Transcription factors regulating terpene synthases in tomato trichomes

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Publication date
2012

Chapter 6

General Discussion

Gene clusters in plant specialized metabolite pathways

Genes involved in different steps of specialized metabolite production pathways are often found in a cluster. Such a feature is quite common in bacterial genomes. In eukaryotes, functionally related genes are thought to be typically dispersed throughout the genome, however cases of gene clusters involved in the synthesis of specialized metabolites in plants have been reported, all of which were implicated in synthesis of defense compounds (Chu *et al*., 2011). For example, in oat (*Avena strigosa*) the biosynthesis of the antibacterial triterpenoid avenacin involves the β-amyrin synthase (*AsbAS1*) that is clustered together with the genes required for 2,3-oxidosqualene cyclization, β-amyrin oxidation, glycosylation and acylation in a region of the genome not conserved in other cereals (Qi *et al*., 2004). In *Arabidopsis* the synthesis and modification of the triterpenes thalianol and marneral is determined by two gene clusters found on chromosome 5 (Field and Osbourn, 2008; Field *et al*., 2011). In rice (*Oryza sativa*) five genes required for the biosynthesis of momilactones, diterpenoid phytoalexins, are clustered on chromosome 4 and their expression is regulated by a bZIP transcription factor (*OsTGAP1*) also located on chromosome 4 (Okada *et al*., 2009). It was therefore intriguing to determine whether such clustering occurs in tomato. With very few exceptions (*SlTPS40*; Chr 6, *SlTPS17* and *SlTPS24*; Chr 7, *SlTPS41*; Chr 8), terpene synthases found on the same chromosome cluster together in a phylogenetic tree (Fig.1 and Chapter 2; Fig.1), which suggests that these clusters have been derived from tandem duplications and functional divergence and could

imply co-ordinate regulation of their production by other genes found in close proximity.

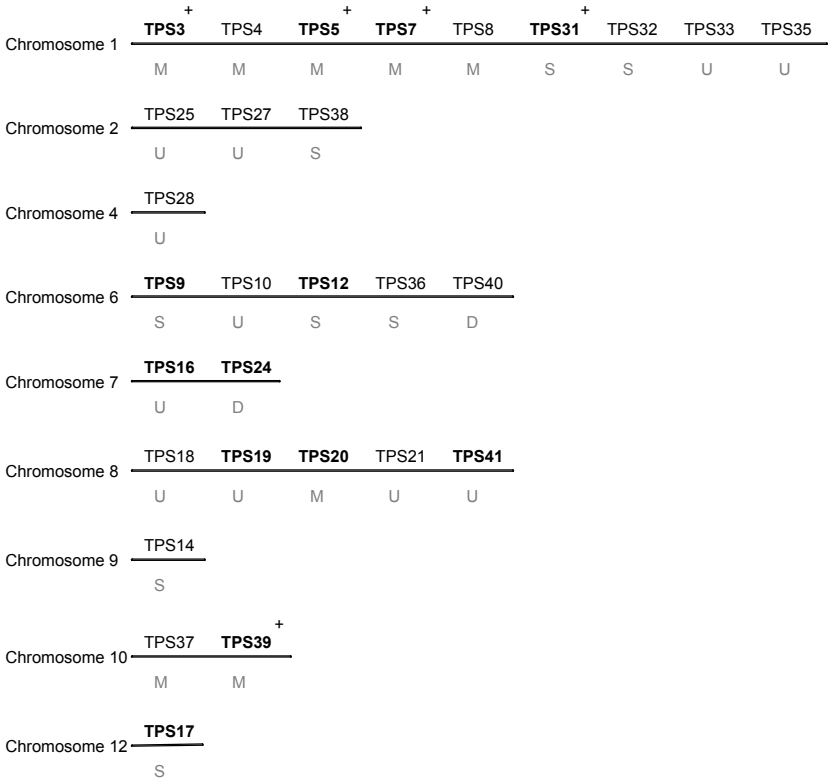

Figure 1. *Functional or potentially functional TPS genes organized per chromosome*. TPSs expressed in trichomes are in bold. For those genes expressed in trichomes, + denotes induction by jasmonic acid treatment. M/S/D or U refers to the biochemical function of the TPSs as (M) mono- / (S) sesqui-/ (D) di-terpene synthases or (U) unknown function when no enzymatic data are available. Compiled from Falara *et al*., 2011 and data from the stem trichome transcriptomic databases (Chapter 5).

The TPS-e/f subclade *SlTPS18*, *SlTPS19*, *SlTPS20* (*PHS1*; Schilmiller *et al*., 2009) and *SlTPS21* genes that group together (Chapter 2; Fig.1) and the TPS-c subclade *SlTPS41* gene are found on chromosome 8 together with the neryl diphosphate synthase (*NDPS1*). NDPS1 catalyzes the formation of NPP- the substrate used by *PHS1* to produce monoterpene products found in type VI leaf trichomes of *S. lycopersicum* (Schilmiller *et al*., 2009). On the same chromosome, genes encoding

terpene-modifying enzymes (one cytochrome P450, one putative alcohol oxidase and two putative acyltransferaces) are also found (Falara *et al*., 2011). Since the enzymatic activities of SlTPS18, 19, 21 and 41 are not known (*SlTPS41* is most homologous with the copalyl diphosphate synthase *CPS1* (*SlTPS40*) that converts GGPP to CPP as part of the gibberellin biosynthesis; Rebers *et al*., 1999), it was suggested that *NDPS1* and *SlTPS41* could encode enzymes that provide substrates for these TPSs, whose products could be further modified by the terpene-modifying enzymes present on chromosome 8 (Falara *et al*., 2011). However, there is also evidence that genes involved in the synthesis of some tomato terpene products are not confined to gene clusters. The chromosomal substitution line IL10-3 contains a segment from chromosome 10 of *S. pennellii* in a *S. lycopersicum* M82 background that results in a reduced level of sesquiterpenes (Schilmiller *et al*., 2010). *SlTPS9* and *SlTPS12* are located on chromosome 6 (Fig.1) but their expression is reduced in the isogenic line IL10-3 (Falara *et al*., 2011). Therefore it is likely that genes involved in the regulation of these two TPSs are located on this segment from chromosome 10.

Most monoterpene synthases (TPS-b clade) are found on chromosome 1 and some (TPS-g clade) are found on chromosome 10 (Fig.1), but the plastidial pathway precursor genes are not all located on one of these chromosomes. However, half of the genes for these precursor enzymes are indeed located on chromosome 1 (Chapter 5; Fig.2). Sesquiterpene synthases (most of which belong to the TPS-a clade) are found on various chromosomes (Fig.1) and, furthermore, the cytosolic pathway precursor genes are also scattered across almost all chromosomes, including chromosomes 3 and 5, which have not yet been shown to contain TPS genes (Chapter 5; Fig.2). In conclusion from the above-mentioned cases from the various plant species, there seems to be clustering of genes coding for specialized metabolite synthases and downstream modifying enzymes, but not with genes coding for precursor enzymes.

Chromosome 1 contains the most TPSs, including the monoterpene synthases studied here (*SlTPS3*, *SlTPS5* (*SlMTS1*), *SlTPS7* and *SlTPS8*) that group together,

whereas the sesquiterpene synthase we investigated, *SlTPS9*, is located on chromosome 6 (Fig.1). The identified transcription factors *SlEOT1*, *SlMYC1* and *SlWRKY4* that can interact with the promoters of some of these terpene synthases are located on chromosomes 2, 8 and 3 respectively and are not part of a gene cluster containing these TPSs.

Finally, it is worth mentioning that although *SlTPS3*, *SlTPS4*, *SlTPS5*, *SlTPS7* and *SlTPS8* are quite homologous and therefore cluster together in the TPS phylogenetic tree (Chapter 2; Fig.1), their promoter regions do not share any significant identity (nucleotide identity ranging from 3,5% between SlTPS5p and SlTPS7p to 16,7% between SlTPS3p and SlTPS8p when comparing sequences of 1200bp upstream of the ATG start codons). This means that there is less sequence conservation in the promoters than in the coding sequences of these genes.

Trichome-specific promoters and *cis*-elements

Relatively few trichome-specific promoters of genes involved in the terpenoid biosynthesis pathway have been described to date (Tissier, 2012) and even fewer have been extensively characterized. *SlMTS1* and *SlEOT1* promoters are specifically expressed in the four secreting cells of tomato glandular trichomes (Chapter 3; Fig.2 and Chapter 4; Fig.1) and, to the best of our knowledge, are the first trichome-specific promoters characterized from *Solanum lycopersicum*. For the promoter of *SlMTS1* 5' sequential deletions were created and fused to two different reporter genes, which allowed the study of the fragments' expression patterns (Chapter 3; Fig.3). Interestingly, all promoter deletion fragments (except the -408bp fragment) retained the trichome-specific expression pattern. A similar case has been reported before for the promoter of the tobacco trichome-specific P450 gene (*CYP71D16*), that catalyzes the hydroxylation of cembratrienol to form cemratriendiol (Wang *et al*., 2002). In that instance all promoter deletion constructs had a trichome-specific expression and the full-length promoter was required for

strong gland expression (Wang *et al*., 2002). In the case of the *SlMTS1* promoter however, the -408bp fragment was unable to drive expression in any tissue. Deletion of a 110bp fragment close to the 5' end of the tobacco trichome-specific cembratriendiol synthase (*NtCBTS2a*) promoter resulted in complete loss of reporter gene expression (*β-glucuronidase* (*GUS*); Ennajdaoui *et al*., 2010). In an elegant study of this promoter, involving sequential and internal deletions, it was also shown that deleting a 360bp promoter fragment (directly downstream of the 110bp fragment) resulted in significant ectopic expression of GUS (Ennajdaoui *et al*., 2010). Ectopic expression was not observed with the *SlMTS1* promoter, for which however internal or 3' sequential deletions were not made. Finally, it is worth pointing out that the 194bp fragment proximal to the start codon of *NtCBTS2a* (which includes the 75bp 5'UTR) did not produce GUS activity (Ennajdaoui *et al*., 2010), unlike the 207bp fragment proximal to the start codon of *SlMTS1* (which includes the 98bp 5'UTR). This fragment not only contained all *cis*-elements necessary for trichome-specific expression, but also contained the binding site of the SlEOT1 transcription factor, possibly found on the 109bp of promoter sequence (Chapter 4). In this 109bp minimal promoter fragment the predicted TATA and CAAT boxes are located (Chapter 3; Fig.1). In comparison, as little as 250bp of the 5'-flanking regions of the *Nicotiana sylvestris* putrescine N-methyltransferase (*PMT*) 1, 2 and 3 genes were able to confer root-specific expression and MeJA responsiveness (Shoji *et al*., 2000). The 111 base pairs upstream of the transcriptional start site of *NsPMT1* in particular, contained three *cis*-elements (G box, GCC-motif and a TA-rich region separating them) that were all required for MeJA-inducible transcription, indicating some level of interaction between these elements and the transcription factors (TFs) that bind to them (Xu and Timko, 2004). The *SlMTS1* promoter contains several regulatory motifs (Chapter 3; Fig.1), including binding sites for MYC, WRKY and MYB transcription factors, which indicates regulation by multiple TFs. Given that *SlMTS1* is a stress-inducible gene (by wounding, spider mite feeding and jasmonate

treatment; van Schie *et al*., 2007), its expression could be liable to strict, coordinate regulation involving multiple transcription factors.

Transcription factors regulating tomato terpene biosynthesis

Three transcription factors (*SlEOT1*; Chapter 4, *SlMYC1* and *SlWRKY4*; Chapter 5) were shown to interact with the *SlMTS1* promoter, although the exact binding sites were not identified. Interaction of the three identified TFs could take place with the native *SlMTS1* promoter in a heterologous system *in planta*. This contrasts with the finding for MYC2 from *Catharanthus roseus*, which only interacts with a tetramerized G box from the *STR* promoter in yeast and in *C. roseus* cells but not with a native *STR* promoter fragment containing the same G box (Zhang *et al*., 2011). SlEOT1 (Chapter 4; Fig.7) and SlMYC1 (Chapter 5; Fig.5) could transactivate SlMTS1 driven reporter gene expression stronger than SlWRKY4 (Chapter 5; Fig.4). SlWRKY4 seemed to have a negative effect on the combinatorial action of the other two TFs exerted on the *SlMTS1* promoter (Chapter 5; Fig.7). WRKY transcription factors are known to function as activators, but also as repressors of transcription (Rushton *et al*., 2010). Nevertheless since SlWRKY4 could transactivate the *SlMTS1* promoter, it most likely functions as an activator and if it exerts any negative effect on the combinatorial action of SlEOT1 and SlMYC1, it might do so by physically blocking one or more binding site(s). However the interaction of SlWRKY4 with *SlMTS1* promoter (fragments) remains to be confirmed in yeast.

Unlike *SlEOT1*, *SlMYC1* and *SlWRKY4* were not specifically expressed in trichomes (Chapter 4; Fig.1&2 and Chapter 5; Fig.3). Nicotine biosynthesis in *Nicotiana tabacum* and artemisinin biosynthesis in *Artemisia annua* takes place in the roots and glandular trichomes respectively, however none of the TFs identified so far involved in these pathways was expressed specifically in these tissues (De Geyter *et al*., 2012). Similarly, the two tomato TFs could both be involved in

regulating terpene biosynthesis in trichomes and have other, yet unidentified roles, in other tissues where they are expressed. Since some of the terpene synthases (with whose promoters the TFs were shown to interact) were expressed in various tissues, SlMYC1 and SlWRKY4 could play a role in regulating those TPSs in the tissues in which they are co-expressed (*see* for overview Chapter 5; Table 5).

SlMYC1 was not significantly induced by JA (Fig.3 and Chapter 5; Table 4). In *Arabidopsis*, *AtMYC3* and *AtMYC4*, which act additively with *AtMYC2* in the activation of JA responses, are only marginally induced by MeJA treatment, unlike *AtMYC2* (Fernandez-Calvo *et al*., 2011). Therefore, SlMYC1 could be involved in the regulation of induced terpene biosynthesis in combination with other, unidentified tomato MYC transcription factors (Fig.2). It should be mentioned nevertheless that SlMYC1 shares higher homology with AtMYC2 than with AtMYC3 and 4. Furthermore, what is worthy of note is that, as indicated in Chapter 5, none of the annotated MYC transcription factors in the transcriptome database showed strong induction by JA (higher at least than 2-fold). Tomato JAMYC2 and JAMYC10 (shown to be induced by JA; Boter *et al*., 2004) were not present in our transcriptomic database nor in the SGN database (http://solgenomics.net/tools/blast/index.pl), as the sequence provided in Genbank (http://www.ncbi.nlm.nih.gov/genbank/) by the authors comes from potato. Nevertheless, it could be that other JA-inducible genes, annotated as bHLH transcription factors, that do not share high homology with the AtMYCs are involved in the process and were not investigated here. Alternatively, it could be that, in tomato, regulation of SlMTS1 activity does not involve a master-regulator MYC transcription factor, unlike *N. tabacum* (Shoji and Hashimoto, 2011) and *C. roseus* (Zhang *et al*., 2011). Still, the MYC-JAZ conserved system could also be present in tomato. *S. lycopersicum* COI-JAZ complex formation, stimulated by JA-Ile has already been shown (Thines *et al*., 2007; Katsir *et al*., 2008), but no targets of SlJAZ proteins have been identified to date. In tomato trichomes transcripts for COI1 and JAZ are present (Chapter 5; Table 3).

SlMYC1 interacted with two trichome-specific JA-inducible terpene synthases (*SlMTS1* and *SlTPS3*), one trichome-specific but not JA-inducible terpene synthase (*SlTPS9*) and one terpene synthase that is very lowly expressed in trichomes and not induced by JA in that tissue (*SlTPS7*; Chapter 2; Fig.2&6 and Chapter 5; Fig.5). It therefore seems to be a regulator of multiple terpene synthases. SlEOT1 on the other hand, could interact only with *SlMTS1* (Chapter 4; Fig.7), which however does not exclude that it could interact with other terpene synthases not investigated here. Furthermore, it is possible that *SlMTS1* (and/or other TPSs) are additionally regulated by some of the other SlEOT1 family members (Chapter 4; Table 1). In *Arabidopsis*, SHI family proteins were shown to act as dimers or multimers (Kuusk *et al*., 2002; Eklund *et al*., 2010). SlEOT1 does not need an interacting family member for binding to the *SlMTS1* promoter (Y1H; Chapter 3&4), however it is possible that interaction with one of the other proteins has an effect on its activity (Fig.2).

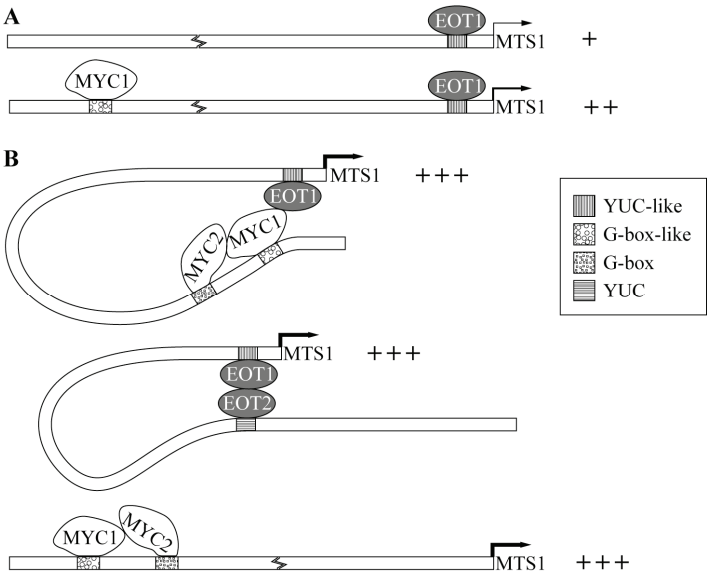

Figure 2. *A model for the transcriptional regulation of SlMTS1*. (**A)** In an uninduced situation SlEOT1 could be involved in the steady-state transcription of *SlMTS1*. Additional binding of SlMYC1 on the *SlMTS1* promoter is enough to increase the expression level. SlEOT1 and SlMYC1 could potentially be interacting with each other. (**B)** In an induced situation SlEOT1 in combination with SlMYC1 and a JA-inducible SlMYC2 or in

combination with the JA-inducible EOT1/SHI family member (SlEOT2) could enhance expression of *SlMTS1*. Assuming binding site similarity between the *Arabidopsis* STY1 and SlEOT proteins, the existence of a YUC and a YUC-like element in the *SlMTS1* promoter indicates interaction with at least two proteins of the EOT1/SHI class. Alternatively, it could be SlMYC1 in combination with a JA-inducible SlMYC2 that regulates induced *SlMTS1* expression. Potential binding sites are shown (YUC, YUC-like, G box, G box-like). This model could be extended to other TPSs, possibly involving additional SlEOT1 family members. SlWRKY4 is not included in the model as its interaction with *SlMTS1* promoter remains to be confirmed in yeast.

A role for EOT1/SHI family proteins in plant defense responses?

The SHI family proteins from various plant species are undoubtedly involved in auxin responses and plant growth (discussed in Chapter 4). AtSTY1 in particular, interacts with the promoter of at least one member of the YUCCA (YUC) family of flavin monooxygenases involved in the tryptophan-dependent auxin biosynthesis pathway (Fig.3A; Sohlberg *et al*., 2006; Eklund *et al*., 2010). Interestingly however, the *Arabidopsis* AP2/ERF transcription factor ORA59 (Pre *et al*., 2008) is also a STY1 target, possibly through a YUC element found in the *ORA59* promoter (Eklund *et al*., 2010). ORA59 is involved in resistance against necrotrophic pathogens, integrating JA and ethylene signals to regulate expression of defense genes like Plant defensin 1.2 (*PDF1.2*) (Fig.3B; Pre *et al*., 2008). ORA59 was also shown to regulate three genes involved in tryptophan biosynthesis (Fig.3A&B; Pre *et al*., 2008). Therefore it is possible that STY1 regulates tryptophan availability and thus could promote either auxin biosynthesis or defense responses. Intriguingly, a yeast-one-hybrid (Y1H) screen with a region from the *ORCA3* promoter with a library of MeJA-treated *Catharanthus roseus* cells identified a clone encoding a protein of the SHI family (Vom Endt *et al*., 2007). The *C. roseus* SHI ortholog (clone 2D81) shares highest sequence identity with SRS5 (http://www.arabidopsis.org/Blast/) and potentially binds in the YUC element found in the *ORCA3* promoter region (Eklund *et al*., 2010). 2D81 could interact with this region also *in vitro* and its binding was not affected by mutations

in the jasmonate-responsive sequences (Vom Endt *et al*., 2007). Binding to a mutated YUC element was not tested.

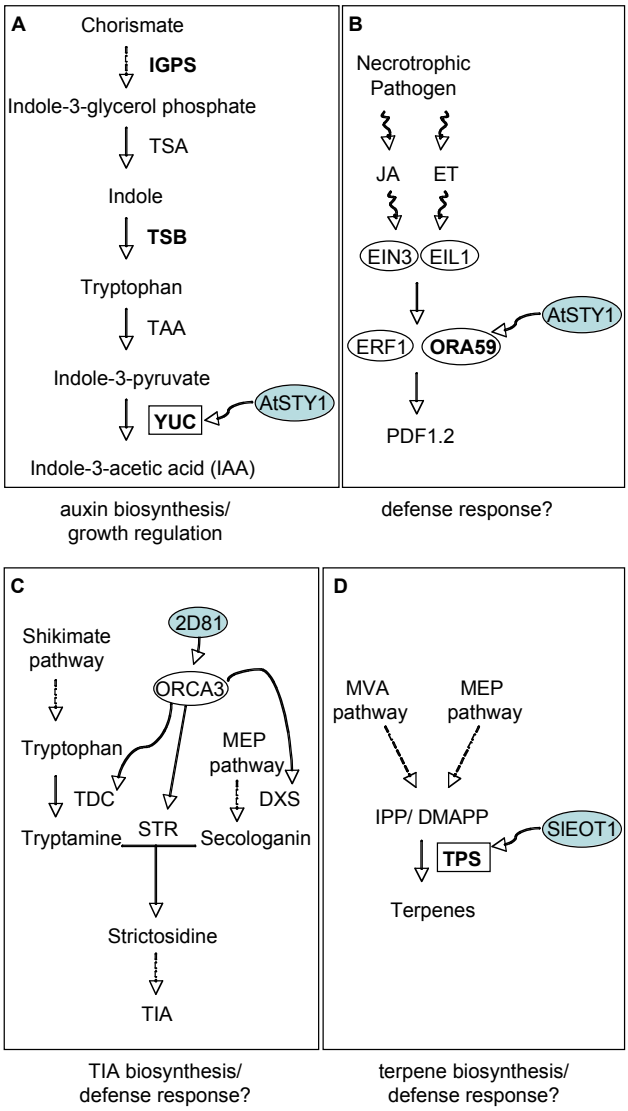

Figure 3. *Interactions and (suggested) roles of EOT1/SHI family members in various plant species*. (**A**), (**B**) *Arabidopsis thaliana* (Eklund *et al*., 2010; Pre *et al*., 2008), (**C**) *Catharanthus roseus* (Vom Endt *et al*., 2007) and (**D**) *Solanum lycopersicum* (this work). Dashed lines indicate more than one enzymatic step. Pathway enzymes regulated by EOT1/SHI proteins are in boxes, transcription factors are in ovals, EOT1/SHI proteins are in shaded ovals. In panel A, enzymes involved in tryptophan metabolism that are regulated by ORA59 (Pre *et al*., 2008) are in bold. The AtSTY1-YUC interaction (panel A) has been

experimentally proven for YUC4 and YUC8 (Eklund *et al*., 2010), the SlEOT1-TPS interaction (panel D) has been experimentally proven for SlMTS1 (this work). The 2D81-ORCA3 interaction (panel C) was detected in a Y1H screen and has been confirmed only *in vitro* (Vom Endt *et al*., 2007; *see* also text). *IGPS*; indole-3-glycerol phosphate synthase, *TSA/B*; tryptophan synthase subunit A/B, *TAA*; tryptophan aminotransferase, *YUC*; YUCCA flavin monooxygenases, *JA*; jasmonic acid, *ET*; ethylene, *EIN3*; ethylene insensitive 3; *EIL1*; EIN3-like 1, *ERF1*; ethylene response factor 1, *ORA59*; octadecanoid-responsive *Arabidopsis* AP2/ERF59, *PDF1.2*; plant defensin 1.2, *TDC*; tryptophan decarboxylase, *STR*; strictocidine synthase, *DXS*; deoxy-xylulose phosphate synthase, *ORCA3*; octadecanoid-derivative responsive *Catharanthus* AP2-domain 3, *TIA*; terpenoid indole alkaloid, *MEP*; methylerythritol, *MVA*; mevalonate, *IPP*; isopentenyl diphosphate, *DMAPP*; dimethallyl diphosphate, *EOT1*; emission of terpenes 1, *TPS*; terpene synthase.

ORCA3 regulates JA-induced terpenoid indole alkaloid (TIA) biosynthesis by controlling expression of tryptophan decarboxylase (*TDC*) that converts tryptophan to tryptamin, as well as expression of strictocidine synthase (*STR*) and deoxy-xylulose phosphate synthase (*DXS*) (Fig.3C; van der Fits and Memelink, 2000). Therefore 2D81, by regulating ORCA3, could also be controlling tryptophan availability or TIA production.

Another potential link with the JA pathway comes from analysis of *AtSRS7*, which is mainly expressed in flower filaments and the shoot apex. In the *srs7-1* mutant expression of the Defective in Anther Dehiscence 1 (*DAD1*) gene was increased and, similarly to JA-deficient mutants, anther dehiscence was inhibited (Kim *et al*., 2010). DAD1 encodes the lipolytic enzyme that catalyzes the release of linolenic acid from cellular lipids in the JA biosynthesis pathway (Ishiguro *et al*., 2001). It was suggested that through a feedback regulation, induction of *DAD1* promotes JA biosynthesis in the mutant. However, it must be noted that exogenously applied JA did not rescue the *srs7-1* floral phenotype, nor was *SRS7* expression affected by JA (Kim *et al*., 2010).

From the above-presented links of SHI family proteins with the jasmonate pathway and production of defense compounds it is already intriguing to speculate an additional role (for at least some of) these proteins in the plant defense system. An extra line of evidence comes from SlEOT1 (Fig. 3D; and possibly other family

members) that interacts with the JA-inducible *SlMTS1* (and potentially other TPSs), strengthening the case that EOT1/SHI proteins could be involved in (in)directly regulating JA-mediated defense responses in various plant species including tomato.

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