should provide a useful genetic tool to assess the physiological roles and nutritional importance of flavonoid production in tomato. Because low levels of some flavonoids (e.g. rutin) were detected in af leaves, we cannot exclude the possibility that another CHI isoform plays a minor role in flavonoid biosynthesis or that naringenin chalcone is converted nonenzymatically to naringenin at very low levels (Bednar and Hadcock, 1988).

Mutations affecting the phenylpropanoid and flavonoid pathways can have pleiotropic effects on plant growth, development, and fertility (Taylor and Grotewold, 2005; Peer and Murphy, 2007; Schilmiller et al., 2009a). Although af abolishes the activity of CHI1 and eliminates anthocyanin production, obvious defects in the growth and development of the mutant were not apparent. This finding is consistent with the view that flavonoids are not essential for the normal growth and development of tomato under optimized growth conditions. We did notice, however, that the fruit size of af plants was smaller than that of cv Red Cherry, which is the parental line employed for the x-ray mutagenesis used to generate the mutant (Burdick, 1958). This
observation raises the possibility that the “wild-type” line (LA0337; cv Red Cherry) that we used for our studies is not isogenic with the *af* accession LA1049. These considerations, together with the fact that the x-ray mutagenesis is likely to have introduced other mutations, highlight the necessity of using genetically complemented lines for comparative analyses of *af*-related phenotypes.

The crystal structures of the functional monomers of CHI from alfalfa (*Medicago sativa*) and Arabidopsis (*Arabidopsis thaliana*) have provided detailed information about the active-site topology of the enzyme, which appears to be highly conserved in a wide range of plant species (Jez et al., 2000; Ngaki et al., 2012). As expected, residues involved in enzyme catalysis and the formation of the active site are well conserved in tomato CHI (Supplemental Fig. S6). Although the C-terminal α-helix (α7) that is truncated by the *af* mutation does not participate directly in substrate binding or catalysis, it does form an α-helical segment (helix-turn-helix α6-α7) that provides structural support to the enzyme’s active site (Jez et al., 2000; Ngaki et al., 2012). Our results show that this mutation not only abolishes the in vitro activity of the enzyme but also prevents the accumulation of the protein in *af* tissues. Accordingly, *af* is a true null mutant with respect to CHI1 function. Our ability to detect CHI1 transcripts but not the corresponding CHI1 protein in *af* leaves suggests that truncation of α7 destabilizes the protein in planta.

**Modulation of Terpenoid Production by CHI1**

Previous studies showed that *af* plants, unlike several other anthocyanin-deficient mutants tested, are highly susceptible to attack by native populations of insect herbivores (Rick et al., 1976). Of particular interest was the finding that increased susceptibility of *af* plants to insect attack was associated with a “lack of foliage aroma” and a striking reduction in the density of type VI trichomes. Based on the results of genetic cosegregation tests, Rick et al. (1976) concluded that the anthocyanin defect and altered reactivity to herbivore attack are most likely conditioned by a single gene (*af*). Our results confirm and extend these findings in several ways. First, we demonstrate that glandular trichomes from *af* leaves are deficient in monoterpens and sesquiterpenes that are derived from the MEP and mevalonic acid pathways, respectively (Schlimmiller et al., 2009a, 2010). This pleiotropic metabolic defect explains the lack of leaf volatiles and presumably accounts for the aroma-related phenotype of *af* leaves. We also found that the size and density of type VI glands on *af* leaves are reduced in comparison with wild-type plants. Under our standard growth conditions, the reduction in trichome density was not as severe as that reported by Rick et al. (1976). These differences may reflect plant growth conditions (e.g. light intensity) or plant developmental stage, which can have strong quantitative effects on trichome characteristics. Finally, our field studies performed in East Lansing, Michigan, like those conducted in Davis, California (Rick et al., 1976), showed that the *af* mutant is highly susceptible to attack by native populations of flea beetles (*Epitrix spp*). We also observed that other species of herbivorous beetles were more prevalent on *af* compared with wild-type plants grown in the same test plot. Increased susceptibility to insect herbivores has been demonstrated for other trichome mutants of tomato, including *hairless* and *odorless2*, which are also deficient in the production of trichome-borne terpenoids (Kang et al., 2010a, 2010b). These collective observations are consistent with the view that tomato leaf volatiles act as repellents of herbivorous beetles (Rick et al., 1976) and, more generally, highlight the importance of glandular trichomes in mediating tomato-herbivore interactions (Kennedy, 2003; Li et al., 2004; Howe and Jander, 2008; Peiffer et al., 2009; Bleeker et al., 2012).

Given the well-established role of CHI in flavonoid biosynthesis and the distinct nature of flavonoid and terpenoid metabolic pathways, the ability of the wild-type CHI1 gene to complement the terpenoid-deficient phenotype of *af* was unexpected. Indeed, the pleiotropic phenotypes of *af* led De Jong et al. (2004) to propose that *af* likely defines a regulatory gene such as *TRANS-PARENT TESTA GLABRA1*, which controls both anthocyanin production and trichome development in Arabidopsis (Walker et al., 1999). There are several hypotheses to explain why the loss of CHI1 results in decreased production of terpenoids in type VI glands. For example, a flavonoid pathway intermediate or end product whose synthesis depends on CHI1 may promote, either directly or indirectly, terpenoid production. This idea is consistent with studies showing that flavonoids can modulate gene expression and various hormone responses (Peer and Murphy, 2007; Ringli et al., 2008; Pourcel et al., 2013).

An alternative hypothesis is that the accumulation of naringenin chalcone (the substrate of CHI1) or another upstream pathway intermediate inhibits terpenoid production in gland cells. This potential mechanism of metabolic cross regulation is analogous to the recently discovered role of a MEP pathway intermediate (methylyerythritol cyclodiphosphate) in controlling the expression of stress-responsive nuclear genes (Xiao et al., 2012). Naringenin chalcone, like other open-chain flavonoids within the chalcone family, possesses an electrophilic α,β-unsaturated carbonyl group that contributes to the broad biological activities of chalcones, including the inhibition of various biosynthetic and regulatory proteins (Sahu et al., 2012). We speculate that reduced CHI activity in glandular trichomes of the *af* mutant may lead to increased levels of naringenin chalcone, which could potentially react with glutathione to alter cellular redox status or modify Cys groups in proteins. In this context, it is noteworthy that naringenin chalcone accumulates in tomato fruit peel, indicating that CHI is a rate-limiting step in flavonol biosynthesis in this tissue (Muir et al., 2001). Similarly, regulation of CHI1 expression in type VI glands by developmental or environmental cues could provide a mechanism to control metabolic flux through both the flavonoid and terpenoid pathways.
terpenoid pathways. Measurement of naringenin chalcone levels in type VI glands from wild-type and mutant lines will provide a critical test of this hypothesis. It is also possible that the regulatory effect of CHI1 on terpenoid production is not dependent on isomerase activity per se but rather on the ability of CHI1 to bind a related small molecule. Interestingly, one of the most highly expressed genes in hop (Humulus lupulus) glandular trichomes encodes a CHI-like protein that is active as a CHI (Wang et al., 2008).

Our genetic analyses suggested that although af is fully recessive with respect to the anthocyanin phenotype, the mutation appears to be semidominant with respect to terpenoid production in type VI trichomes. Given our uncertainty about the genetic relationship between af and cv Red Cherry (see above), this conclusion awaits confirmation through detailed characterization of genetically complemented af lines. Nevertheless, a semidominant effect of af on terpenoid production would suggest that a single functional allele of CHI1 is insufficient to meet the metabolic demand for terpenoid production in cells of the trichome gland. Haploinsufficiency in plants often involves multiprotein complexes associated with metabolic processes (Meinke, 2013). Indeed, there is evidence that CHI and other flavonoid biosynthetic enzymes assemble into multiprotein complexes (Burbulis and Winkel-Shirley, 1999). The dual cytosolic/nuclear localization of CHI has also led to the suggestion that CHI-derived compounds may regulate gene expression in Arabidopsis (Saslowsky et al., 2005). These collective studies raise the possibility that CHI1 may modulate terpenoid production through interaction with proteins involved in terpenoid production or with proteins required for the expression of genes encoding terpenoid biosynthetic enzymes. Given the prevalence of transcription factor binding to exons (Stergachis et al., 2013), we cannot exclude the possibility that the af deletion mutation indirectly affects the activity of regulatory proteins that control the expression of terpenoid pathway genes.

Flavonoid and terpenoid biosynthesis dominate the metabolic activity of glandular trichomes in many plant species (Lange et al., 2000; Aziz et al., 2005; Nagel et al., 2008; Wang et al., 2008, 2009; Sallaud et al., 2009; Chatzopoulou et al., 2010; Dai et al., 2010; Schilmiller et al., 2010; McDowell et al., 2011; Bleeker et al., 2012; Tissier, 2012). Although genes encoding many of the respective biosynthetic enzymes have been identified and characterized in detail, it remains to be determined how metabolic flux through these two apparently disparate pathways is coordinated. That such coordination exists is supported by studies showing that the emission of phenylpropanoid/flavonoid- and terpenoid-derived components of scent in snapdragon (Antirrhinum majus) flowers is controlled by similar developmental and environmental (e.g. diurnal) cues (Dudareva et al., 2000, 2003). More intriguingly, recent transgenic studies provide evidence for the existence of transcription factors that promote metabolic flux through both pathways (Bedon et al., 2010; Ben Zvi et al., 2012). Our characterization of pleiotropic metabolic defects in the tomato af mutant is consistent with the emerging role of secondary metabolites as lineage-specific signals that coordinate responses to changing environments (Clay et al., 2009; Jander and Clay, 2011; Xiao et al., 2013). Currently, we have no evidence that the af mutation impairs terpenoid production in cell types other than type VI glands. It is possible, therefore, that the modulation of terpenoid metabolism by CHI1 reflects a mechanism to coordinate the development and biosynthetic capacity of this specialized defensive structure (Kliebenstein, 2013). The enhanced susceptibility of af plants to attack by coleopteran herbivores further suggests that flavonoid-terpenoid cross-regulation impacts species interactions in an ecologically meaningful way (Stam et al., 2013). Future studies are needed to address the mechanisms by which secondary metabolites, or the enzymes that produce them, exert specific effects on seemingly unrelated metabolic pathways.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Tomato (Solanum lycopersicum) ‘Red Cherry’ (LA0337) was used as the wild type for all experiments. Seeds for the wild type, af (LA1049), and the ILS-2 introgression line (LA4055) were obtained from the C.M. Rick Tomato Genetics Resource Center (University of California, Davis). Seedlings were grown in Jiffy peat pots (Humert International) and maintained in a growth chamber as described previously (Chen et al., 2006; Kang et al., 2010b). Three- to 4-week-old plants were used for metabolite profiling and analysis of trichome morphology and density.

Metabolite Analysis

For quantification of anthocyanins, leaves from 4-week-old plants were placed in a 2-mL microcentrifuge tube (Dot Scientific) with 1 mL of methanol containing 1% (v/v) hydrochloric acid and incubated overnight at 4°C. Anthocyanin pigments in the resulting extract were measured spectrophotometrically at 530 and 657 nm. Anthocyanin levels were calculated as previously described (Rabino and Mancinelli, 1986).

Leaves from 4-week-old plants were used to prepare leaf-dip or type VI trichome extracts as described previously (Kang et al., 2010b). For the analysis of flavonoids and other nonvolatile compounds, single leaflets were placed in 1 mL of methanol:acetic acid:water (9:3:1) containing 10 μM propyl-4-hydroxybenzoate as an internal standard and incubated overnight in the dark with gentle shaking. Alternatively, a defined number of type VI glands, collected with a Pasteur pipette, were dissolved in 100 μL of methyl tertiary-buty1 ether containing tetradecane (10 ng μL−1) as an internal standard. Alternatively, type VI glands collected with a Pasteur pipette were dissolved in 100 μL of methyl tertiary-buty1 ether containing the internal standard. Quantification of metabolites was performed as described previously using HPLC/time-of-flight MS for non-volatile metabolites and GC-MS for volatile terpenes (Kang et al., 2010b).

Trichome Density and Morphology

A dissecting microscope (Leica MZ16) equipped with KL 2500 LED light sources and a Leica DFC 290 camera was used to document trichome morphology, size, and density. Scanning electron microscopy was performed as described previously (Kang et al., 2010b). All measurements were performed on wild-type and af plants grown together in the same growth chamber.

Analysis of Insect Herbivory in Field Plots

Field experiments were performed in the summer of 2008 and 2009. Four-week-old plants grown in the greenhouse were transferred to a test plot at the...
Map-Based Cloning of Af

Fine-mapping of Af was performed with an F2 population derived from a cross between af (LA1049) and ILS-2 (LA4055) and was facilitated by the tomato genome sequence (Tomato Genome Consortium, 2012). A population of 1,255 F2 plants was scored for the anthocyanin phenotype (AC or ac) and subsequently genotyped with PCR-based conserved ortholog set markers located within the introgressed region of chromosome 5. Specifically, 5. pennellii EST sequences generated in the Solanum Trichome Project (http://www.trichome.msu.edu) were used to convert the RFLP markers T1790 and CT93, and the tomato unigene SGN-U564842, to PCR-based markers. Primer sequences used for mapping are listed in Supplemental Table S1. The PCR amplification conditions were as described previously (Kang et al., 2010a).

PCR Analysis

RNA extracted from leaves (Plant RNeasy Kit; Qiagen) was used for cDNA synthesis (Thermoscript RT-PCR system; Invitrogen) according to the manufacturer’s protocol. Full-length cDNAs corresponding to SICHI1 and SICHI2 were amplified by PCR (DNA Engine Dyad Thermal Cycler; Bio-Rad) using the primer set (CHI1 and CHI2) listed in Supplemental Table S2. A cDNA encoding elongation factor 1A was PCR amplified using the elf4a primer (Supplemental Table S2) and used as a loading control. RT-PCR (25 μL) contained 2 μL of cDNA, 1 μL of a 10 μM solution of each primer, 0.75 μL of 10 mM deoxyribonucleotide triphosphate mix, 5 μL of 5× KAPA buffer, and 0.5 μL of KAPA DNA polymerase (KAPAHFi HiStart; Kapa Biosystems). The amplification protocol included an initial 4-min denaturation step at 95°C, followed by 25 cycles in which the template was denatured for 20 s at 95°C, annealed for 1 s at 52°C, and extended for 40 s at 72°C, followed by a final incubation for 2 min at 72°C. Amplified products were separated on a 1% agarose gel. Full-length genomic DNA corresponding to SICHI1 and SICHI2 from wild-type and af plants was PCR amplified using the primer sets listed in Supplemental Table S2. The PCR-amplified fragments were cloned into the pGEM-T-Easy plasmid vector (Promega). Automated nucleotide sequencing was performed at the Michigan State University Genomics Technology Support Facility (http://rtst.msu.edu/).
Regenerated T0 transgenic plants containing the CHI1-CHI2 transgene were potted in soil and transferred to a growth chamber for preliminary biochemical analyses. These plants were subsequently transferred to a greenhouse for the collection of seeds (T1 generation) from individual lines. T1 seedlings exhibiting an AC+ phenotype were selected from lines in which the ratio of AC+ to AC- was 3:1. These AC+ plants were grown in a greenhouse for the collection of T2 seeds. Lines in which all T2 progeny exhibited the AC+ phenotype were selected as being homozygous for the transgene.

Supplemental Data

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

Supplemental Figure S1. RT-PCR for CHI1 and CHI2 from wild-type and dfl leaves.

Supplemental Figure S2. Affinity purification of CHI1WT and CHI1fl expressed in Escherichia coli.

Supplemental Figure S3. β-Phellandrene levels in F1 and F2 plants derived from a cross between the dfl mutant (LA1049) and its reported wild-type parent (cv Red Cherry; LA0337).

Supplemental Figure S4. Terpenoid accumulation on the leaf surface of CHI1::CHI1 transgenic lines.

Supplemental Figure S5. Phylogenetic tree of CHI and CHI-fold proteins from cultivated tomato.

Supplemental Figure S6. Amino acid sequence alignment of CHIs from several plant species.

Supplemental Table S1. Description of PCR-based markers for genetic mapping.

Supplemental Table S2. Description of oligonucleotide primers used for PCR.

ACKNOWLEDGMENTS

We thank Eric Czuprenski, Razi Shaha, and Eric Kastory for assistance with plant growth and genetic mapping experiments. Dr. Cornelius Barry for helpful advice with genetic mapping, Ray Hammerschmidt and Gary Zehr for providing field space, the Michigan State University Center for Advanced Microscopy for assistance with scanning electron microscopy, the Michigan State University Mass Spectrometry and Metabolomics Core for GC-MS resources, the SOL Genomics Network for bacterial artificial chromosome clones and bioinformatics resources, and the C.M. Rick Tomato Genetics Resource Center (University of California [Davis]) for kindly providing tomato seed stocks.

Received December 2, 2013; accepted January 13, 2014; published January 14, 2014.

LITERATURE CITED


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