The *FATTY ACID DESATURASE2* Family in Tomato Contributes to Primary Metabolism and Stress Responses[1][OPEN]

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The conversion of oleic acid (C18:1) to linoleic acid (C18:2) in the endoplasmic reticulum is critical to the accumulation of polyunsaturated fatty acids in seeds and other tissues, and this reaction is catalyzed by a Δ12-desaturase, FATTY ACID DESATURASE2 (FAD2). Here, we report that the tomato (*Solanum lycopersicum*) genome harbors two genes, *SlFAD2-1* and *SlFAD2-2*, which encode proteins with in vitro Δ12-desaturase activity. In addition, tomato has seven divergent FAD2 members that lack Δ12-desaturase activity and differ from canonical FAD2 enzymes at multiple amino acid positions important to enzyme function. Whereas *SlFAD2-1* and *SlFAD2-2* are downregulated by biotic stress, the majority of divergent *FAD2* genes in tomato are upregulated by one or more stresses. In particular, *SlFAD2-7* is induced by the potato aphid (*Macrosiphum euphorbiae*) and has elevated constitutive expression levels in *suppressor of prosystemin-mediated responses2* (*spr2*), a tomato mutant with enhanced aphid resistance and altered fatty acid profiles. Virus-induced gene silencing of *SlFAD2-7* in *spr2* results in significant increases in aphid population growth, indicating that a divergent *FAD2* gene contributes to aphid resistance in this genotype. Thus, the *FAD2* gene family in tomato is important both to primary fatty acid metabolism and to responses to biotic stress.

Fatty acids are essential components of cellular membranes and storage lipids and are also precursors for a wide variety of plant metabolites, including signaling molecules and phytoalexins (Ohlrogge and Browse, 1995; Lim et al., 2017). The relative abundance of different fatty acid species and their derivatives in plants is regulated in part through the action of fatty acid desaturases (FADs) and related enzymes. FADs add double bonds at specific positions within the acyl chain of the fatty acid, thereby shaping many of the chemical properties of the molecule, including its Tm (Aguilar and de Mendoza, 2006), its oxidative stability (Shahidi and Zhong, 2010), and its ability to act as a substrate for synthesis of other metabolites (Chehab et al., 2007). Duplication of genes encoding FADs has also enabled the functional diversification of these enzymes in some plant species, giving rise to divergent catalytic capabilities and synthesis of novel fatty acids or their derivatives (Cao et al., 2013). Because of their impacts on the chemical properties of membrane and storage lipids and on the accumulation of secondary metabolites derived from fatty acids, standard and divergent FADs influence many important physiological and agronomic properties of crops. For example, they impact seed oil quality (Haun et al., 2014), fruit aromas (Domínguez et al., 2010), and abiotic and biotic stress resistance (Upchurch, 2008).

This study focuses on members of the FAD2 family, which includes Δ12-desaturases and also enzymes with divergent activities. Although this family occurs throughout the plant kingdom, our knowledge of these enzymes is based largely on research on the model plant Arabidopsis (*Arabidopsis thaliana*) and on oil seed crops. The Arabidopsis *FATTY ACID DESATURASE2* (*AtFAD2*) gene was the first *FAD2* gene to be described and encodes a Δ12-desaturase localized in the

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endoplasmic reticulum (ER; Browse et al., 1989; Falcone et al., 1994; Okuley et al., 1994). Together with FAD6, a Δ12-FAD localized in the chloroplast, the FAD2 enzyme enables the accumulation of polyunsaturated fatty acids in plants by adding a second double bond to the monounsaturated fatty acid oleic acid ($18:1^{\Delta 9}$) at the Δ12 position in the acyl chain (Ohlrogge and Browse, 1995). This yields linoleic acid ($18:2^{\Delta 9,12}$), which can in turn be converted to linolenic acid ($18:3^{\Delta 9,12,15}$) by Δ15 (i.e. *ω*3) FADs (namely FAD3 in the ER and FAD7 and FAD8 in the chloroplast; Ohlrogge and Browse, 1995). Linoleic acid and linolenic acid are among the most abundant polyunsaturated fatty acids in plants, and their accumulation is strongly influenced by levels of *FAD2* gene expression and enzyme activity (Meï et al., 2015; Dar et al., 2017).

Homologs of *AtFAD2* have been identified in a diverse array of oil seed crops because of commercial interest in modifying the fatty acid content of oil by manipulating FAD2 activity in seeds. Numerous studies have demonstrated that suppressing the Δ12-desaturase activity of FAD2 isomers in seeds reduces the abundance of linoleic and linolenic acid relative to oleic acid in the storage lipids of the seed (Dar et al., 2017). This decrease in polyunsaturation is considered a desirable trait in oil seed crops because it enhances the oxidative stability and shelf life of the oil, reducing rancidity and improving the industrial properties of the oil (Clemente and Cahoon, 2009). Because of this, the oil seed quality of canola (*Brassica napus*; Schierholt et al., 2000), peanut (*Arachis hypogae*; Jung et al., 2000; López et al., 2000), cotton (*Gossypium hirsutum*; Chapman et al., 2001), soybean (*Glycine max*; Buhr et al., 2002), sunflower (*Helianthus annuus*; Schuppert et al., 2006), safflower (*Carthamus tinctorius*; Hamdan et al., 2012), and other oil seed crops has been enhanced by decreasing endoplasmic Δ12-desaturase activity through means such as genome editing (Haun et al., 2014), chemical mutagenesis (Lee et al., 2018), RNA interference (Buhr et al., 2002), and selection for naturally occurring allelic variation in *FAD2* genes (López et al., 2000; Thambugala et al., 2013), and several of these high-oleic acid crops are commercially widespread.

Despite the predominant research focus on seed-localized FAD2 isomers with known Δ12-desaturase activity, other studies indicate that the FAD2 family also contains considerable diversity in function and expression patterns. This diversification is enabled by the presence of multigene families; whereas the Arabidopsis genome carries only one copy of *AtFAD2*, comparative analysis of other species reveals that many plant genomes carry multiple *FAD2* homologs. For example, soybean has seven *FAD2* gene family members (Lakhssassi et al., 2017), cotton has nine (Feng et al., 2017), safflower has 11 (Cao et al., 2013), and parsley (*Petroselinum crispum*) and carrot (*Daucus carota* ssp. *sativus*) have 17 and 24, respectively, the highest number of family members documented so far, indicating that functional divergence of *FAD2* is not limited to oil seed crops (Somssich et al., 1989; Busta et al., 2018). Although the functions of the majority of *FAD2* homologs are untested, analyses of plant species that produce unusual fatty acids or fatty acid derivatives have identified so-called divergent FAD2 proteins with novel enzymatic capabilities. *FAD2* family members that encode hydroxylases (van de Loo et al., 1995; Broun et al., 1998a), epoxidases (Lee et al., 1998), acetylenases (Cahoon et al., 2001; Okada et al., 2013; Busta et al., 2018), or conjugases (Cahoon et al., 1999; Liu et al., 2001; Dyer et al., 2002; Reed et al., 2002; Cahoon and Kinney, 2004) have been identified in medicinal species from a diversity of taxa, including the Apiaceae, Asteraceae, Balsaminaceae, Brassicaceae, Cucurbitaceae, Euphorbiaceae, and Santalaceae. Despite the evidence for functional diversification of FAD2 in multiple plant families, the potential for such diversification in major crops has been largely unexplored.

In addition to encoding catalytic diversity, *FAD2* homologs also show diversity in their expression patterns. While some *FAD2* genes are targeted to the seeds, others are ubiquitous or specific to other organs; for example, transcript profiling of the *FAD2* gene family in cotton and safflower revealed that developing seeds, roots, stems, and leaves expressed overlapping but distinct sets of *FAD2* homologs (Cao et al., 2013; Feng et al., 2017). In addition, different members of the *FAD2* family in soybean varied in their responsiveness to chilling and salinity (Feng et al., 2017), two abiotic stresses known to upregulate *FAD2* expression (Wang et al., 2004; Kargiotidou et al., 2008; Teixeira et al., 2009; Zhang et al., 2012). In other species, some *FAD2* genes are upregulated by biotic stresses, including viroid infection (Gadea et al., 1996), fungal infection (Wang et al., 2004), or application of fungal elicitors (Kirsch et al., 1997; Cahoon et al., 2003). *FAD2* family members that confer desaturase activity are thought to contribute to adaptation to abiotic stress by altering the properties of cell membranes (Zhang et al., 2012), and divergent *FAD2* genes that encode acetylenases are reported to synthesize secondary metabolites with antibiotic properties (Cahoon et al., 2003). Furthermore, transient silencing of a *FAD2* homolog in wheat (*Triticum aestivum*) results in increased susceptibility to powdery mildew (Li et al., 2011). Together, the diversity of enzymatic capabilities and expression patterns within the *FAD2* family suggest a diversity of functions that have not yet thoroughly been explored, including possible roles in biotic stress resistance.

The current study characterizes the FAD2 family in tomato (*Solanum lycopersicum*), which has not been previously described. This work expands our understanding of FAD2 family members to a major crop other than oil seed producers and examines their stress-responsive expression patterns and functions in vegetative tissues. Genomic analysis identified nine *FAD2* genes in tomato. Of these nine genes, only two had Δ12-desaturase activity when ectopically expressed in yeast (*Saccharomyces cerevisiae*), indicating that *SlFAD2-1* and *SlFAD2-2* are the family members responsible for linoleic acid synthesis in tomato. Pathway analysis and

reverse-transcription quantitative PCR (RT-qPCR) further indicated that the nine genes had distinctly different expression patterns and that some family members are stress responsive in foliage. Whereas *SlFAD2-1* and *SlFAD2-2* were unresponsive to mechanical wounding or aphids (*Macrosiphum euphorbiae*) and were downregulated by a bacterial pathogen (*Pseudomonas syringae* pv. DC3000), five out of seven of the other family members were transcriptionally upregulated by one or more of these stresses. *SlFAD2-7* showed particularly strong and broad stress responses; it was induced in foliage by wounding, aphid infestation, bacterial infection, and exogenous application of salicylic acid (SA), a key signaling molecule in biotic stress. Levels of constitutive foliar expression of *SlFAD2-7* were also enhanced by *spr2*, a mutation in *FATTY ACID DESATURASE7* (*SlFAD7*) that increases accumulation of linoleic acid and hexadecadienoic acid ($16{:}2^{\Delta 9,12}$) and promotes aphid resistance. Furthermore, virus-induced gene silencing (VIGS) of *SlFAD2-7* in the *spr2* mutant compromised aphid resistance in this genotype, resulting in increased aphid population growth on silenced plants. These results indicate that *SlFAD2-7* can promote plant defenses against aphids in certain genetic backgrounds. In summary, our findings demonstrate that the *FAD2* gene family in tomato includes multiple divergent members, in addition to two standard Δ12-desaturases, and that a divergent family member influences plant interactions with aphids and other biotic stresses.

RESULTS

The Tomato Genome Contains Nine Homologs of the Arabidopsis *AtFAD2* Gene and One Homolog of *AtFAD6*

A search of the tomato genome (International Tomato Annotation Group release 2.40) for homologs of the Arabidopsis *AtFAD2* gene identified nine homologs, which are located on chromosomes 1 (*SlFAD2-1*), 3 (*SlFAD2-2*), 4 (*SlFAD2-3* and *SlFAD2-4*), and 12 (*SlFAD2-5*, *SlFAD2-6*, *SlFAD2-7*, *SlFAD2-8*, and *SlFAD2-9*). Based on alignment of their predicted amino acid sequences (Fig. 1), their percents of shared identity with *AtFAD2* ranged from 52% to 76% (Table 1). For comparison, we also searched for homologs of the Arabidopsis chloroplastic Δ12-desaturase *AtFAD6*. In contrast to the *SlFAD2* gene family, the FAD6 enzyme in tomato appears to be encoded by a single gene, *SlFAD6*, which is located on chromosome 7 and shares about 81.56% sequence identity with *AtFAD6*. A phylogenetic analysis of the deduced polypeptide sequences for these 10 tomato genes and for *AtFAD2* and *AtFAD6* revealed that these sequences separate into five distinct groups (Fig. 2A). As expected, *SlFAD6* clusters with *AtFAD6* and forms a unique group separate from all *FAD2* sequences. Of the *SlFAD2* family members, *SlFAD2-1* and *SlFAD2-2* are the most closely aligned to *AtFAD2* and form a discrete cluster with *AtFAD2* (Fig. 2A).

Members of the *SlFAD2* Family Share Transmembrane Domains and Common Motifs Associated with Iron Binding and ER Localization

Regardless of their specificity, almost all membrane-bound desaturases in plants contain three His boxes, which are hypothesized to serve as ligands for a diiron cluster at the active site of the enzyme (Shanklin and Cahoon, 1998). Consistent with this, a tripartite motif of conserved histidines was identified in the predicted amino acid sequences of *SlFAD6* and all members of the *SlFAD2* family with the exception of *SlFAD2-5* (Fig. 1). As expected for membrane-associated desaturases, all of the predicted amino acid sequences also contained between four and six predicted transmembrane domains, with the exception of the SlFAD2-5 sequence, which only contained one. The predicted SlFAD2-5 protein is highly truncated compared to other family members, lacking all three His boxes and nine amino acid positions previously described to be important to enzyme activity (Fig. 1; Broun et al., 1998b; Gagné et al., 2009); therefore, it may potentially be a pseudogene. In all other genes in our analysis, the locations of the three His boxes align, and their predicted amino acid sequences distinguish *FAD2* homologs from members of the *FAD6* family. For *AtFAD2* and all eight full-length *SlFAD2* genes, the consensus sequences for the three His boxes are HEXGH, HXRHH, and HVXHH (Fig. 1). In contrast, the first and third His boxes in *SlFAD6* and *AtFAD6* are HDCAH and HIPHH, which match motifs (HDCXH and HXPHH) previously reported to be characteristic of *FAD6* genes in multiple plant species (Chi et al., 2011). Most of the full-length *SlFAD2* genes are also distinguished from *SlFAD6* by the presence of an ER retrieval motif at the C terminus (positions 456–460 in Fig. 1). *SlFAD2-1*, *SlFAD2-2*, *SlFAD2-3*, *SlFAD2-4*, *SlFAD2-6*, and *SlFAD2-8* all contain a conserved sequence (Y-X-X-K/R/D/E- ϕ, where ϕ represents a large hydrophobic amino acid such as L, F, M, or I) that is absent in *SlFAD6* and that has previously been shown to be necessary and sufficient to localize the FAD2 protein in the ER (McCartney et al., 2004). These results support the categorization of *SlFAD2* genes as members of the *FAD2* family.

Members of the *SlFAD2* Family Display Sequence Divergence at Sites Associated with Enzyme Function

Previously, targeted mutagenesis identified four amino acid positions that, when modified, could convert the Arabidopsis FAD2 Δ12-desaturase into a hydroxylase (Broun et al., 1998b; Broadwater et al., 2002). A comparative analysis of FAD2 Δ12-desaturases and FAD2 acetylenases from multiple plant species also identified five amino acid positions that consistently differed between these two groups of enzymes and that, when mutagenized, modified the chemoselectivity, stereoselectivity, and substrate recognition of desaturases and acetylenases (Gagné et al., 2009).

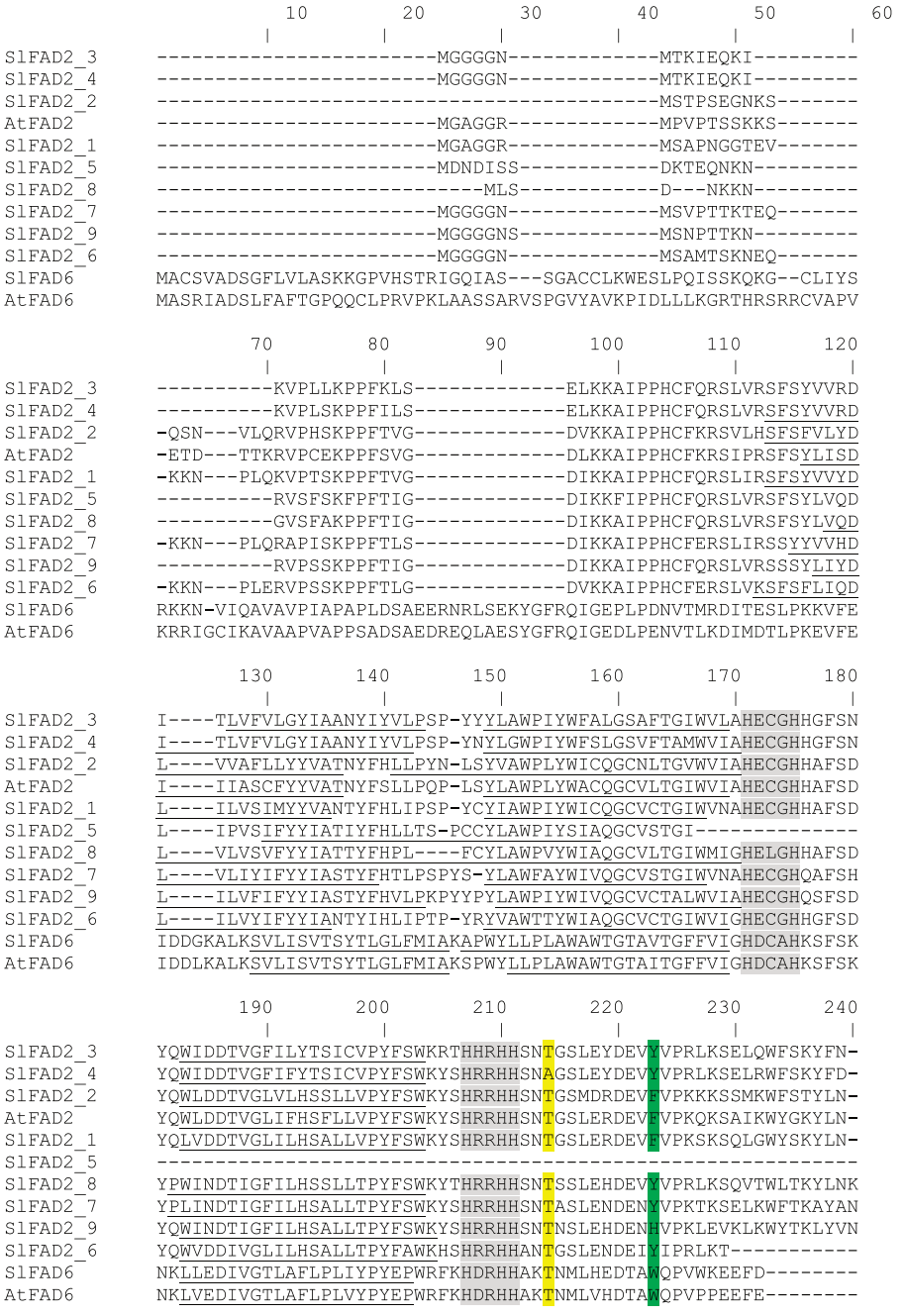

Figure 1. Comparison of tomato transcripts with Arabidopsis *FAD2* and *FAD6*. Nine homologs of the Arabidopsis *FAD2* gene (*AtFAD2*, At3g12120) were identified in the tomato genome: *SlFAD2-1* (Solyc01g006430), *SlFAD2-2* (Solyc03g058430), *SlFAD2-3* (Solyc04g040120), *SlFAD2-4* (Solyc04g040130), *SlFAD2-5* (Solyc12g036520), *SlFAD2-6* (Solyc12g044950), *SlFAD2-7* (Solyc12g049030), *SlFAD2-8* (Solyc12g100230), and *SlFAD2-9* (Solyc12g100250). An alignment of their predicted amino acid sequences was performed in ClustalW, and the Arabidopsis *FAD6* gene (*AtFAD6*, At4g30950) and its homolog in tomato (*SlFAD6*, Solyc07g005510) were included for comparison. His boxes are highlighted in gray, and ER retrieval motifs are highlighted in blue. Putative transmembrane domains calculated by TMPred are underlined. Residues highlighted in yellow were previously demonstrated to contribute to catalytic differences between canonical FAD2 Δ12-desaturases and divergent FAD2 hydroxylases (Broun et al., 1998b). Residues highlighted in green are variable between FAD2 Δ12-desaturases and divergent acetylenases (Gagné et al., 2009).

Therefore, to investigate the potential functions of members of the *SlFAD2* family, we compared their amino acid sequences with respect to these nine positions known to regulate enzymatic activity. *SlFAD2-1* and *SlFAD2-2* are the only members of the *SlFAD2* family that are identical to *AtFAD2* at all nine of these critical residues (Fig. 1), and their predicted amino acid sequences differed from all of the other FAD2 proteins in tomato at four of these sites (positions 223, 333, 340, and 392 in Fig. 1). Conversely, *SlFAD4* is the most divergent of the full-length members of the *SlFAD2* family and differs from *AtFAD2* at seven out of nine of these residues, including two amino acid positions reported by Broadwater et al. (2002) to be particularly important to catalytic specificity (Fig. 1, positions 214 and 392 in our alignment). Although most members of the *SlFAD2* family diverged from the expected amino acid sequence for a Δ12-desaturase, they also diverged from the sequences reported for known hydroxylases and acetylenases at these nine critical amino acid positions (Broun et al., 1998b; Broadwater et al., 2002; Gagné et al., 2009), matching at most one out of four positions that confer hydroxylase activity and two out of five positions important to acetylenase activity. This sequence analysis suggests that *SlFAD2-1* and *SlFAD2-2* are the most likely candidates for Δ12-desaturases in

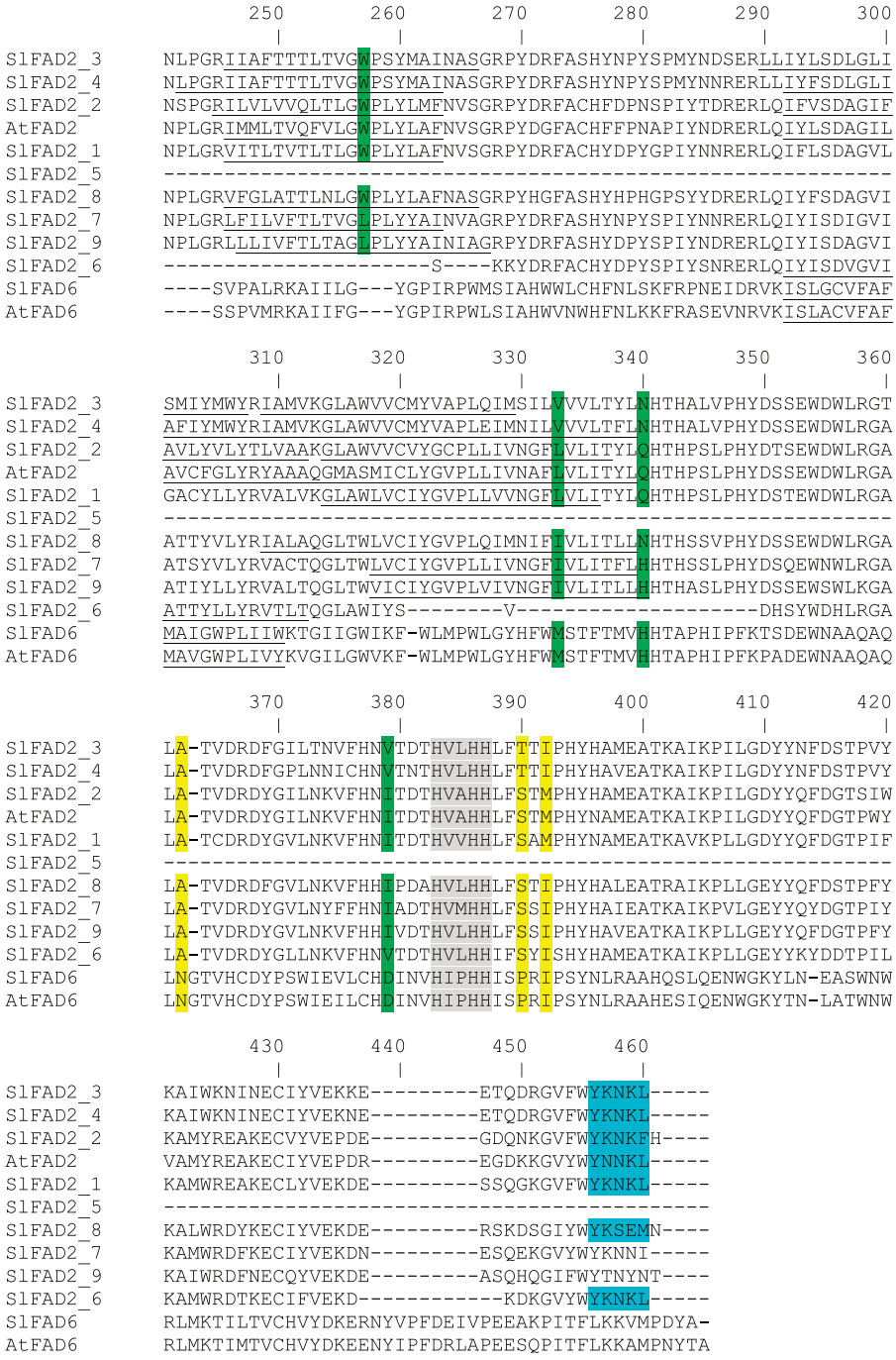

Figure 1. Continued.

tomato, while *SlFAD2-3* through *SlFAD2-9* may have alternative functions.

Members of the *SlFAD2* Family Differ in Their Tissue Expression Patterns

To explore when and where members of the *SlFAD2* family are expressed, digital expression analysis of tomato expressed sequence tags (ESTs; The Institute for Genomic Research tomato gene index release 9.0) was performed to assess expression profiles of this family in various tissues, including seed, fruit, flower, leaf, shoot, root, callus, and suspension culture. Transcripts were detected for all genes except *SlFAD2-3* and *SlFAD2-8* and varied greatly in the tissues in which they were detected (Fig. 2B). *SlFAD2-1* and *SlFAD6* were the most abundant and ubiquitous transcripts and showed strong expression levels in seeds, a primary site of fatty acid synthesis. This digital expression analysis was also

Table 1. *Gene identities and primer sequences for cloning and gene expression analysis*

Gene	SOL Genomics Sequence	% Amino Acid Identity[a]	*E* value[b]	Primers for Cloning (5′ Forward 3′; 5′ Reverse 3′)	Size[c] (bp)	Primers for RT-qPCR	Size[c] (bp)
SlFAD2-1	Solyc01g006430	73.89	~0.0[d]	F: CACCATGGGAGCTGGTGGTCGTAT; R: TCAGAGCTTGTTTTTGTACC	1,152	F: TCCTTGTCCTGATCACCTAC; R: GAGATGATTCGTCTTTCTCG	323
SlFAD2-2	Solyc03g058430	75.86	~0.0	F: CACCATGTCCACTCCTTCTGAGGGC; R: CTAATGAAACTTGTTTTTATACC	1,137	F: GTTGTCCAGCTCACTCTAGG; R: TGTCATAATGAGGCAATGAA	310
SlFAD2-3	Solyc04g040120	58.49	e−173	F: CACCATGGGAGGTGGTGGTAATATGA; R: TTAGAGTTTGTTTTTATACCAAA	1,128	F: TGGAAACGTACTCATCATCGTC; R: TGAAAAACGTTAGTTAGTATACCAA	509
SlFAD2-4	Solyc04g040130	57.96	e−172	F: CACCATGGGAGGTGGTGGTAATATGA; R: TTAGAGTTTGTTTTTATACCA	1,128	F: GAAGGCTATTCCTCCTCATT; R: ACGACGATGACTGTATTTCC	319
SlFAD2-5	Solyc12g036520	59.55	5e−28	F: CACCATGGACAACGATATATCATCCGA; R: CTATATTCCAGTAGAAACACAA	282	F: TGGACAACGATATATCATCC; R: ACCTTGAGCAATCGAGTAAA	280
SlFAD2-6	Solyc12g044950	52.48	e−141	F: CACCATGGGAGGTGGTGGTAATATGTC; R: TCAAAGCTTGTTTTTGTACCAA	951	F: CTTCAAAGCCTCCTTTTACA; R: GTGGAGGATAAGACCAACAA	323
SlFAD2-7	Solyc12g049030	63.80	~0.0	F: CACCATGGGAGGTGGTGGTAATATGTC; R: TCAAATATTGTTTTTGTACCA	1,155	F: ACTGCTTCCCTTGAGAACGA; R: GCTCCTCGTAGCCAATTCCA	441
SlFAD2-8	Solyc12g100230	65.49	~0.0	F: CACCATGTTGTCTGATAACAAGAAAAA; R: TTAATTCATCTCACTTTTATAC	1,107	F: ATGGCTTTGCAAGTCACTAT; R: GAATGTGGTGGAAGACCTTA	325
SlFAD2-9	Solyc12g100250	63.71	~0.0	F: CACCATGGGAGGTGGTGGTAATTCTAT; R: TTATGTGTTGTAATTTGTATAC	1,140	F: ACCCCTTGTAATTGTGAATG; R: AGATGCTTCATCTTTCTCCA	355
SlFAD6	Solyc07g005510	82	~0.0	F: CACCATGGCTTGCAGTGTTGCAGACTCT; R: TCAAGCGTAGTCAGGCATTACT	1,326	F: TGTTGGAACACTAGCCTTTT; R: TCCAAAAATGATAACCCAAC	438

[a]Identity in common with the nearest homolog in Arabidopsis, which is *AtFAD2* (GenBank accession At3g12120) for all *SlFAD2* family members and *AtFAD6* (GenBank accession At4g30950) for *SlFAD6*. [b]Statistical significance of homology to closest Arabidopsis homolog according to BLASTp (*AtFAD6* for *SlFAD6*; *AtFAD2* for all others). [c]Amplicon size. [d]*E* values $<\sim 5 \times 10^{-324}$ are rounded to zero.

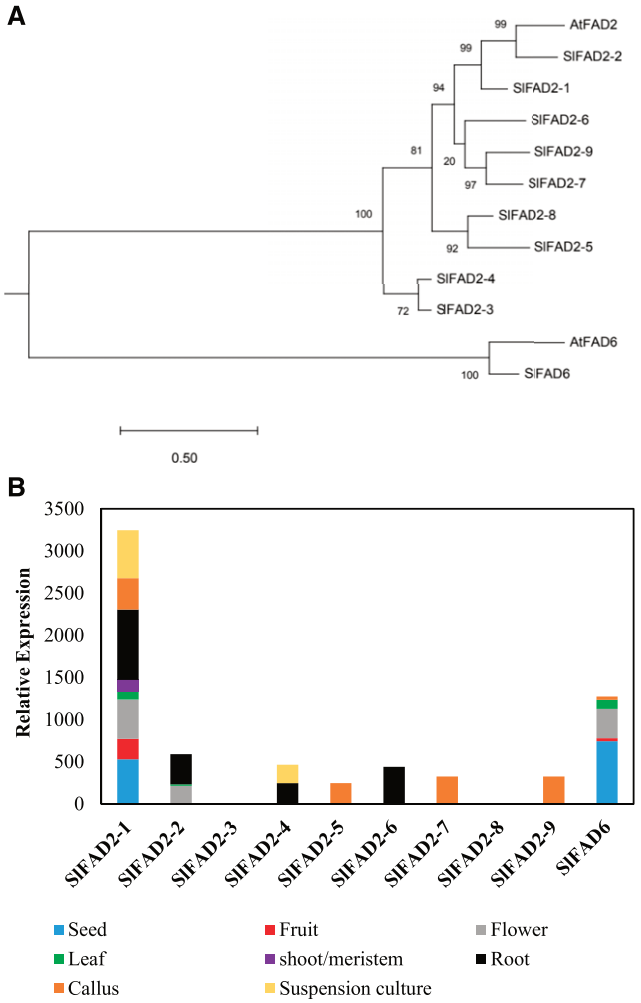

Figure 2. Comparison of *FAD2* family members in tomato. A, A phylogenetic tree based on predicted amino acid sequences was assembled for the nine members of the *SlFAD2* gene family in tomato, and *AtFAD2*, *AtFAD6*, and *SlFAD6* were included for comparison. The tree was inferred using the nearest-neighbor interchange algorithm without bootstrapping, and visualized using MEGA5. Values on branches indicate statistical support estimated using a Shimodaira-Hasegawa-like procedure. Branch length is indicated by the scale bar shown below, which represents the number of nucleotide replacements per site. B, Publicly available RNA sequencing libraries were also searched to identify the tissues in which each of the 10 tomato genes (*SlFAD2-1* through *SlFAD2-9*, as well as *SlFAD6*) are expressed.

consistent with the RNA sequencing data set from developing tomato fruit published by Pattison et al. (2015). All 10 of our genes of interest were present in the data set, but only three (*FAD2-1*, *FAD2-2*, and *FAD6*) showed marked expression levels in the fruits; of these, *FAD2-1* and *FAD6* were among the top 10% of most highly expressed genes in the embryo and endosperm (Pattison et al., 2015). These data support the hypothesis that, of the *FAD2* family members, *FAD2-2*, and particularly *FAD2-1*, are the most likely candidates to be involved in tomato seed oil synthesis.

SlFAD2-1 and *SlFAD2-2*, But Not Other Family Members, Confer Δ12-Desaturase Activity When Expressed in Yeast

To determine whether any of the *SlFAD2* genes encode functional Δ12-desaturases, these genes were expressed in *S. cerevisiae* under the control of the Gal-inducible *GALACTOKINASE 1* (*GAL1*) promoter, and fatty acid methyl ester (FAME) profiles of the yeast cultures were analyzed by gas chromatography-mass spectrometry (GC-MS) to detect the presence of the Δ12-desaturase products C16:2$^{\Delta 9,12}$ and C18:2$^{\Delta 9,12}$ when cultures were grown in the presence of Gal. *S. cerevisiae* is a suitable system in which to test for Δ12-desaturase activity of transgenes because this yeast lacks native Δ12-desaturases but accumulates the necessary C16:1$^{\Delta 9}$ and C18:1$^{\Delta 9}$ substrates for these enzymes (Kajiwara et al., 1996). Yeast cells transformed with the vector expressing the GUS enzyme served as a vector control and lacked any peaks corresponding to C16:2$^{\Delta 9,12}$ or C18:2$^{\Delta 9,12}$ (Fig. 3A). In contrast, yeast cells transformed with *AtFAD2* served as a positive control and accumulated C16:2$^{\Delta 9,12}$ and C18:2$^{\Delta 9,12}$ (Fig. 3B). Of the nine *FAD2* homologs from tomato, *SlFAD2-1* and *SlFAD2-2* yielded C16:2$^{\Delta 9,12}$ and C18:2$^{\Delta 9,12}$ when expressed in yeast (Fig. 3, C and D), whereas all other family members lacked detectable Δ12-desaturase activity (Fig. 3, E and F; Supplemental Table S1). The identities of C16:2$^{\Delta 9,12}$ and C18:2$^{\Delta 9,12}$ in all samples were confirmed by comparing the retention times of their GC peaks to those of purified standards and also through analysis of their mass spectra (Supplemental Fig. S1). In summary, these results indicated that Δ12-desaturase activity was limited to SlFAD2-1 and SlFAD2-2. This is consistent with our prior observations that within the *SlFAD2* gene family, *SlFAD2-1* and *SlFAD2-2* had the highest percent shared identity with the Arabidopsis *AtFAD2* gene (Fig. 2A) and the highest degree of conservation at amino acid positions that have previously been identified as important to enzyme activity (Fig. 1).

Members of the *SlFAD2* Family Have Overlapping But Distinct Protein Interaction Networks

To gain further insights into the potential functions of *SlFAD2* genes, including those that do not appear to encode Δ12-desaturases, we searched the tomato protein-protein interaction (PPI) network in the STRING database and extracted all putative interaction partners linked with each *SlFAD2* gene. This database represents functional relatedness among genes derived by integrating information from multiple functional genomics data across many organisms (Szklarczyk et al., 2017). In the tomato PPI network, *SlFAD2* genes are connected to 172 other genes on average (range = 157–204; Supplemental Table S2), with extensive overlap among the interaction profiles of all *SlFAD2* genes (Fig. 4A). As expected, the interaction profiles of all family members are significantly enriched in gene

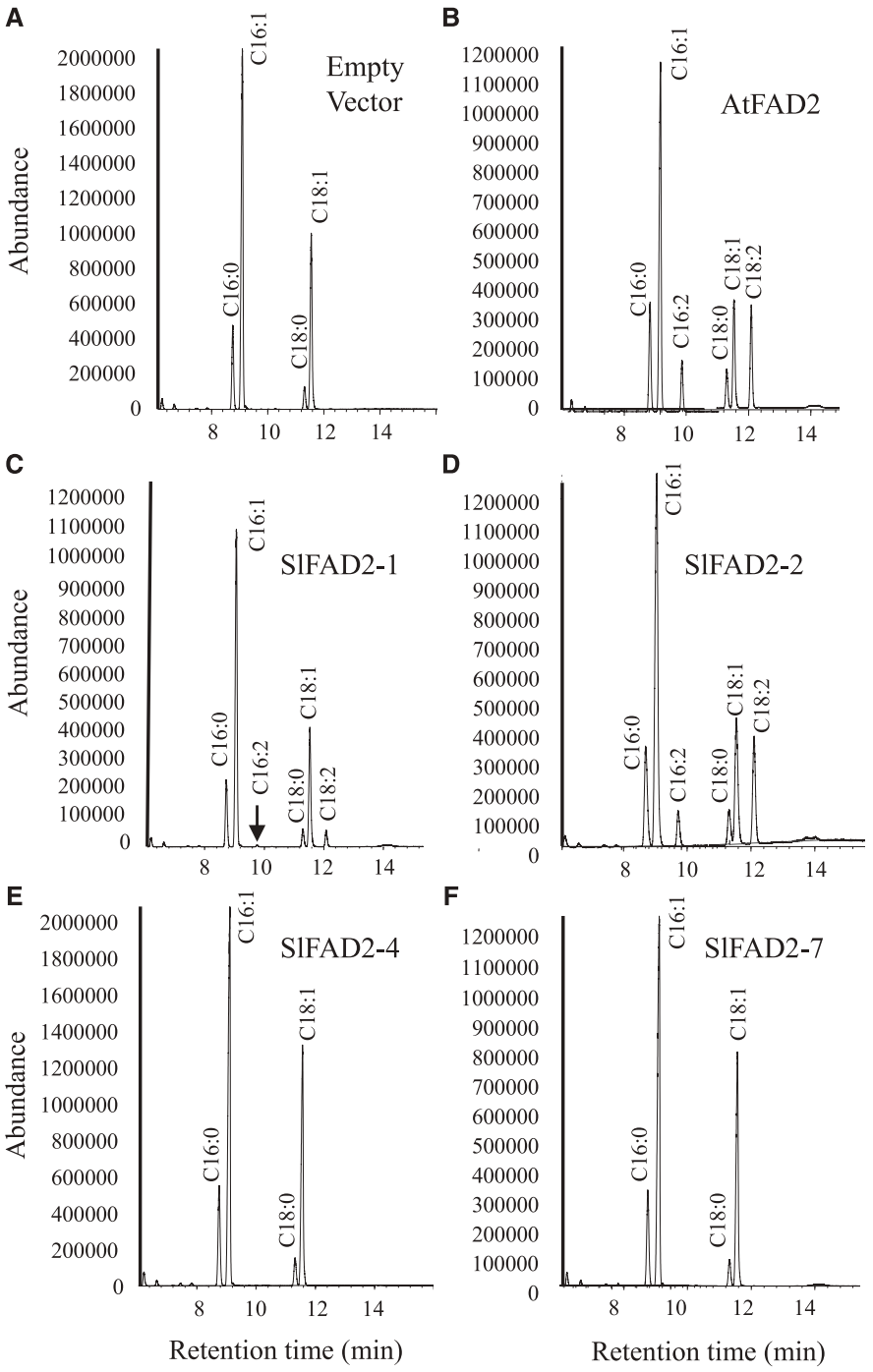

Figure 3. Δ12-Desaturase activity of *SlFAD2* family members expressed in yeast. Each member of the *SlFAD2* family in tomato was expressed in *S. cerevisiae*, which lacks native Δ12-desaturases, and the FAME profiles of the yeast cultures were analyzed by GC-MS to determine whether heterologous expression of the putative desaturases from tomato would yield C16:2 and C18:2. The vector pYES-DEST52/*GUS* (A) provides a negative control, and pYES-DEST52/*AtFAD2* (B), expressing an Arabidopsis protein with known Δ12-desaturase activity, is a positive control. Plasmids expressing tomato *SlFAD2* family members shown include pYES-DEST52/*SlFAD2-1* (C), pYES-DEST52/*SlFAD2-2* (D), pYES-DEST52/*SlFAD2-4* (E), and pYES-DEST52/*SlFAD2-7* (F).

ontology (GO) biological process (BP) terms associated with lipid, oxoacid, and small molecule metabolism (Fig. 4B). Compared to other family members, *SlFAD2-2* is connected to the largest number of other genes (204) and the highest number of BP terms, suggesting that the FAD2-2 protein may be particularly important in primary metabolism. Interestingly, the BP term "response to stress" is distinctly overrepresented among genes that connect with *SlFAD2-4*, the family member with the second highest number of interaction partners (connected to 195 genes). These results suggest that *SlFAD2-4* may be important in plant stress responses.

In Silico Promoter Analysis Identifies Multiple Hormone- and Stress-Responsive cis-Regulatory Elements for *SlFAD2* Family Members

To investigate the putative regulatory mechanisms of the *SlFAD2* gene family, we scanned the upstream promoter regions of the nine *SlFAD2* genes and

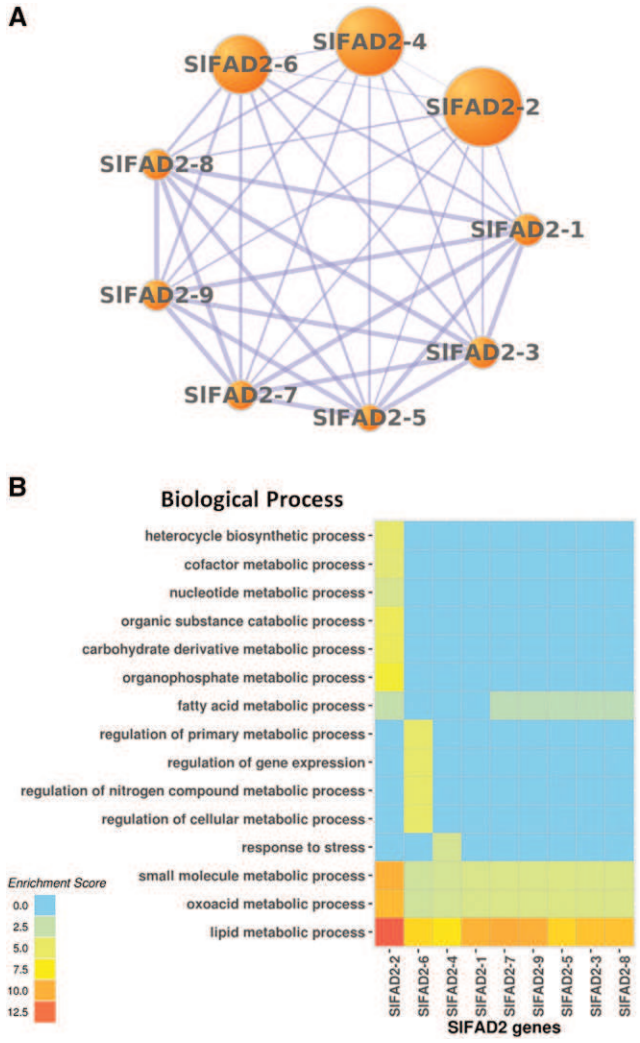

Figure 4. Network-based predicted interaction profiles of the nine *SlFAD2* genes. A, Visualization of the *SlFAD2* interconnected network. Each circle represents a *SlFAD2* gene, with the size of the circle proportional to the number of genes it interacts with (network degree). Blue lines indicate overlaps between two connected *SlFAD2*s, with the thickness of the link proportional to the overlap score. B, A heatmap showing functional terms from the GO BP categories overrepresented in the interaction profile of each *SlFAD2* gene. The cells are colored according to the enrichment score (range indicated in the legend). Enrichment scores were calculated as the $-\log_{10}$ (*q* value), where the *q* value is the hypergeometric test *P*-value corrected for false discoveries using the Benjamini-Hochberg method. Blue grids indicate no significant overlap between the GO BP term on the *y* axis and the corresponding *SlFAD2* gene on the *x* axis, whereas yellow to red gradient indicates significant overlaps, with darker colors indicating higher statistical significance.

identified 32 known plant cis-regulatory elements (CREs) over- or underrepresented in comparison to the tomato genomic background (Supplemental Table S3). Four of nine members of the *SlFAD2* family (*SlFAD2-1*, *SlFAD2-4*, *SlFAD2-5*, and *SlFAD2-8*) were significantly overrepresented with CREs associated with responsiveness to abscisic acid, a key hormone in plant development and environmental stress responses. Other hormone response elements that were overrepresented included CREs for auxin (in *SlFAD2-7* and *SlFAD2-9*) and gibberellins (in *SlFAD2-2* and *SlFAD2-3*). A majority of the *SlFAD2* family was also enriched in stress-responsive regulatory elements, including CREs associated with drought (*SlFAD2-1*, *SlFAD2-4*, *SlFAD2-5*, *SlFAD2-7*, and *SlFAD2-8*), UV irradiation (*SlFAD2-7*), and fungal elicitors (*SlFAD2-9*). In particular, *SlFAD2-4*, *SlFAD2-5*, *SlFAD2-7*, and *SlFAD2-8* all contained CREs with homology to the MYCATERD1 motif, which was first identified as a MYC recognition motif involved in transcriptional responses to water stress in Arabidopsis (Simpson et al., 2003). These results suggest that abscisic acid, giberellins, and auxin play roles in regulating the *SlFAD2* family and that multiple members of this family may be stress responsive.

Multiple Stresses Strongly Upregulate Expression of *SlFAD2-4* and *SlFAD2-7*

Because PPI network and promoter analysis suggested that certain members of the *SlFAD2* family may be stress responsive, we evaluated their expression patterns in tomato foliage ('Castlemart') in response to three different stresses: a virulent bacterial pathogen, *P. syringae*; an insect pest (the potato aphid); and mechanical wounding (Table 2). *SlFAD6*, which encodes a putative chloroplast-localized Δ12-desaturase, was also included in the expression analysis for comparison to the *SlFAD2* family. *SlFAD6* and the two *SlFAD2* family members with Δ12-desaturase activity, *SlFAD2-1* and *SlFAD2-2*, were all significantly downregulated 5 d after pathogen infection, whereas *SlFAD2-4*, *SlFAD2-5*, *SlFAD2-6*, *SlFAD2-7*, and *SlFAD2-9* were significantly upregulated. *SlFAD2-4* and *SlFAD2-7* were also upregulated by mechanical wounding and by the potato aphid. Foliar expression values for *SlFAD2-3* and *SlFAD2-8* were low, and these genes were not consistently detected in all samples. Our results indicate that the majority of *SlFAD2* family members that lack in vitro Δ12-desaturase activity are upregulated by one or more stresses, whereas family members with confirmed Δ12-desaturase activity are downregulated or unresponsive to these stresses. Furthermore, of the stress-responsive members of the *SlFAD2* family, *SlFAD2-4* and *SlFAD2-7* appear to be responsive to the broadest range of stresses and were the only family members upregulated by aphid infestation. For these reasons, our subsequent experiments focused on *SlFAD2-4* and *SlFAD2-7*.

SlFAD2-4 and *SlFAD2-7* Are Upregulated by SA, and Their Expression Is Independent of JA Synthesis

Because *SlFAD2-4* and *SlFAD2-7* were strongly upregulated by mechanical wounding, aphid infestation, and *P. syringae* infection, we also investigated how

Table 2. *Relative gene expression in tomato foliage*

Expression of *SlFAD2* family members as well as another Δ12-desaturase gene, *SlFAD6*, were measured by RT-qPCR in a wild-type tomato cultivar ('Castlemart') in response to three stresses: infestation by the potato aphid (*Macrosiphum euphorbiae,* measured at 2 dpi), infection by a virulent bacterial pathogen (*Pseudomonas syringae* pv. *tomato* DC3000 [*Pst*], measured at 5 dpi), and mechanical wounding (measured at 24 h after wounding). Gene expression was also measured in a mutant with enhanced linoleic acid content (*spr2*) compared to wild-type plants. Relative expression values for each treatment were calculated relative to the respective controls, normalized using the *RPL2* housekeeping gene, and analyzed by one-way ANOVA. Single and double asterisks represent fold changes that are statistically significant at $\alpha = 0.1$ and $\alpha = 0.05$, respectively. $n = 5$ for aphid-infested plants and uninfested controls, $n = 6$ for *Pst*-infected and mock-inoculated controls, $n = 3$ for wounded and unwounded controls, and $n = 5$ for the comparison between *spr2* and wild-type controls.

Genes	Aphid	*Pst*	Wounding	*spr2*/Wild Type
SlFAD2-1	−1.6	−3.3*	−0.87	−0.5
SlFAD2-2	−0.88	−3.8*	−0.9	2.1
SlFAD2-3	1.26	–[a]	–[a]	–[a]
SlFAD2-4	6**	12**	80**	8.78**
SlFAD2-5	1.02	9.5**	1.28	2.1
SlFAD2-6	1.71	2.1*	−0.69	1.3
SlFAD2-7	8**	37**	3.4**	7.5**
SlFAD2-8	−0.95	–[a]	−0.83	−0.97
SlFAD2-9	−1.04	12.7**	9.39**	47**
SlFAD6	1.74	−9**	−3*	1.68

[a]No transcripts observed.

expression of these genes is influenced by salicylate and jasmonate signaling. These pathways are both activated by *P. syringae* infection and aphid infestation (Moran and Thompson, 2001; Betsuyaku et al., 2018), and jasmonates also regulate wound responses (Wasternack et al., 2006). Exogenous SA application significantly upregulated both *SlFAD2-4* and *SlFAD2-7* expression (Fig. 5, A and B), indicating that both genes are SA responsive. In contrast, constitutive *SlFAD2-4* and *SlFAD2-7* expression was enhanced, not inhibited, by *suppressor of prosystemin-mediated responses2* (*spr2*; Table 2), a mutation that inhibits jasmonic acid (JA) synthesis (Li et al., 2003). Moreover, mechanical wounding significantly upregulated *SlFAD2-4* and *SlFAD2-7* in *spr2* as well as in wild-type plants (Fig. 5, C and D), indicating that wound-responsive induction of these genes is independent of jasmonate signaling. Prior studies have shown that foliar expression of *AtFAD7* and certain lipases in Arabidopsis can also be upregulated by wounding in mutants with impaired JA synthesis or perception (Nishiuchi et al., 1997; Ruduś et al., 2014). Thus, wound-responsive changes in lipid metabolism, including induction of divergent *FAD2* family members, appears to involve JA-independent wound signaling.

VIGS of *SlFAD2-7* Compromises Aphid Resistance in the *spr2* Mutant and Has Suppressive Effects on Aphids on Susceptible Wild-Type Tomato Plants

Of the stress-responsive members of the *SlFAD2* family, *SlFAD2-7* was the most strongly induced by biotic stress, including aphids; therefore, we chose to focus on this gene for functional characterization. The *spr2* mutation, which enhances constitutive expression of the aphid-inducible and SA-responsive *SlFAD2-7* gene (Fig. 5), is also known to enhance aphid resistance in an SA-dependent manner (Avila et al., 2012). Therefore, we utilized VIGS to assess whether *SlFAD2-7* contributes to aphid resistance in the *spr2* mutant. A tobacco rattle virus (TRV) vector was used to silence *SlFAD2-7* in *spr2*, as well as in a near-isogenic, aphid-susceptible wild-type cultivar (cv Castlemart), and population growth of the potato aphid was compared on silenced plants and plants infiltrated with vector controls (*TRV::GUS*). In both genetic backgrounds, the *SlFAD2-7* silencing vector achieved ∼50% to 60% reduction in expression of the target gene ($P = 0.0186$), but did not significantly inhibit expression of other *FAD2* family members according to RT-qPCR analysis; in fact, expression of *FAD2-1* and *FAD2-2* increased in response to silencing of *FAD2-7* (Supplemental Table S4). On the *spr2* mutant (Fig. 6A), aphid reproduction was significantly higher on plants in which *SlFAD2-7* was silenced compared to plants that received a vector control, with a 45% increase in offspring numbers. These data suggest that *SlFAD2-7* contributes to plant defenses against aphids in *spr2*. In contrast, silencing *SlFAD2-7* in the near-isogenic aphid-susceptible cultivar caused a modest but statistically significant reduction in aphid population growth (Fig. 6B). For plants infiltrated with the vector control, the wild-type genotype cv Castlemart supported more than twice as many aphids as *spr2* (compare Fig. 6, A and B), which is consistent with our previous observations of aphid resistance in *spr2* (Avila et al., 2012). In summary, *SlFAD2-7* contributed to aphid resistance in *spr2* but had a stimulatory effect on aphid reproduction on a wild-type susceptible cultivar.

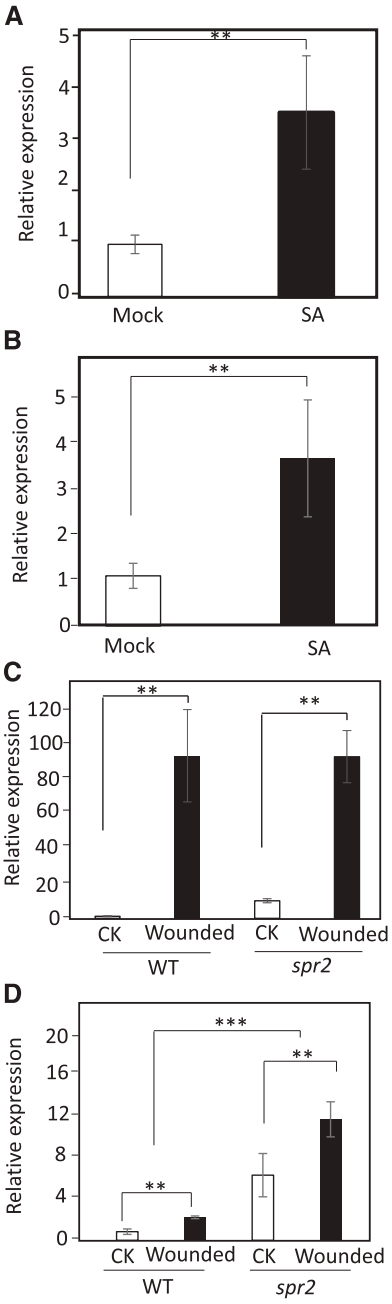

Figure 5. Transcriptional regulation of *SlFAD2-4* and *SlFAD2-7* in response to SA and wounding. RT-qPCR was used to measure the relative transcript abundance of *SlFAD2-4* (A) and *SlFAD2-7* (B) in tomato foliage ('Castlemart') treated with 100 μM SA or a blank carrier solution (Mock). Expression levels of *SlFAD2-4* (C) and *SlFAD2-7* (D) were also compared in mechanically wounded plants and untreated checks (CK) in two genetic backgrounds: Wild-type (WT) plants (cv Castlemart) and the *spr2* mutant, which is impaired in jasmonate signaling due to a deficiency in C18:3 for jasmonate synthesis. Relative expression was measured 24 h after treatment, and expression was normalized relative to *RPL2*. Error bars indicate the SE. Data were analyzed by one-way ANOVA (A and B) or two-way ANOVA (C and D), and treatments labeled with double or triple asterisks are significantly different at α = 0.05 and α = 0.001, respectively. n = 8 (A and B) and n = 3 (C and D).

DISCUSSION

We report here that *FAD2* in tomato represents a multigene family, including two family members encoding confirmed Δ12-desaturases (*SlFAD2-1* and *SlFAD2-2*), one potential pseudogene (*SlFAD2-5*), and six additional divergent *FAD2* genes. We also established that the tomato genome contains a single gene, *SlFAD6*, with homology to the *AtFAD6* chloroplastic Δ12-desaturase gene.

Identifying which genes in tomato encode Δ12-desaturases is an important advance because it provides a necessary foundation for improving the fatty acid content of tomato, the world's most widely grown horticultural crop. Nearly 38 million tons of processing tomatoes were produced worldwide in 2017 (WPTC, 2019), and the United States crop that year was valued at over 900 million dollars (USDA NASS, 2018). Tomato seeds represent a costly waste product of processing tomatoes, representing about 10% of fruit weight and ~60% of processing waste, and the use of tomato seeds for oil production has been proposed as a means of increasing the sustainability and profitability of tomato production (Schieber et al., 2001; Eller et al., 2010). Tomato seed oil is reported to be similar in taste to olive oil (*Olea europaea*; Yilmaz et al., 2015), and it is

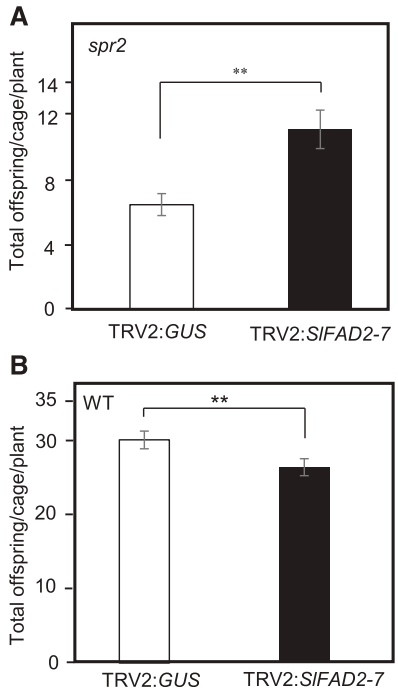

Figure 6. Effects of silencing *SlFAD2-7* on aphid infestation. A TRV-based VIGS system was used to suppress expression of *SlFAD2-7* in the *spr2* mutant (A), which is resistant to the potato aphid, and in wild-type (WT) tomato plants ('Castlemart'; B), which are susceptible to this aphid species. Plants were challenged with aphids (three cages/plant; four adults/cage), and offspring numbers were measured at 4 d postinoculation (dpi). Error bars indicate the SE. Double asterisks represent significant differences at α = 0.05 (one-way ANOVA). n = 20.

also marketed as a beauty product because it contains lycopene, β-carotene, α-tocopherol, and other antioxidants (Eller et al., 2010; Zuorro et al., 2013). However, the composition of tomato seed oil is problematic from the perspective of oxidative stability, which is important to shelf life, as well as from the perspective of stability at high temperatures, which is important for cooking oils or industrial applications. The most abundant fatty acid in tomato seed oil is linoleic acid (C18:2), which represents ~37% to 57% of its total fatty content (Botineştean et al., 2015). Linoleic acid has relatively low heat stability, is prone to oxidation, and is a major contributor to rancidity in foods (Haman et al., 2017). In nearly all oil seed crops, cultivars that are low in linoleic acid and high in its monunsaturated precursor oleic acid are popular and have been developed by selecting for or engineering reduced FAD2 activity. In tomato, *SlFAD2-1* and *SlFAD2-2* could be similarly targeted to enhance oil seed quality and facilitate the use of the nearly four million tons of tomato seeds generated annually as a waste product of processing tomato production.

Characterization of the *FAD2* family in tomato is also important because of the relevance of these genes to plant stress responses. Most *SlFAD2* family members were differentially regulated in response to at least one of the stresses tested here (Table 2), and *SlFAD2-6* (formerly called *CEV19*) is also induced by viroid infection (Gadea et al., 1996). Whereas family members with in vitro Δ12-desaturase activity (*SlFAD2-1* and *SlFAD2-2*) were downregulated in response to bacterial infection, five of the seven divergent members were upregulated in response to one or more biotic stresses (Table 2), suggesting that canonical and divergent *FAD2* genes play distinctly different roles in plant stress responses. In Arabidopsis, the FAD2 Δ12-desaturase contributes to adaptation to cold stress and salinity, probably by modulating membrane fluidity and ion transport across membranes (Miquel and Browse, 1994; Zhang et al., 2012). While Δ12-desaturases influence levels of abiotic stress tolerance by modifying the physical properties of membranes, certain divergent FAD2 enzymes contribute to biotic stress responses by participating in the synthesis of defensive secondary metabolites. The antimicrobial polyacetylenes falcarindiol and falcarinol accumulate in the vasculature of tomato in response to fungal infection (De Wit and Kodde, 1981; Elgersma et al., 1984), and data from three other plant families (Apiaceae, Asteraceae, and Araliaceae) indicate that pathogen-inducible divergent FAD2-like acetylenases synthesize necessary precursors for these phytoalexins (Kirsch et al., 1997; Cahoon et al., 2003; Busta et al., 2018). In avocado (*Persea americana*), pathogen-inducible divergent *FAD2* expression is also correlated with accumulation of an antimicrobial diene, (Z,Z)-1-acetoxy-2-hydroxy-4-oxoheneicosa-12,15-diene (Wang et al., 2004). Thus, FAD2 acetylenases and possibly other divergent FAD2 enzymes contribute to the synthesis of defensive secondary metabolites in response to pathogen infection. Here, we demonstrate that a divergent *FAD2* gene in tomato (*SlFAD2-7*) is also responsive to challenge by a piercing-sucking insect (the potato aphid) and can influence host plant susceptibility to this pest (Fig. 6; Table 2).

Interestingly, the influence of *SlFAD2-7* on plant-aphid interactions varied depending upon the tomato genotype. When this gene was silenced in the aphid-resistant mutant *spr2*, aphid infestations increased significantly, indicating that *SlFAD2-7* contributed to aphid resistance in this genotype. In contrast, when this gene was silenced in the susceptible isogenic wild-type cultivar (cv Castlemart), silencing had deleterious effects on aphid population growth. The effects of individual genes on biotic interactions can often vary in different plant genotypes; for example, the efficacy of many plant genes for virus resistance is strongly influenced by their genetic background (Gallois et al., 2018). One potential explanation for differences in how *SlFAD2-7* impacts aphids on *spr2* versus the wild-type genetic background is that these two genotypes differ in the availability of substrates for divergent FAD2 enzymes. Compared to wild-type foliage, the *spr2* mutant accumulates more than 3-fold higher levels of linoleic acid, one of the primary substrates for acetylenases and other divergent FAD2 enzymes (Li et al., 2003). The identity or abundance of metabolites generated by divergent FAD2 enzymes could vary in different plant genotypes depending upon substrate availability, and this could in turn influence the impact of these enzymes on biotic interactions. Similarly, we previously observed that silencing *α-DIOXYGENASE1* (*SlDOX1*), a gene involved in oxylipin synthesis, caused greater increases in aphid infestation on *spr2* than on wild-type plants (Avila et al., 2013). Thus, metabolites generated from linoleic acid by divergent members of the FAD2 family as well as other enzymes, such as α-DOX1, may potentially contribute to aphid resistance in *spr2*.

By demonstrating a role for a divergent *FAD2* gene in insect resistance, our data add to a growing body of evidence indicating that plant fatty acid desaturation plays important roles in plant interactions with aphids, a large and economically important group of sap-feeding insects. Infestations by the soybean aphid (*Aphis glycines*) reduce the abundance of polyunsaturated fatty acids in the foliage and seeds of a susceptible soybean cultivar, indicating that desaturation is responsive to aphid infestation (Kanobe et al., 2015). Desaturase activity in host plants also influences aphid population growth; loss of function of *AtFAD7* or another desaturase gene, *SUPPRESSOR OF SA INSENSITIVITY2* (*SSI2*), decreases aphid infestation on Arabidopsis, apparently by modifying defensive signaling against this pest (Pegadaraju et al., 2005; Louis et al., 2010; Avila et al., 2012). Other desaturases have a positive impact on plant defense; for example, synthesis of anacardic acid by a Δ9 14:0-acyl carrier protein FAD contributes to aphid- and mite resistance in geranium (*Pelargonium* × *hortorum*; Schultz et al., 1996). Thus, FADs in plants can impact aphid infestation levels by influencing defense signaling and by producing

defensive secondary metabolites. Further study is warranted to investigate the pathways through which fatty acid metabolism in host plants impacts aphid infestations and the roles that divergent FAD2 enzymes play in these interactions.

CONCLUSION

In summary, the *FAD2* family in tomato includes two Δ12-desaturase genes (*SlFAD2-1* and *SlFAD2-2*) capable of contributing to synthesis of linoleic acid in the ER, as well as five stress-responsive divergent *FAD2* genes, at least one of which (*SlFAD2-7*) contributes to aphid resistance in a mutant (*spr2*) with modified fatty acid content. These data provide a foundation for manipulating oil seed content in tomato and also for enhancing aphid resistance.

MATERIALS AND METHODS

Biological Materials

This study utilized the tomato (*Solanum lycopersicum* 'Castlemart') and a mutagenized tomato line, *suppressor of prosystemin-mediated responses2* (*spr2*), which was developed in the cv Castlemart genetic background (Li et al., 2003). Seeds for these genotypes were originally obtained from Dr. Gregg Howe (Michigan State University). All plants were germinated under stable greenhouse conditions (~21°C to 27°C; 16 h light/8 h dark photoperiod) in LC1 Sunshine potting mix (Sungro Horticulture) supplemented with 15-9-12 Osmocote Plus slow-release fertilizer (Scotts-MiracleGro Company) and were maintained and watered with a dilute nutrient solution containing 0.1% (w/v) $CaNO_3$ (Hydro Agri North America), 0.05% (w/v) $MgSO_4$ (Giles Chemical Corp), and 0.05% (w/v) 4-18-38 Gromore fertilizer (Gromore; Avila et al., 2012). The potato aphid (*Macrosiphum euphorbiae*) was maintained in growth chambers (Conviron; 20°C; 16 h light/8 h dark photoperiod) on tomato seedlings ('Castlemart'). *Pseudomonas syringae* pv *tomato* DC3000 was provided by Dr. Yinong Yang (Pennsylvania State University) and cultured on King's B broth with rifampicin 50 μg/mL. All bioassays or tissue collection experiments were performed in Conviron growth chambers under standard conditions (24°C; 16 h light/8 h dark photoperiod; 220 μM m^{-2} s^{-1} light intensity) unless otherwise noted.

Identification of Tomato *FAD2* Homologs

BLASTp (Altschul et al., 1990) was used to search for homologs of the Arabidopsis (*Arabidopsis thaliana*) *AtFAD2*-predicted amino acid sequence (GenBank accession number At3G12120) in the tomato genome (International Tomato Annotation Group release 2.40) using proteome and genome files downloaded from the Sol Genomics Network (SGN; http://solgenomics.net). For comparison, homologs of *AtFAD6* (GenBank accession number At4G30950), which encodes a Δ12-FAD localized to the chloroplast rather than the ER, were also searched. All of the Δ12-FAD protein sequences identified in tomato were aligned using the ClustalW algorithm with default parameters (https://www.genome.jp/tools-bin/clustalw). A phylogenetic tree was inferred using the nearest-neighbor interchange algorithm without bootstrapping, and visualized using Molecular Evolutionary Genetics Analysis (MEGA5) sotware (https://www.megasoftware.net/; Tamura et al., 2011); statistical support was estimated using a Shimodaira-Hasegawa-like procedure. Transmembrane domains were identified within these sequences using the TmPred tool from the ExPASy Bioinformatics Resource portal (Hofmann and Stoffel, 1993).

Analysis of the *FAD2* Gene Subnetwork in the STRING Database

The tomato PPI network was downloaded from the STRING database (protein.links.v11.0.txt.gz file available at https://string-db.org/cgi/download.pl; Szklarczyk et al., 2017). Genes linked with each *SlFAD2* gene were extracted from the network, and the overlaps among the interaction profiles of each *SlFAD2* gene were quantified as Jaccard coefficients (JCs; Brohée et al., 2008). The resulting *SlFAD2* interconnected network was plotted in Cytoscape (Shannon et al., 2003). Tomato GO was downloaded from the plant GSEA server, available at http://structuralbiology.cau.edu.cn/PlantGSEA/download.php (Yi et al., 2013). Given the nature and structure of the GO graph, annotations from child terms were propagated upwards to all parent terms satisfying the "is a" and "part of" relationships (Ambavaram et al., 2014). Then, redundant terms (terms with high overlaps in the genes they annotate) were identified by estimating JCs between every pair of GO BP terms. Within each GO term pair with JC > 0.8, the GO term with the lesser number of annotations was removed. Finally, GO terms with >1,000 genes and <3 genes were removed, and hypergeometric tests were performed on the remaining terms for enrichment analysis of each set of *SlFAD2*-linked genes. The resulting *P*-values were corrected for false discoveries using the Benjamini-Hochberg method and expressed as negative logarithms (Benjamini and Hochberg, 1995). These $-\log_{10}$ values (*q* values) were used to create a heatmap in R using the ggplots package (https://www.rdocumentation.org/packages/ggplot2).

Analysis of Promoter Motifs

The 2,000-bp upstream regions of all nine *SlFAD2* genes were extracted from the *Solanum lycopersicum*.SL2.50.40 reference genome using the Regulatory Sequence Analysis Tools webserver (Nguyen et al., 2018). In each promoter, oligomers of 6, 7, and 8 bp in length were identified and the number of occurrences counted using the oligo-analysis tool in Regulatory Sequence Analysis Tools (http://rsat.eead.csic.es/plants/oligo-analysis_form.cgi). The expected number of occurrences was calculated from the predefined background frequencies model consisting of all upstream regions, clipping overlaps with upstream open reading frames. The probability of having at least the number of observed occurrences of each motif (overrepresentation) or less than the observed number of occurrences (underrepresentation) was calculated using the binomial formula. The resulting *P*-values were converted to *E* values, and the $-\log_{10}$ (*E* value) was expressed as the significance index of identified DNA motifs. Motifs reported with significance index >0 were then compared to known plant CREs listed in the PLACE database using the STAMP server (Higo et al., 1999; Mahony and Benos, 2007).

Heterologous Expression of *SlFAD2* Genes from Tomato in Yeast

For each of the nine *SlFAD2* homologs identified in tomato, primers were designed to target the entire open reading frame (Table 1), which was amplified from complementary DNA (cDNA) synthesized from total RNA extracted from foliage of the tomato cultivar 'Castlemart'. A clone of the *AtFAD2* gene from Arabidopsis (clone U12792) was obtained from The Arabidopsis Information Resource (TAIR). The blunt-end PCR products containing the tomato cDNAs were cloned into the pENTR TOPO vector (Invitrogen) and then cloned into the destination vector pYES-DEST52 (Thermo Fisher Scientific) using Gateway cloning recombination technology (Invitrogen). The pYES-DEST52 expression vector includes the *GALACTOKINASE1* (*GAL1*) promoter for inducible gene expression in yeast (*Saccharomyces cerevisiae*). The resulting plasmids and a vector control consisting of the pYES-DEST52 vector containing a GUS cDNA insert were introduced into *S. cerevisiae* INVSc1 cells (Invitrogen). Transformants were first grown in minimal medium lacking uracil and containing Glc at 28°C. After overnight culture, the cells were collected, washed one time with water, and diluted to an optical density (OD) of 0.4 in minimal media containing Gal to induce expression of the transgenes.

Fatty Acid Analysis

The fatty acid profiles of yeast lines expressing *SlFAD2* homologs were analyzed by GC-MS of FAMEs using protocols adapted from Cao et al. (2013). In brief, 10 mL saturated cultures were grown for 3 d at 15°C, and cells were collected, washed three times with water, pelleted, and dried under vacuum. Lipids were extracted and transmethylated as follows. Two milliliters of 1 N methanolic HCl was added to the materials, sealed in an 8-mL glass tube, and heated to 80°C for 1 h. After cooling on ice, 2 mL of 0.9% (w/v) NaCl was added, and the mixture was extracted three times with 2 mL of hexane. The FAMEs were analyzed using GC-Agilent 6890N/MS-Agilent 5937 with FAMEWAX columns (30m × 0.25-μm film thickness; Restek). One microliter of sample was

injected and 14:1 of split ratio was applied. The oven temperature was raised from 130°C to 225°C at a rate of 7°C/min and then maintained at 225°C for 12 min. Peaks were identified by comparing the retention times with those of the corresponding standards (Nu-Chek-Prep Inc), and their identities were also confirmed by comparing mass spectra to the National Institute of Standards and Technology mass spectral library.

Stress Challenge and SA Treatment

To test the influence of stresses on *SlFAD2* gene expression, 4-week-old tomato plants ('Castlemart') were challenged with *P. syringae*, potato aphid infestation, or mechanical wounding. *P. syringae* inoculation was performed according to previously described protocols (Uppalapati et al., 2011). In brief, plants were spray-inoculated with a *P. syringae* bacterial suspension (OD_{600} = 0.005) in 10 mM $Mgcl_2$ containing 0.025% (v/v) Silwet L-77 (OSI specialties Inc). Plants were then incubated in growth chambers at 100% relative humidity (RH) for the first 24 h and at 70% RH for the remainder of the experiment. Control plants were mock-inoculated using the same protocols with corresponding solutions that lacked bacteria (six replicate plants/treatment). Leaf samples for gene expression analysis were flash-frozen in liquid nitrogen 5 d after inoculation, the earliest time point to observe consistent symptoms of infection. To measure gene expression in response to infestation by the potato aphid, aphids were confined to cloth sleeve cages (each enclosing two leaflets) placed on the second and third leaf below the meristem of 4-week-old plants (two cages/plant; 60 aphids/cage), while mock-challenged plants received empty cages (five replicate plants/treatment). Cages and aphids were removed 48 h after infestation, and leaves were then flash-frozen for RNA extraction. To measure gene expression in response to mechanical wounding, a leaf of each plant was crushed with a hemostat to produce a row of punctures that crossed the midvein. Leaves were harvested and flash-frozen 24 h after wounding. To assess gene expression in response to SA, the foliage of 4-week-old tomato plants was sprayed with 100 μM SA in 0.1% (v/v) ethanol. Equal volumes of 0.1% (v/v) ethanol solution were applied to mock controls (eight replicate plants/treatment). Leaves were harvested and flash-frozen 24 h after hormone application.

Gene Expression Analysis

Tissue for gene expression experiments was collected from the youngest leaf that was fully expanded, which was typically the third or fourth leaf below the meristem. After flash-freezing, total RNA was purified from tomato foliage using TRIzol according to the manufacturer's instructions (Invitrogen). First-strand cDNA was synthesized from total RNA using the Superscript III First-Strand Synthesis System and oligo dT primers (Invitrogen). Gene expression analysis was performed by RT-qPCR using a StepOnePlus real time PCR system (Applied Biosystems) and the QuantiTect SYBR Green PCR kit (Qiagen). Each experiment included at least three biological replicates per treatment and two technical replicates per biological sample. Gene-specific primer sets were designed to measure expression of each of the nine *SlFAD2* genes as well as *SlFAD6* for comparison (see Table 1 for primer sequences). *RIBOSOMAL PROTEIN L2* (*RPL2*; GenBank accession number GBX64562) was included as an endogenous housekeeping control gene and quantified using the following primers: forward (5′-GAGGGCGTACTGAGAAACCA-3′) and reverse (5′-CTTTTGTCCAGGAGGTGCAT-3′). *RPL2* was chosen as a reference gene on the basis of previous GeNorm pilot studies comparing the stability of multiple housekeeping genes in tomato (Defibaugh-Chávez, 2008); furthermore, in the current study, the cycle threshold (C_t) values for this reference gene were confirmed as not significantly affected by aphid infestation, *P. syringae* infection, wounding, SA treatment, or infiltration with VIGS vectors ($P > 0.10$). The PCR conditions used for all primer sets were 95°C for 3 min, followed by 40 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s, with data acquisition at the end of each cycle and a final data acquisition step to generate melting curves from 65°C to 95°C every 0.3°C. The efficiency of amplification of each primer set was calculated by applying the formula $E = 10[-1/C_t \text{ slope}]$ to data generated using serial dilutions of a sample pooled from aliquots of all cDNA samples (Rasmussen 2000). Data for each sample were normalized to the expression levels of the endogenous control *RPL2*, and gene expression levels in treated plants were calculated relative to the untreated wild-type control group in each experiment.

VIGS

Expression of *SlFAD2-7* (locus name Solyc12g049030) was transiently suppressed in tomato foliage using a TRV vector for VIGS according to previously described protocols (Liu et al., 2002a, b). The TRV1 and TRV2 VIGS vectors were kindly provided by S.P. Dinesh-Kumar (University of California at Davis). A 440-bp fragment of *SlFAD2-7* (bases 442–881 in the SGN sequence Solyc12g049030.1.1) was amplified using a forward primer containing an *Eco*RI restriction site (5′-CGGGAATTC ACTGCTTCCCTTGAGAACGA-3′) and a reverse primer containing an *Xho*I restriction site (5′-CGGCTCGAG GCTCCT CGTAGCCAATTCCA-3′). The PCR-amplified fragment was cloned into the TRV2 vector pYL156 and electroporated into *Agrobacterium tumefaciens* GV3101. Cultures of *A. tumefaciens* harboring TRV1 or the *TRV2::SlFAD2-7* construct were grown overnight at 28°C in lysogeny broth with 50 μg kanamycin and 50 μg rifampicin. One-milliliter overnight cultures were transferred into 40 mL of fresh lysogeny broth and shaken overnight at 28°C. Bacterial cells were harvested and resuspended in 40 mL induction medium (10 mM $MgCl_2$ and 10 mM MES, pH 5.6) supplemented with 150 μM acetosyringone and shaken at room temperature for 5 h. Bacterial cultures containing TRV1 and *TRV2::SlFAD2-7* were mixed in equal ratios (OD_{600} = 1.0) and infiltrated into tomato plants at the two-leaf stage (~10 d after germination) using a needleless syringe (Velásquez et al., 2009). Additional sets of plants were also infiltrated with a mix of TRV1 and constructs that silence *GUS* or *PHYTOENE DESATURASE* (*SlPDS*). Since *GUS* is absent in the tomato genome, the *TRV::GUS* construct is a useful negative control, because it is similar in size to our experimental construct but does not cause any silencing (Wu et al., 2011). Silencing of *SlPDS* results in photobleaching of the leaves, providing a useful indicator of the timing of silencing (Ruiz et al., 1998). After Agroinfiltration, plants were maintained at 20°C and 50% RH with a 16 h light/8 h dark photoperiod (220 μM m^{-2} s^{-1} light intensity) in a growth chamber (Conviron). Plants infiltrated with *TRV::SlPDS* were monitored visually to assess the onset of silencing, and all other plants were used for bioassays only after the *TRV::SlPDS* plants showed extensive photobleaching (3–3.5 weeks after infiltration).

Assay to Measure Aphid Infestation on VIGS-Treated Plants

Adult potato aphids of uniform age (collected within 24 h after emergence to adulthood) were confined to individual leaflets using clip cages (20 replicate plants per treatment group; three cages/ plant; four aphids/cage). Cages were placed on the youngest leaf that was fully expanded, which was typically the third or fourth leaf below the meristem. At 4–5 d after inoculation, total offspring were counted. Individual plants were treated as biological replicates, and replicate cages on the same plant were treated as subsamples and averaged before analysis. Silencing of *SlFAD2-7* was confirmed in a subset of plants (20 replicates/treatment group) at the end of the bioassay. After aphid removal, three leaf punches (10 mm) per infected leaf (three leaves/plant) were collected and flash-frozen. Leaf discs were pooled into a single sample per plant, and RNA extraction and RT-qPCR analysis of *SlFAD2-7* expression was performed as described above.

Statistical Analysis

Bioassay data and gene expression data were analyzed by one-way ANOVA (for experiments with a single variable) or two-way ANOVA (for experiments with a full-factorial combination of two variables) using JMP Pro 13 (SAS Institute).

Accession Numbers

Sequence data for the genes examined in this study can be found in the GenBank database or in the Solanaceae Genomics Network database under the identifiers listed in Table 1.

SUPPLEMENTAL DATA

The following supplemental materials are available.

Supplemental Figure S1. Identification of fatty acids produced by heterologous *SlFAD2* expression in yeast.

Supplemental Table S1. Fatty acid composition of yeast cells expressing *SlFAD2* genes.

Supplemental Table S2. PPI network predicted for *SlFAD2* genes according to the STRING database.

Supplemental Table S3. Cis-elements over- or underrepresented in the 2000-bp upstream promoters of *SlFAD2* genes.

Supplemental Table S4. Effects of VIGS targeting of *SlFAD2-7* on expression of the *FAD2* gene family.

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