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Trichome Specific Expression: Promoters and Their Applications

Alain Tissier
Department of Cell and Metabolic Biology, Leibniz Institute of Plant Biochemistry, Weinberg, Germany

1. Introduction

As often reminded to the readers in articles or reviews which deal with plant adaptation to their environment, higher plants are sessile organisms, a life habit which does not allow them to escape danger or to move to avoid adverse conditions. This environmental pressure has led to a myriad of adaptations, which are reflected in the vast diversity of plant habitats, morphologies, life cycles and physiological adaptations among others. The surface of the aerial parts of plants is a major interaction domain between the plant and its environment and as such is the site of many adaptations, be they chemical or anatomical. Among those adaptations, the leaf hairs or trichomes, which cover the surface of a large number of plant species, play a prominent role. Plant trichomes constitute a world of their own, so great is their diversity. In a review published in 1978 and entitled "A glossary of plant hair terminology", Payne compiles a comprehensive list of more than 490 terms used to describe trichome morphology (Payne, 1978). Despite this extensive diversity, two major classes of trichome may be distinguished on the basis of their capacity to produce and secrete or store significant quantities of secondary metabolites, namely glandular or non-glandular. Non-glandular trichomes, or leaf hairs, are poorly metabolically active and provide protection mainly through physical means, for example by restricting access to insects, but also by preventing water losses, or protecting against UV radiation. *Arabidopsis thaliana* has been a model for the study of non-glandular trichome development and many genes involved in non-glandular trichome initiation and development could be identified and characterized (Uhrig and Hulskamp, 2010). The metabolic activity of these non-glandular trichomes is however fairly limited and offers little potential for metabolic engineering. A particular class of hairs is the fibers which are present in various species. Cotton seed trichomes are the most economically important since they are the basis of the cotton fiber, but other species such as cottonwood also have fiber hairs. Glandular trichomes are present in many different plant families and can also be divided in two main classes. The capitate trichomes typically have 1 to 10 glandular cells located at the tip of the trichome stalk, and the secretion is directly exuded from the top cells. The secreted material is in general fairly viscous, and in many cases it makes the leaves sticky. Those trichomes are encountered for example in the Solanaceae (tobacco, tomato, potato, etc.) and in some Lamiaceae species (e.g. *Salvia*). Peltate trichomes have the capacity to synthesize and store volatile compounds (mono- and sesquiterpenes, phenylpropenes) in a subcuticular cavity. Typical representative examples

are those from mint and other Lamiaceae, which are valued for the essential oil produced in their trichomes. In both cases, the massive metabolic fluxes that take place in the secretory cells may lead to the accumulation of metabolites which represent up to 10-15% of the leaf dry weight (Wagner *et al.*, 2004). These cells can thus be considered like true cell factories and therefore constitute attractive targets for metabolic engineering (Schilmiller *et al.*, 2008).

1.1 Why trichome specific promoters?

Whether they are cotton fibers or glandular trichomes producing essential oils or resins, the availability of genes and promoters which are specifically expressed in those structures provides material both for more in-depth studies of trichome specific processes and for high precision engineering of trichome traits. A number of genes which are highly expressed in trichomes may also be expressed in other organs because they are involved in similar processes there. The promoters from these genes are not ideal for the study of trichome specific processes for obvious reasons. Using these promoters will lead to expression outside of the trichomes and may lead to undesirable effects because of the toxicity of the compounds produced. A trichome specific promoter may be used in several ways to further investigate trichome processes. One is to search for transcription factors by one-hybrid screening or other related methods. Unbiased search for upstream regulators may also be achieved in mutant screens in plants expressing promoter:reporter gene fusions. Although not necessarily practical in the species of interest (for example in mint which is a sterile polyploidy species), a convenient host with conserved features but which is more amenable to transformation and screening, may be chosen for this purpose.

Another major motivation to isolate and characterize trichome specific promoters is genetic engineering, in particular for the expression of metabolic pathway genes. When expressed under a strong ubiquitous promoter, like the Cauliflower Mosaic Virus (CaMV) 35S, perturbation of metabolic pathways in the whole plant may have deleterious consequences on plant development and physiology. The trichomes, as a distinct entity with restricted communication to the rest of plant, represent therefore a particularly interesting target for metabolic engineering.

Besides metabolic engineering, the availability of trichome specific regulators may help to modify trichome related traits. For example, modulating the expression of transcription factors specifically controlling trichome differentiation and/or development could lead to an increase in trichome density, an improvement of the productivity of trichome-based secretions (e.g. essential oils) or a boost in trichome-mediated resistance to insect pests or other pathogens.

2. Cotton

2.1 Genomics of cotton fibers

Cotton fibers are specialized single-celled hairs which develop on ovules. The cotton hairs are among the longest plant cells reported and are coated with cellulose fibers which confer its value to the cotton crop. Because cotton hairs are single-celled, it has been proposed that their development is controlled by similar gene networks as those of Arabidopsis leaf trichomes, which are also single-celled but branched. It should be noted however, that Arabidopsis seeds do not have trichomes and thus cannot be considered as an ideal

surrogate model to evaluate the specificity of expression of cotton fiber genes. The development of seed trichomes is a synchronized process with several easily distinguishable phases. These have been well documented in previous reviews and will be briefly summarized here. The initiation of fiber cells takes place early on at the onset of anthesis, which is conveniently used as the reference time point expressed in days post anthesis (DPA) (Lee *et al.*, 2007). Already after 2 DPA, the fibers start elongating, a process which lasts until 20 DPA. This is followed by secondary wall biosynthesis until 45-50 DPA and concluded by the maturation phase. The synchronized process has allowed the preparation of RNA from these different phases. Initially, fiber specific genes were isolated by differential screening of cDNA library. This led to the successful identification of several genes with strong and specific expression in fibers, including E6, genes encoding Lipid Transfer Proteins (LTPs), a Proline Rich Protein and other genes with no obvious sequence similarity (John and Crow, 1992; Ma *et al.*, 1995; Orford and Timmis, 1995; Rinehart *et al.*, 1996; Orford and Timmis, 1997; Orford and Timmis, 1998; Orford *et al.*, 1999). Already, Northern or RT-PCR analysis showed that genes can be expressed during distinct phases of development of the fiber cells or throughout the life of these cells. This is relevant since the promoters from these genes should allow to direct the expression of transgenes during given stages of development of the fibers, which may have important practical consequences depending on the engineering objective. These early studies were followed by genomics approaches, including Expressed Sequence Tag (EST) library sequencing and microarray hybridization. In particular, EST libraries corresponding to various stages of development were produced and these provide invaluable resources for the identification of fiber specific genes (Li *et al.*, 2002a; Arpat *et al.*, 2004; Udall *et al.*, 2006; Yang *et al.*, 2006). As genes from these EST collections start being characterized, more information has become available on the pattern of expression and the importance of some transcription factors in fiber development (Lee *et al.*, 2007). For some of the genes, the promoters have been cloned and characterized by transgenesis or transient assays. Because cotton transformation is a lengthy process, alternative hosts have been used to characterize cotton promoter:GUS fusions. In most cases, these are either *Arabidopsis thaliana* or tobacco (*Nicotiana tabacum*). These hosts are far from ideal when it comes to characterize seed fiber specific expression because they are both devoid of seed trichomes. Arabidopsis is perhaps a little better because its trichomes are single-celled, like those of cotton, whereas those of tobacco are typically multicellular. There is, in addition, evidence that single celled trichomes from Arabidopsis, which, like cotton, belongs to the Rosids, and multicellular trichomes of the Solanaceae or other Asterids (Antirrhinum) are under the control of distinct regulatory network (Serna and Martin, 2006). A list of available cotton fiber promoters is provided in Table 1. This list is probably not exhaustive, but contains already 28 promoters, underscoring the high interest in characterizing such promoters. The expression range, expressed in DPA was compiled, and illustrates the diversity of promoters available, from the differentiation stage to the late secondary wall synthesis phase. Thus, targeting engineering to specific phases of fiber development is theoretically possible. It is difficult to compare the strength of these promoters between them, as they were often assessed in independent studies using different methods (Northern, semi-quantitative and quantitative RT-PCR,). Nonetheless, it can be assumed that genes with a function in cell wall biosynthesis, e.g. cellulose synthase, are probably among the most highly expressed.

Gene	Protein description	Expression measured by RT-PCR or Northern	Expression window in cotton fibers (in days)	Expression in other tissues	References
GhE6	hypothetical	Y	15-24	N	(John and Crow, 1992)
GhLTPx_GH3	Lipid transfer protein	Y	5-20	N	(Ma *et al.*, 1995)
FbL2a	Hypothetical	Y	25-45	N	(Rinehart *et al.*, 1996)
pGhEX1	Expansin	Y	6-20	N	(Orford and Timmis, 1998; Harmer *et al.*, 2002)
GhLTP6	Lipid transfer protein	N	10-20	N	(Ma *et al.*, 1995; Hsu *et al.*, 1999)
GhLTP3	Lipid transfer protein	N	5-20	N	(Liu *et al.*, 2000)
GhTUB1	beta-tubulin	Y	0-14	early seedling development (cotyledons, root tips)	(Li *et al.*, 2002b)
GhCTL1-2	Chitinase-like	Y	8-31	xylem, pollen, cells with secondary walls (weak)	(Zhang *et al.*, 2004)
GaRDL1	RD22_like	Y	3-12	N	(Wang *et al.*, 2004)
GhACT1	Actin	Y	4-21	Cotyledons	(Li *et al.*, 2005)
GhDET2	Steroid reductase	Y	3-14	Roots	(Luo *et al.*, 2007)
GhGlcAT1	glucuronosyltransferase	N	NA	NA	(Wu *et al.*, 2007)
Fsltp4	Lipid transfer protein	Y	6-14	N	(Delaney *et al.*, 2007)
GhTUA9	alpha-Tubulin	Y	5-10	N	(Li *et al.*, 2007)
GaHOX1/2	Transcription factor	Y	3-12	N	(Guan *et al.*, 2008)
GaMYB2	Transcription factor	Y	0-9	trichomes in other organs	(Wang *et al.*, 2004; Shangguan *et al.*, 2008)
GhMYB109	Transcription factor	Y	4-8	N	(Suo *et al.*, 2003; Pu *et al.*, 2008)
GhSCFP	Protease	N	2-25	N	(Hou *et al.*, 2008)
GhH6L	Arabinogalactan	Y	3-20	N	(Wu Y, 2009)
GhMYB25	Transcription factor	Y	0-5	trichomes of other tissues, pollen, anthers, root epidermis, root initials	(Machado *et al.*, 2009)

Gene	Protein description	Expression measured by RT-PCR or Northern	Expression window in cotton fibers (in days)	Expression in other tissues	References
GhSUS3	Sucrose synthase	Y	0-5	NA	(Ruan *et al.*, 2009)
GhXTH1	Xyloglucan endotransglycosylase/hydrolase	Y	10-25	N	(Michailidis *et al.*, 2009)
GbML1	Transcription factor	Y	-3-8	Petal	(Zhang *et al.*, 2010)
GhRING1	Ubiquitin Ligase	Y	0-20	NA	(Ho *et al.*, 2010)
GhXTH1	Xyloglucan endotransglycosylase/hydrolase	Y	10-15	Petal	(Lee *et al.*, 2010)
ADPGp_SSU2	ADP-glucose pyrophosphorylase	Y	10	meristem, immature stem, roots	(Taliercio, 2011)
GhCesA4	Cellulose synthase	Y	16-24	root vascular tissue	(Wu *et al.*, 2009; Kim *et al.*, 2011)

Table 1. Promoters expressed in cotton fibers. DPA: days post-anthesis. Y: yes; N: no; NA: not available

2.2 Examples of engineering of cotton trichomes

The first attempts at genetic engineering of cotton fibers were performed in the late 1990s, soon after the first specific promoters were identified. The objective was to introduce poly-hydroxybutyrate (PHB) into cotton fibers, via the expression of two genes *phaB* and *phaC* from the bacterium *Alcaligenes eutrophus*, which naturally produces PHB in inclusion bodies. *phaB* encodes the acetoacetyl-CoA reductase and *phaC* the PHB synthase. Expression of both genes in *Arabidopsis thaliana* was previously shown to support *de novo* biosynthesis of PHB in plants for the first time (Poirier *et al.*, 1992). In cotton, this was achieved by expressing *phaB* under the control of the promoters from the fiber specific genes FbL2a or E6, and *phaC* with the FbL2a or 35S promoters. Since the substrate for the PHB synthase does not occur naturally in plants, the expression of *phaC* under 35S should not have deleterious effects on whole plants. The transgenic plants were reported briefly in a first paper (Rinehart *et al.*, 1996) and analyzed in more detail in a second article (John and Keller, 1996). Production of PHB in the lumen of cotton fiber cells could be shown as evidenced by staining, electron microscopy, HPLC and GC-MS. PHB accumulated in the form electron-translucent granules. Quantification of crotonic acid released after hydrolysis indicated levels of up to 3440 µg/g dry fiber in the best lines. The majority of the PHB produced (68.3 %) had a MW above 0.6×10^6 Da, which is similar to PHB produced in bacteria. PHB synthesis peaked at 10 DPA and did not increase nor decrease afterwards, indicating the absence of major PHB degrading activity in cotton fibers. The thermal properties of the transgenic fibers were also assessed and indicated that they had higher heat retention capacity (John and Keller, 1996). However, although promising, those modified properties were apparently not significant enough to warrant commercialization. This was due to the relatively low level of PHB produced (0.34% of fiber weight), which would need to increase several fold to be considered for commercialization.

In a more recent attempt at metabolic engineering, melanin biosynthesis was introduced in cotton fibers (Xu *et al.*, 2007). Dyeing cotton fibers has a heavy imprint on the environment and solutions to reduce its polluting impact are desirable. Naturally colored cotton fibers exist but the choice of colors is limited and the colored cotton varieties have low producing capacity. An alternative is to use biotechnology to engineer colors into cotton fibers. As a proof of concept, Xu and co-workers (2007) expressed two genes, *TyrA* and *ORF438*, from *Streptomyces antibioticus*, which are required and sufficient to synthesize melanin. Both genes were codon optimized for expression in cotton, fused to a vacuolar targeting peptide and cloned under the control of a fiber specific promoter from the *Ltp3* gene (Liu *et al.*, 2000). The same construct was used to transform tobacco and cotton. Both in tobacco and in cotton transgenic plants the change in color in the leaf trichomes (tobacco) or in the seed fibers (cotton) was distinctly visible although no dosage of melanin was reported (Xu *et al.*, 2007). In addition to its color, melanin also absorbs UV light and could therefore provide UV-protection properties to cotton fabrics.

3. Tobacco

Tobacco (*Nicotiana tabacum*) is an allotetraploid species which is grown worldwide for its leaf which is processed and used for various products, from which the most widely sold and consumed are cigarettes. It is well established that regular tobacco smoking is a health-damaging habit with associated increased risks of cancer and cardio-vascular diseases. Health-promoting uses of tobacco could provide alternative revenue sources for tobacco farmers, for example by producing pharmaceutical ingredients in tobacco through genetic engineering. Plant Made Pharmaceuticals (PMPs) have mostly concerned therapeutic proteins, such as antibodies or hormones like insulin. Plants are also known to provide many natural small molecules to the pharmacopeia or as drug leads. These belong to the secondary, or specialized, as they are now sometimes called, classes of metabolites. The huge diversity of these compounds provides a phenomenal reservoir of chemical structures whose biosynthesis pathways are now beginning to be elucidated thanks to the contribution of genomics approaches in plant biochemistry studies. One issue which is frequently raised about plant natural products is the availability of the raw material and the cost associated to extraction and purification of the compound. Pharmaceutical companies will shy away from substances whose supply cannot be safely guaranteed, which is likely to be the case if the chemical is produced in one rare plant of the Amazon forest for example. But the plant does not need to come from tropical forest to be endangered. The story of Taxol is a good example in this respect. Taxol is a diterpenoid extracted from yew tree with potent anticancer activity. Initially, Taxol was extracted from the barks of pacific yew trees (*Taxus brevifolia*), where it was present in less than 0.01% of the dry matter, with many related taxoids to separate it from, making it an extremely expensive chemical to produce. Chemical synthesis was too complex to be exploited commercially. Since the extraction was destructive, natural populations of Taxus were threatened through commercial exploitation of the trees. Fortunately, a semi-synthetic method starting from a precursor abundant in the twigs, 10-deacetyl-baccatin III, was developed. This allowed a durable and renewable procedure since twigs can be harvested without felling trees.

The presumed progenitors of *N. tabacum* are *N. sylvestris* and *N. tomentosiformis*. All three species have glandular capitate trichomes on their leaf and stem surfaces, with distinct exudate profiles. Cultivated tobacco (*Nicotiana tabacum*) and its wild relatives, *Nicotiana sylvestris* and *N. tomentosiformis* produce diterpenes in large amounts in their glandular capitate trichomes. *N. tomentosiformis* secretes large quantities of labdanoid diterpenes. In *N. tabacum*, these may have two types, either macrocyclic cembranoid or bicyclic labdanoids. The cembranoids are also produced by *N. sylvestris* trichomes and include the cembratrien-diols (α- and β-CBT-diols) and their precursors the cembratrien-ols (α- and β-CBT-ols). Labdanoids include Z-abienol and labdene-diol. Depending on the variety, these diterpenoids may be present in varying amounts and combinations. The terpenoid biosynthesis capacity of tobacco glandular trichomes is massive. In the appropriate conditions, the amount of CBT-diols produced by a *N. sylvestris* leaf may represent up to 10% of the leaf dry weight. Early studies performed on tobacco glandular trichomes concluded that the biosynthesis of the diterpenes takes place in the trichome heads themselves (Keene and Wagner, 1985; Kandra and Wagner, 1988; Guo *et al.*, 1994). This, together with the high productivity of tobacco trichomes makes them an ideal target for terpenoid metabolic engineering.

Terpenes are hydrocarbon molecules whose structure is based on repeated units of isoprene. They are derived from two C5 precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). IPP units can be sequentially added to DMAPP by isoprenyl transferases, thus leading to the major short chain isoprenyl diphosphates, geranyl diphosphate (GPP- C10), farnesyl diphosphate (FPP – C15) and geranylgeranyl diphosphate (GGPP – C20). These isoprenyl diphosphates are the substrates of terpene synthases, which in many cases make cyclic products. These are the origin of the skeletal diversity of terpenes. In tobacco, the pathway to the cembratrien-diols was elucidated and shown to involve two steps. The first is encoded by a multigene family of diterpene synthases, the cembratrien-ol synthases (CBTS), which altogether account for the mix of the two stereoisomers of CBT-ol (α and β) (Wang and Wagner, 2003; Ennajdaoui *et al.*, 2010). The second step is carried out by a cytochrome P450 mono-oxygenase which hydroxylates the CBT-ols at a specific position (Wang *et al.*, 2001; Wang and Wagner, 2003). Since the biosynthesis of trichome diterpenoids specifically takes place in the glandular cells, one way to get trichome specific promoters is to clone the corresponding genes.

3.1 Tobacco trichome specific promoters

The first tobacco trichome specific promoter identified was that of the *CYP71D16* gene, which encodes the CBT-ol hydroxylase (Wang *et al.*, 2002). The gene was itself identified through subtractive cDNA library construction which was followed by the cloning of the promoter. 1.8 kb of the promoter was sufficient to confer a highly specific expression of the GUS reporter gene to the trichomes. Remarkably, only the glandular cells were stained, highlighting the distinct differentiation status between the glandular cells of the head and the non-glandular cells of trichome stalk.

The CBTS genes provided another set of trichome specific promoters. It was found that the CBT-ol synthase activity is encoded by a family of 3 closely related genes, which arose via recent duplication event. These genes share over 90 % identity at the nucleotide level, including in the promoter regions. One of those promoters (pCBTS2a) was further studied

with sequential and internal deletions. This allowed the identification of a positive regulatory region and a negative regulatory region. When the inhibitory region is deleted, expression can be detected in the whole leaf epidermis as well as in patches in roots. On the contrary, when the activating region is deleted, no expression at all can be detected (Ennajdaoui *et al.*, 2010). This indicates that the cell specific expression is the result of the unique combination of a broad activating region with an inhibitory region which restricts expression to the desired cells.

Genes involved in the other tobacco labdanoid pathway have been recently identified and one promoter was also identified and characterized as trichome specific (Tissier, unpublished results).

The availability of several distinct promoters with identical specificity and different strengths should broaden the possibilities for metabolic engineering.

3.2 Strategies and example for tobacco trichome engineering using specific promoters

Since tobacco produces diterpenoids, one logical possibility for metabolic engineering is to use tobacco trichomes for the production of heterologous diterpenoid. The substrate, namely GGPP, should be available in non-limiting quantities and in addition, the glandular cells have a machinery which allows them to excrete hydrophobic compounds like diterpenes. To facilitate the detection of heterologous terpenoids, it may be useful to eliminate or reduce the endogenous diterpenoids. This can be achieved by inactivating the *CBTS* genes. Because they are members of a multigene family which are most likely located at the same chromosomal locus, the most efficient way to achieve this is to use gene silencing technologies. This was done with an antisense construct under the control of a 35S promoter (Wang and Wagner, 2003). However, more efficient silencing was obtained with intron-hairpin constructs targeting the exon 2 of the *CBTS* genes, under the control of the *CBTS2a* gene itself. In this case, the best transgenic lines had almost no CBT-diols detectable (Ennajdaoui *et al.*, 2010). There is also the possibility to exploit natural variation in *N. tabacum*. During a survey of the metabolic profiles of tobacco leaf exudates we have noticed that some cultivars produce labdanoids and no cembranoids, while others produce cembranoids and no labdanoids. Thus, by crossing these cultivars it is theoretically possible to breed new varieties which produce no diterpenoids at all, but which still have the capacity to produce new ones. Once a diterpene-free background has been established by either of these approaches, heterologous diterpene synthases may be cloned behind trichome specific promoters for targeted expression to the glandular cells. This was successfully done for taxadiene synthase (Rontein *et al.*, 2008) with yields of up to 10 µg/g fresh weight.

3.3 Glandular trichome expression as a gene function discovery tool

Several steps of Taxol biosynthesis have been investigated, including the early oxidations of taxadiene which lead to the synthesis of the important semi-synthesis precursor, 10-DABIII (Croteau *et al.*, 2006). The first of these oxidations was shown to be at the C5 position of the taxadiene core, which is necessary to form the so-called oxetane ring (Hefner *et al.*, 1996). Subsequently, a gene encoding taxadiene 5-α-hydroxylase (*T-5-OH*) was identified and

characterized (Jennewein *et al.*, 2004). In order to reconstitute these early steps of the taxol biosynthesis pathway, both genes were expressed in tobacco under the control of trichome specific promoters. Taxadiene synthase (TS) was cloned downstream of the CBTS2a promoter as described above while the T-5-OH was cloned 3' of the CYP71D16 promoter. Surprisingly, no taxadien-5-α-ol could be identified in the transgenic plants expressing both genes. Instead, a new product, which was later found to be 5(12)-oxa-3(11)-cyclotaxane, derived from a complex rearrangement of the taxadiene core upon oxidation, could be identified (Rontein *et al.*, 2008). The activity of the enzyme was then further proved from a protein expressed in yeast. This shows that the tobacco trichome platform was useful to produce sufficient quantities of the product to be structurally characterized. The initial functional assignment was likely misguided by the presence of small amounts of T-5-OH as a by-product in the enzyme assays. Since T-5-OH was the compound that was looked for, the major product may have been ignored. The novel assignment derived from the initial tobacco expression, was later confirmed by expression in *E. coli*, although in this case significant amounts of T-5-OH could be detected (Ajikumar *et al.*, 2010).

In another example, the function of the genes required for the biosynthesis of *Z*-abienol in tobacco could be confirmed by expression in *N. sylvestris* trichomes. *Z*-abienol is a labdane diterpenoid whose biosynthesis was predicted to require two successive enzymatic steps (Guo *et al.*, 1994), first a copalyl-diphosphate synthase like then a kaurene synthase like enzyme (Peters, 2010). Two candidate genes were thus identified and expressed in *N. sylvestris*, which does not produce *Z*-abienol. The exudate of these transgenic plants contained significant amounts of *Z*-abienol of up to 100 µg/g FW (Sallaud and Tissier, unpublished results).

The supply of isoprenyl diphosphates IPP and DMAPP in tobacco trichomes should allow also engineering of other terpenoid classes, like mono- or sesquiterpenes. In those cases, the appropriate isoprenyl transferases (i.e. geranyl diphosphate or farnesyl diphosphate synthases) should be expressed in addition to the terpene synthases. This was done for a sesquiterpene synthase from tomato, the santalene and bergamotene synthase, which uses an unusual isoprenyl diphosphate precursor, *Z,Z*-FPP (Sallaud *et al.*, 2009). Both enzymes are naturally targeted to the plastids, which is where IPP and DMAPP from the methyl-erythritol pathway (MEP) are synthesized. In this case, the sesquiterpenes could not be identified in the leaf exudate, rather in the headspace collected from transgenic plants (Sallaud *et al.*, 2009). This indicates that tobacco trichomes are suitable for the biosynthesis of sesquiterpenes, but not for their storage. For these, and other volatile compounds, such as monoterpenes or phenylpropenes, glandular trichomes with a storage compartment for volatile compounds, such as the peltate trichomes of mint, should be used (see below).

3.4 Other examples of tobacco trichome engineering

As in cotton, the genes for the biosynthesis of the pigment melanin were expressed in tobacco under the control of the cotton trichome promoter LTP3 (Xu *et al.*, 2007). This promoter was previously shown to be active in tobacco trichomes (Liu *et al.*, 2000). Based on the color of trichomes, the presence of melanin could be detected, however no quantification was performed. This, however, shows that glandular trichomes which are normally producing terpenoids may also be used as an engineering platform for other classes of compounds.

4. Mint and other lamiaceae

4.1 Mint

Peppermint (*Mentha x piperita*) is an aromatic plant which is grown worldwide for its essential oil whose distinctive character is imparted by its most well known compound, (-)-menthol. The essential oil of mint, and of many other aromatic plants form the Lamiaceae, is stored in glandular trichomes of the peltate type (Gershenzon *et al.*, 1987; Gershenzon *et al.*, 1989). Peltate trichomes are composed of 8 glandular cells topped by a subcuticular space where the secretion products are stored. When the cuticule is ruptured, by pressing the leaf between the fingers, or by an insect, the volatile compounds are released and may reach their target. It was shown that the peltate trichomes are not just a site of storage, but also that the terpenoids are produced in the peltate glandular cells (Gershenzon *et al.*, 1989). A technique for the purification of intact peltate glands was developed to allow the production of a trichome specific EST library (Gershenzon *et al.*, 1992). This EST library provided sequence information for the characterisation of the (-)-menthol biosynthetic pathway, which was completely elucidated over the years by the research group from Prof. Croteau (Alonso *et al.*, 1992; Gershenzon *et al.*, 1992; Rajaonarivony *et al.*, 1992; Lupien *et al.*, 1999; Turner *et al.*, 1999; Gershenzon *et al.*, 2000; McConkey *et al.*, 2000; Bertea *et al.*, 2001; Wust *et al.*, 2001; Croteau *et al.*, 2005). Thus, peltate trichomes are extremely well adapted for the production and storage of volatile compounds, in particular mono- and sesquiterpenoids. Metabolic engineering of mint trichomes should therefore yield particularly interesting results for these volatile compounds. Mint transformation by *Agrobacterium tumefaciens* was independently reported by several groups (Diemer *et al.*, 1998; Weller *et al.*, 1998). However, although the use of trichome specific promoters was proposed as early as 1999 as a pre-requisite for metabolic engineering in mint (Lange and Croteau, 1999), to our knowledge no characterization of trichome promoters from mint has been published to date. The promoter of the Arabidopsis GL1 transcription factor was shown to be functional in tobacco and peppermint (Gutierrez-Alcala *et al.*, 2005). However, whether the strength of this promoter will be sufficient for metabolic engineering remains to be seen. Nonetheless, the whole menthol pathway from spearmint provides a set of genes with trichome specific expression and the identification of their promoters should not raise major difficulties.

4.2 Basil

Like mint, Basil (*Ocimum basilicum*) is grown for its aromatic properties which are due to volatile compounds produced in similar peltate trichomes. Following the successful approach developed in mint, trichome specific EST libraries from different cultivars of basil, corresponding to distinct chemotypes, were produced. These were used to elucidate the pathways to volatile phenylpropenes and monoterpenes (Gang *et al.*, 2001; Gang *et al.*, 2002a; Gang *et al.*, 2002b; Iijima *et al.*, 2004a; Iijima *et al.*, 2004b). As in mint, the enzymes of the pathway are likely to be highly specific to the peltate glandular cells, and therefore the promoters of the corresponding genes should drive specific expression to these cells. Like mint, basil could prove an interesting host for the metabolic engineering of volatile compounds, with the additional option of the capacity to engineer phenylpropanoid metabolism in accessions which produce phenylpropenes. However, to date no promoters of basil trichome genes have been characterized. One could also assume that promoters from mint should operate in basil, and reciprocally, because of the similarity of their trichomes and the fact that mint and basil both belong to the Lamiaceae.

4.3 Sage

Sage (*Salvia sp.*) is a large genus with a number of species which are grown commercially for the extraction of fragrant or aromatic oils. One of the most important is *Salvia sclarea* (clary sage), a biennial plant which produces both an essential oil rich in linalyl acetate and linalool, and a concrete with high amounts of the labdanoid diterpene sclareol. Sclareol is currently used in the fragrance industry as a synthesis precursor for Ambrox®, a highly valued compound with amber-like fragrance und excellent fixative properties (Decorzant *et al.*, 1987; Martres *et al.*, 1993; Koga *et al.*, 1998; Moulines *et al.*, 2001; Barrero *et al.*, 2004; Moulines *et al.*, 2004). *Salvia sclarea* possesses two types of glandular trichomes, capitate and peltate. The capitate trichomes are likely to produce sclareol, which is secreted onto the surface of the inflorescences while the volatile compounds like linalyl acetate are more likely to be produced in peltate trichomes (Lattoo *et al.*, 2006; Schmiderer *et al.*, 2008). The productivity of sclareol by *Salvia sclarea* is very high, making it an attractive target for metabolic engineering of terpenoids. Recently, massive sequencing of calyx RNA, where peltate and capitate glands are highly abundant, was carried out (Legrand *et al.*, 2010). A number of genes encoding proteins with clear similarities to terpene synthases and enzymes isoprenoid metabolism could be identified, thus providing genes with potentially highly specific pattern of expression, notably restricted to trichome glandular cells. Although transformation and regeneration of transgenic *Salvia sclarea* plants has not been achieved to date, hairy root cultures were established (Kuzma *et al.*, 2006; Kuzma *et al.*, 2008), and transformation of a related species (*Salvia miltiorrhiza*) by *Agrobacterium tumefaciens* could be successfully demonstrated (Yan and Wang, 2007; Lee *et al.*, 2008). These results suggest that trichome specific metabolic engineering of clary sage is technically feasible.

4.4 Lavender

Lavender (*Lavandula angustifolia, L.* x *intermedia* and other species) is a perennial plant grown in the Mediterranean area for its highly fragrant and characteristic essential oil, which is a complex mixture of mono- and sesquiterpenoids. As for other Lamiaceae species discussed above, the essential oil is produced in peltate glandular trichomes located mostly on the inflorescences (Guitton *et al.*, 2010). Here also, genomics approaches have been initiated to better understand the molecular basis of essential oil production. In one study, EST libraries from flowers and leaves were sequenced by the Sanger method to yield a total of 14,000 sequences (Lane *et al.*, 2010), thus providing the foundation to identify trichome specific genes. A recent study also showed that the oil profile changes over the course of flower development correlated with changes in expression of certain terpene synthases, providing important information regarding harvest time (Guitton *et al.*, 2010). In addition, a trichome specific promoter from the linalool synthase of *L. angustifolia* (LaLIS) was recently isolated and characterized (Biswas *et al.*, 2009). Lavender transformation is also well established, having been reported by two independent groups (Mishiba *et al.*, 2000; Nebauer *et al.*, 2000). Attempts were also made at metabolic engineering of essential content by overexpressing 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) and a limonene synthase with a constitutive 35S promoter (Munoz-Bertomeu *et al.*, 2007; Munoz-Bertomeu *et al.*, 2008). Overexpression of HMGR lead to an increase of both monoterpenes and sesquiterpenes, indicating that the cytosolic mevalonate pathway may contribute to both types of terpenes, although monoterpenes are synthesized in the plastids (Munoz-Bertomeu *et al.*, 2007). Overexpression of the spearmint limonene synthase on the other hand led to a strong

increase in limonene while not affecting the other constituents of the oil, indicating that the supply of isoprenyl diphosphate precursors is likely not to be limiting in glandular trichomes (Munoz-Bertomeu *et al*., 2008). These results bode well for the metabolic engineering of lavender trichomes, and no doubt that the availability of specific promoters should allow more precise manipulation of essential production in these species.

5. Tomato

Like tobacco, tomato (*Solanum lycopersicum*) belongs to the family Solanaceae, which is rich in species with trichomes. Wild species of tomatoes, such as *Solanum pennellii*, *S. habrochaites*, and *S. peruvianum* among others, have different trichome types including non-glandular and glandular types. Altogether up to 7 different types could be described, of which 3 main glandular types could be described (Luckwill, 1943)(See Figure 1).

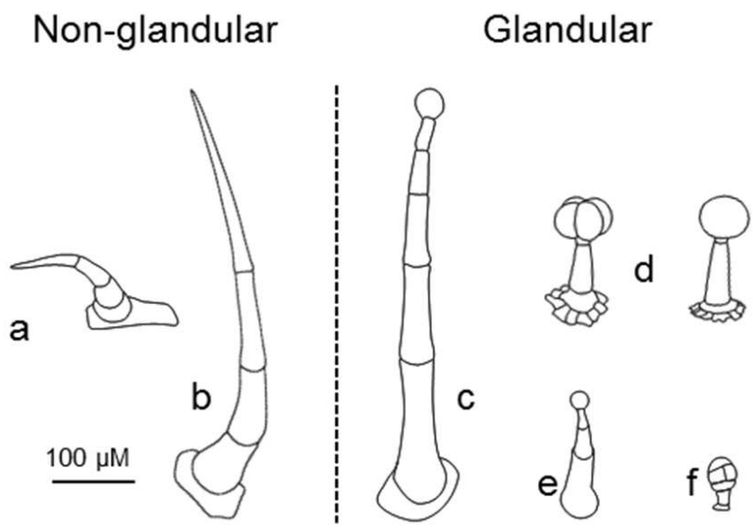


Fig. 1. Trichome types from various tomato species. a. type II non-glandular trichomes from *S. lycopersicum*. b. Type III long hairs (non-glandular). c. Type I long glandular trichomes with single secretory cell at the tip. d. Type VI glandular trichomes. On the left, trichomes from the cultivated tomato, *S. lycopersicum*. On the right, trichomes from the wild species *S. habrochaites*. The type VI trichomes from *S. lycopersicum* have four secretory cells on one plane, which can be easily distinguished from each other. The type VI trichomes from *S. habrochaites* also have four secretory cells, but they are wrapped in a common cuticular envelope, making it look like a single cell from the outside. In addition there is an intercellular space in the middle of these type VI trichomes, where the metabolites are stored. e. type IV trichomes. In some species, like *S. habrochaites*, these trichomes have a single glandular head, while in others like *S. pennellii* they look more like tobacco glandular trichomes with several glandular cells. F. type VII short glandular trichomes.

The type VII are short glandular trichomes with a single stalk cell and a berry-shaped glandular head composed of 7-10 cells. Tobacco also has similar trichomes, and it was shown in tobacco that these trichomes secrete short proline rich proteins, called phylloplanins which have antifungal activities (Shepherd *et al*., 2005). These trichomes do not appear to secrete small metabolites, and thus seem to be specialized for peptide

synthesis. Type I and type IV are capitate trichomes with few or a single glandular cell at the tip. The Type I are extremely long trichomes which can be easily seen with a naked eye, while the type IV trichomes are shorter. Type I trichomes are rare in cultivated tomatoes, while fairly abundant in some wild species, like *S. habrochaites*. Type IV trichomes are absent from cultivated tomatoes, and very abundant in *S. habrochaites*. These trichomes seem to be involved in the synthesis and secretion of secondary metabolites, mostly terpenoids (McDowell *et al.*, 2011). Type VI trichomes are present in both *S. lycopersicum* and *S. habrochaites* but they present distinct morphologies in each species. In *S. lycopersicum*, the four secretory cells are distinctly visible, forming a four-leaf clover shape when viewed from above with a total width of ≈ 60 μM. In *S. habrochaites*, the four secretory cells are encased in an envelope made of cuticule and cell wall materials, so that they appear as a single unit from the outside. The diameter of this ball-shape structure is also about 60 μM, and in contrast to the type VI trichomes from the cultivated tomato, it contains a cavity, most likely of intracellular space resulting from cell wall degradation, between the 4 cells. This storage cavity is reminiscent of the subcuticular space of the peltate trichomes of mint and is likely to contain the secretion products of the glandular cells.

Tomato trichomes have attracted major interest because of their roles in biotic interactions, in particular with arthropods. There are many reports of the roles of trichome secretions in the resistance to insect or arthropod pests (Kennedy, 2003). Most of the resistances to insects are found in wild species, like *S. pennellii* and *S. habrochaites*. For example the white fly *Bemisia tabacci*, which can transfer viruses, can be overcome thanks to glandular trichome secretions (Heinz and Zalom, 1995; Rubinstein and Czosnek, 1997; Snyder *et al.*, 1998; Vendramim *et al.*, 2009). *Tuta absoluta* is another important pest which is recently causing increasing damages to tomato crops. Again sources of resistance have been identified in wild accessions of *S. habrochaites* (Gilardon *et al.*, 2001; Maluf *et al.*, 2010).

The origin of the resistance lies both in the nature and in the quantity of the chemicals secreted. These wild species can be crossed to *S. lycopersicum*, and they can be used to introgress agriculturally relevant traits (disease and abiotic stress resistance, flavor, yield, etc.) into the cultivated tomato genome. However, introgression of complex traits which involve not only biosynthetic pathways, but also regulatory factors controlling may prove difficult and could lead to the introduction of undesirable genes from the wild species which adversely affect yield traits for example, and which may be difficult to eliminate because of the lower level of recombination between wild and cultivated tomato genomes. An alternative is then to introduce the required genes by genetic engineering. To avoid the synthesis of these compounds in the whole plant, which may cause undesirable side effects, trichome specific promoters are required.

In early studies, it was found that polyphenol oxidases are strongly expressed in tomato type VI glandular trichome (Kowalski *et al.*, 1992; Yu *et al.*, 1992; Thipyapong *et al.*, 1997). However, the promoters of this complex multigene family are not specific to trichomes and are also expressed in many other tissues upon stress (Yu *et al.*, 1992; Thipyapong *et al.*, 1997).

5.1 Omics of tomato glandular trichomes

Subsequently, as interest in elucidating the biosynthesis pathways of tomato glandular trichomes increased, trichome specific EST libraries from tomato were produced, in

particular from the wild species with abundant trichome secretions. The first libraries were released in 2001 from *S. habrochaites* (accession LA1777))(van Der Hoeven *et al.*, 2000) and *S. pennellii* (accession LA716). They were produced by Sanger sequencing and contained around 2000 sequences each (see Table 2).

Species	accession	Trichome type	Sequencing type	# of ESTs	Reference
S.habrochaites	LA1777	mixed	Sanger	2,656	(van Der Hoeven *et al.*, 2000; Fei *et al.*, 2004)
S. habrochaites	PI126449	mixed	Sanger	5,494	(Fridman *et al.*, 2005)
S. lycopersicum	NA	mixed	Sanger	7,254	(Besser *et al.*, 2009)
S. pennellii	LA716	mixed	Sanger	2,917	(Fei *et al.*, 2004)
S. lycopersicum	LA3475	mixed stems	NGS	278,000	(McDowell *et al.*, 2011)
S. lycopersicum	LA3475	type VII	Sanger	791	(McDowell *et al.*, 2011)
S. lycopersicum	LA3475	type VI	NGS	225,000	(McDowell *et al.*, 2011)
S. lycopersicum	LA3475	type I	Sanger	831	(McDowell *et al.*, 2011)
S. habrochaites	LA1777	mixed leaves	NGS	108,000	(McDowell *et al.*, 2011)
S. habrochaites	LA1777	type I	Sanger	978	(McDowell *et al.*, 2011)
S. habrochaites	LA1777	type IV	Sanger	1,425	(McDowell *et al.*, 2011)
S. habrochaites	LA1777	type VI	NGS	224,000	(McDowell *et al.*, 2011)
S. habrochaites	PI126449	Type VI	Sanger	15,000	(McDowell *et al.*, 2011)
S. pimpinellifolium	LA1589	type VI	NGS	227,000	(McDowell *et al.*, 2011)
S. pennellii	LA0716	type IV	Sanger	1,277	(McDowell *et al.*, 2011)
S. pennellii	LA0716	type VI	Sanger	1,137	(McDowell *et al.*, 2011)
S. pennellii	LA0716	mixed leaves	NGS	275,000	(McDowell *et al.*, 2011)
S- arcanum	LA1708	mixed stems	NGS	415,000	(McDowell *et al.*, 2011)
Total				**1,791,760**	

Table 2. A summary of currently available EST libraries from tomato trichomes. The accession numbers are those according to the Tomato Genetics Resource Center nomenclature (preceded with LA), or from the USDA germplasm collection (preced with PI).

Other similar sequence libraries were produced (Fridman *et al.*, 2005; Slocombe *et al.*, 2008), followed by the recent release of trichome specific libraires from several Solanum species and from distinct trichome types (McDowell *et al.*, 2011). Some of these were sequenced by next generation sequencing technologies, thus affording much larger numbers of EST sequences (up to 278 000 in some cases) (McDowell *et al.*, 2011). These sequence databases have been extremely useful in identifying and characterizing genes for the trichome specific biosynthesis pathways, in particular for terpenes (van Der Hoeven *et al.*, 2000; Sallaud *et al.*, 2009; Schilmiller *et al.*, 2009) and methylketones (Fridman *et al.*, 2005; Ben-Israel *et al.*, 2009; Yu *et al.*, 2010). A summary of the available EST sequences from various tomato species and trichome types is provided in Table 2. With a total of 1 791 760 ESTs, tomato trichomes are probably the trichomes with the best sequence resources currently available.

The availability of the tomato (*S. lycopersicum*) genome sequence makes it possible now to rapidly have access to promoter sequences, although the most interesting promoters will undoubtedly come from the wild species. However, the high sequence similarity between *S. lycopersicum* and *S. pennellii* or *S. habrochaites* should allow the facile identification of the promoters in those wild species.

There is at this stage and to the best of our knowledge, no reported example of metabolic engineering in tomato trichomes. Since tomato is a food crop grown for its fruit, much more has focused on fruit metabolism. There was even a report of taxadiene synthase (TS) expression in tomato fruit under the control of a fruit specific promoter (Kovacs *et al.*, 2007). From a purely metabolic point of view, this makes sense since tomato fruits are rich in carotenoids which derive from the same substrate as diterpenes, namely GGPP. However, the overexpression of TS caused sterility and growth defects which are undesirable side effects. In addition, the presence of potentially toxic secondary compounds in edible vegetables or fruits is a potential source of incidents by contamination of the food supply chain which must be avoided.

6. Artemisia

Artemisia annua, or sweet wormwood, a biennial plant from the Asteraceae family, has attracted attention as the source of an alternative to quinoline drugs for the treatment of malaria. The emergence of foci of resistance to quinine and related drugs in strains of *Plasmodium falciparum* requires the use of durable alternative treatments. Sweet wormwood was long known in Chinese traditional medicine to treat fevers. It was rediscovered in the 1970s for the treatment of malaria. The active ingredient is artemisinin, a sesquiterpene lactone, but semi-synthetic derivatives (Artemeter, Artesunate) have been developed as drugs by the pharmaceutical industry. Artemisia, like many other species from the Asteraceae, produces sesquiterpene lactones in glandular capitate trichomes localized on the leaves, stems and flowers. As with other trichome specific biosynthetic pathways, the elucidation of the first steps of the artemisinin biosynthesis pathways was made possible after sequencing trichome specific cDNA libraries, with the exception of the very first committed step, the sesquiterpene amorphadiene synthase (AaAS). AaAS was initially identified from a leaf cDNA library by similarity to known sesquiterpene synthases from plants (Mercke *et al.*, 2000; Wallaart *et al.*, 2001) and its specific pattern of expression in trichomes was later confirmed (Bertea *et al.*, 2005; Olofsson *et al.*, 2011). A succession of oxidation steps requiring a P450 mono-oxygenase (CYP71AV1) and an aldehyde dehydrogenase leads to artemisinic acid, while the synthesis of de-hydro-artemisinic acid requires the intervention of reductase (DBR2) (Zhang *et al.*, 2008; Liu *et al.*, 2009; Teoh *et al.*, 2009; Wang *et al.*, 2009; Zhang *et al.*, 2009; Weathers *et al.*, 2011). Although much progress has been achieved in the elucidation of the artemisinin pathway, relatively little was done with regards to promoter identification. Although most genes of the pathway are likely to be trichome specific (Liu *et al.*, 2009; Olsson *et al.*, 2009; Wang *et al.*, 2009), only one study reports on the cloning of the *AaAS* promoter and the identification of a WRKY transcription factor (AaWRKY1) which binds to the promoter of *AaAS* (Ma *et al.*, 2009). So far, to the best of our knowledge, no attempt at engineering of *A. annua* trichome metabolism has been reported. Given the importance of this compound as a pharmaceutical ingredient, attempts

at metabolic engineering of the artemisinic acid pathway in other plants and in microorganisms will be here briefly reviewed. Artemisinic acid can be used as a precursor for the semi-synthesis of artemisinin and related compounds. In parallel to these pathway elucidation efforts, different approaches were proposed and undertaken to improve the supply of the ingredient. Increasing demand, as well as requirements of reliable quality and supply to maintain stable prices, have spurred the search for either improvement of the available crop plant or transferring the production in heterologous hosts by metabolic engineering.

The first strategy is plant-based with the objective of improving artemisinin production by breeding using existing natural variation or induced mutagenesis. To reach this goal, a high density genetic map based on markers derived from transcriptome deep sequencing was created and used to map QTLs for artemisinin production (Graham *et al.*, 2010). This work showed that next generation sequencing technologies allow the rapid production of dense genetic maps in species where there is little or no prior genetic knowledge.

Another set of approaches is based on the expression of artemisinin biosynthesis genes in heterologous hosts. Reconstitution of the pathway to artemisinic acid was tested in tobacco but using ubiquitous promoters such as CaMV 35S (Zhang *et al.*, 2011). Previous sesquiterpene engineering studies in tobacco had revealed that targeting a FPP synthase together with a sesquiterpene synthase to plastids gave the best results (Wu *et al.*, 2006). With the same strategy and using different combinations of genes, it could be shown that amorphadiene, artemisinic and de-hydroartemisinic alcohol could be produced in the range of µg/g FW, but no artemisinic aldehyde or acid could be detected (Zhang *et al.*, 2011). Further analysis indicated that an endogenous reductase in tobacco prevents accumulation of artemisinic aldehyde and acids (Zhang *et al.*, 2011), thus questioning the relevance of tobacco for such metabolic engineering. The use of trichome specific promoters may solve this issue, or perhaps an even better solution would be to use other Asteraceae hosts which are able to accumulate sesquiterpene lactones in large quantities, such as chicory for example. Another explored strategy was the transient expression in *N. benthamiana*. This system was shown to be quite successful for the transient expression of proteins at very high levels (Marillonnet *et al.*, 2005), however the requirements for successful metabolic engineering are likely to be different. Nonetheless, transient expression of AaAS with CYP71AV1, together with HMGR to increase isoprenyl precursor supply, resulted in the production of artemisinic acid-12-β-diglucoside at levels up to 39.5 mg/kg FW (equivalent 16.6 mg/kg artemisinic acid). This indicated that artemisinic acid is indeed produced to significant levels, and highlights the importance of the host and the tissues targeted for expression. While altogether these results are promising and suggest that metabolic engineering of advanced terpenoid metabolites in plants is feasible, much progress is required to reach levels which will make commercial exploitation a reality. Many combinations of constructs with different promoters, sub-cellular targeting (plastids, cytosol, mitochondria), and hosts will have to be tested to identify the best solutions. However, in plants, even with transient expression systems, this is a highly time consuming tasks.

In comparison, micro-organisms allow a much higher throughput to test a multiplicity of constructs in a short time frame. Highly successful engineering endeavors have been achieved in *E. coli* and yeast by Keasling and co-workers. Through introduction of

mevalonate pathway genes in *E. coli*, production levels of amorphadiene of up to 0.5 g/L could be reached (Martin *et al.*, 2003; Newman *et al.*, 2006). Even artemisinic acid could be produced in *E. coli* after extensive modification of the P450 CYP71AV1 (Chang *et al.*, 2007). However the best results were obtained in yeast, where production levels of up to 100 mg/L could be reached (Ro *et al.*, 2006).

7. Conclusion

Trichomes have been used as a model to study cell differentiation and organ development in *Arabidopsis thaliana*, where the power of molecular genetics and genomics has made possible numerous advances in this area. However, Arabidopsis trichomes offer little opportunities for the development of novel products or applications, essentially because Arabidopsis trichomes are devoid of metabolic or structural properties of interest. However, trichomes play important roles in several crop species, where they are at the origins of important agricultural derived products, like cotton fibers and essential oils and fragrance ingredients of the Lamiaceae. In addition, trichome-borne resistances to insects and microorganisms in plants like tomatoes have attracted interest to restrict the use of pesticides. In those species (cotton, tomatoes, Lamiaceae), extensive EST resources were created and have proved valuable tools to identify and characterize trichome specific genes involved in development or metabolic pathways. Nonetheless, examples of trichome engineering using trichome specific promoters are still scarce and are limited to a handful of cases in cotton and tobacco. It seems that one limitation is to reach levels of productions for the metabolite of interest which are in the same range as those of endogenous metabolites. To reach those levels, it is necessary to understand more about how gene expression in those specialized cells is regulated, so as to be able to design and construct appropriate expression vectors enabling to reach these targets.

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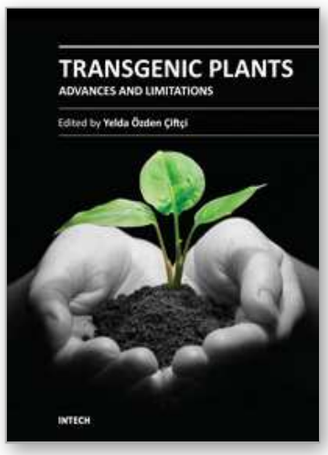
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Development of efficient transformation protocols is becoming a complementary strategy to conventional breeding techniques for the improvement of crops. Thus, Transgenic Plants - Advances and Limitations covers the recent advances carried on improvement of transformation methods together with assessment of the impact of genetically transformed crops on biosafety. Each chapter has been written by one or more experienced researchers in the field and then carefully edited to ensure throughness and consistency.

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Phone: +86-21-62489820
Fax: +86-21-62489821

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