Chapter 12

IDENTIFYING SUBSTRATES AND PRODUCTS OF ENZYMES OF PLANT VOLATILE BIOSYNTHESIS WITH THE HELP OF METABOLIC PROFILING

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Abstract: Ongoing efforts in metabolic profiling of both cultivated and wild plants continue to identify new plant compounds, many of them unique to a single species or found only in closely related species. Such compounds are defined as specialized, or secondary, metabolites and they play many physiological and ecological roles, including in plant-insect and plant-pathogen interactions. To date, only a few of the enzymatic reactions leading to the synthesis of such compounds have been elucidated and the enzymes responsible identified. Our group has concentrated on the biosynthesis of plant volatiles. We present several examples in which metabolic profiling together with gene expression profiling and biochemical methods have led to the identification of enzymes responsible for the synthesis of volatile terpenes in Arabidopsis flowers, benzenoid esters in Arabidopsis leaves, and terpenes and methylated phenylpropenes in glands of sweet basil.

Key Words: Secondary metabolites; gene expression profiling; bacterial expression system; biochemical assays; methylation; terpenes; esters; phenylpropenes.

1 INTRODUCTION

Achieving the goal of cataloguing all the components of the cell – genes, enzymes (surely the majority of the cellular proteins), and other types of proteins, and metabolites – and elucidating all the causal relationships

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among them will require a vast effort. While most biological research to date has been the piecemeal elucidation of components and causal relationships of a very small and circumscribed subset of cellular pathways, several recent approaches have been based on "systems biology" in which a very large number of components are catalogued and statistical methods are used to try to infer correlations, which in turn suggest further types of investigation (Fiehn and Weckwerth, 2003; and see this volume). This approach has been most prominent in the sequencing of whole genomes, including two plant genomes (Arabidopsis and rice) followed by computer analysis of the coding information of these genomes, and the analysis of the expression of the entire set of genes by means of DNA microarrays.

DNA, RNA, and proteins each constitute a class of compounds with some structural properties in common, thus allowing for the development of analytical methods that apply to basically all members of the class. As pointed out by Trethewey (2004), the metabolites found in the cell have no shared chemical features on which general, combined isolation-separation-identification methods can be based – at least, no such features have been recognized so far. Analysis of metabolites typically starts with some method of extraction from the tissue, and different methods have to be used to extract different classes of compounds. In the next steps, compounds have to be separated and identified, and these processes too may involve different methodologies for different groups of compounds. Current metabolic profiling techniques are primitive and allow for the extraction and separation of only a small fraction of plant metabolites and only a fraction of those have been identified. Thus, it is not surprising that at present we have probably not yet identified the majority of compounds of plant primary metabolism, and our knowledge of specialized (secondary) metabolism in any species is either severely limited or non-existent.

2 PLANT VOLATILES: CHEMISTRY AND FUNCTION

Plant volatiles constitute a small segment of the total plant metabolite output, and they do share chemical properties – mainly their volatility – that allows us to apply common analytical techniques. Plant volatiles are organic molecules (typically less than 300 Da) that often contain oxygen functionalities and sometimes nitrogen or sulfur (Figure 12-1). These compounds are lypophylic in nature and although their boiling point is typically above ambient temperature, they have high vapour pressure and therefore easily vaporize.

Plant volatiles serve many functions (Pichersky and Gershenzon, 2002; Dudareva et al., 2004). Many floral scents are emitted to attract pollinators,

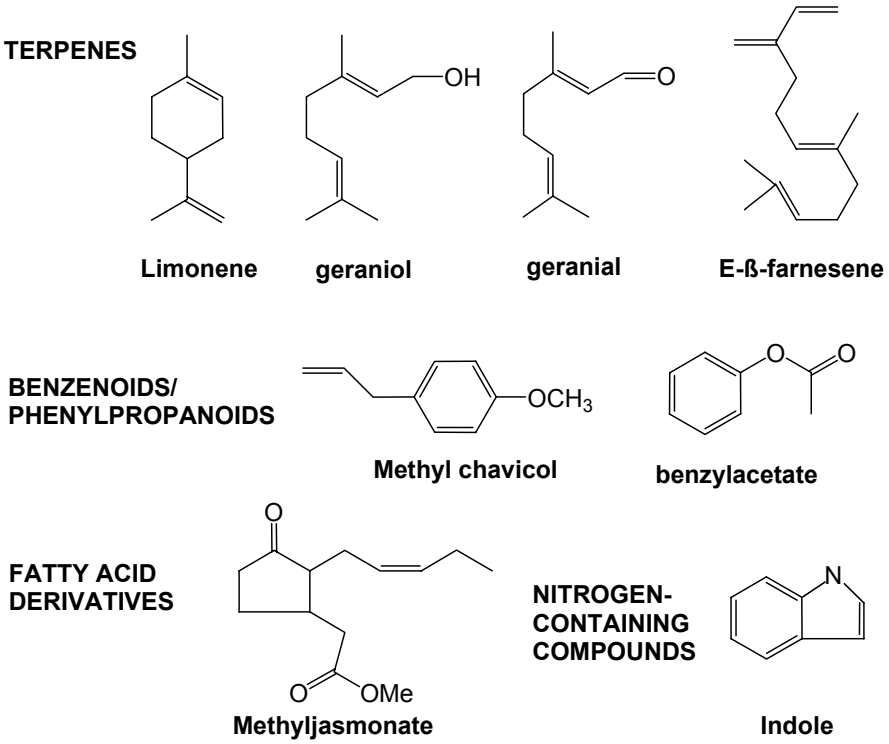

Figure 12-1. Examples of plant volatiles.

mostly but not only insects, although some odours may be used to deter unwanted visitors. Volatiles in fruits may directly attract animals, insects, or birds that eat the fruit and thereby disperse the seeds, or they may constitute a reward by contributing to the flavour. In vegetative tissues, volatiles are emitted following injuries inflicted during insect herbivory, and the emitted volatiles can attract predators of the herbivores. Some data even suggest that neighbouring plants are capable of detecting such "distress signals" and turn their own defense systems on. Finally, some volatiles are simply toxic compounds (and may be stored in specialized vegetative tissues or cells, such as glands), and exert their effect on herbivores after they are ingested when the herbivores feed on the plant. Some compounds can serve as both attractants and repellants/toxins, depending on which insect/animal is involved and even whether the same insect/animal is interacting with them through the olfactory or the digestive systems.

3 PLANT VOLATILES ARE DERIVED FROM A FEW BASIC PATHWAYS BY A LIMITED NUMBER OF MODIFICATION REACTIONS

Little is known about the pathways leading to the synthesis of the majority of plant volatiles. Our laboratories have focused on identifying and characterizing the enzymes that make volatiles and the genes encoding these enzymes. Our long-term goal has been to understand how the myriad species of the plant kingdom have evolved the ability to make so many different volatile compounds, estimated to be in the thousands. Our results, and the results from several other laboratories, have shown that while volatiles are diverse chemicals, most are derived from just a few modified biochemical pathways.

One such pathway is the terpene pathway in which a carbon skeleton is built up first into isoprene diphosphate (C5) units that are condensed into C10 or C15 diphosphate intermediates, which are finally converted into monoterpene (C10) and sesquiterpene (C15) volatiles, respectively, by enzymes encoded by a large family of genes termed terpene synthases (Figure 12-2) (larger terpenes are also produced in other branches of the pathway, but they are not generally volatiles). In contrast to the biosynthetic terpene pathway, most other volatile compounds are derived from two other classes of compounds, phenylpropanoids and fatty acids, through the shortening of a carbon skeleton, often followed by further modification, or simply by modification of the existing carbon skeleton. Compounds that are already somewhat volatile may also be modified, resulting in enhanced volatility or changed olfactory properties. The majority of these modifications involve the reduction or removal of carboxyl groups, the addition of hydroxyl groups, and the formation of esters and ethers (Figure 12-2). Each type of modification is catalysed by a group (or several groups) of related enzymes constituting protein families. Typically, these protein families contain enzymes involved in the synthesis of both volatile and non-volatile compounds. Some of our investigations into the identification of the enzymatic functions of specific members of such families are described below.

4 IDENTIFICATION OF THE ENZYMATIC FUNCTION OF MEMBERS OF THE TERPENE SYNTHASE FAMILY IN *ARABIDOPSIS THALIANA*

Until recently it was believed that *Arabidopsis thaliana*, a small weedy plant that is used as a model plant organism, produces only a few secondary

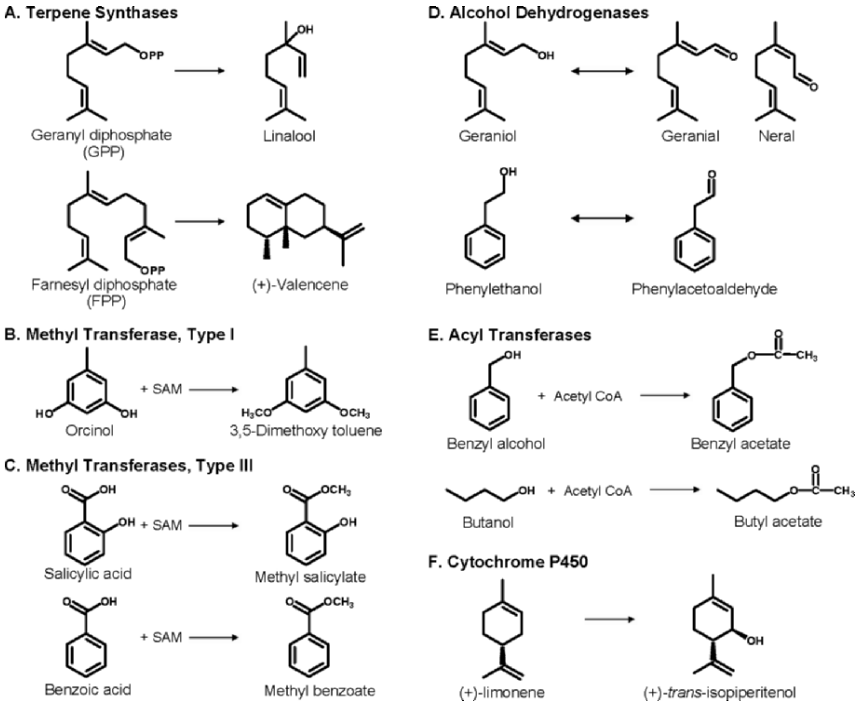

Figure12- 2. Some reactions catalysed by representatives of enzyme families.

metabolites. In particular, because it is believed to be mostly self-pollinating, it was thought to produce no floral volatiles, and casual sniffing of the flowers by the human nose does not indeed detect a noticeable bouquet. However, the recent determination of the complete genome sequence of Arabidopsis revealed about 30 genes with sequence similarity to known terpene synthases (TPSs) from other species (Aubourg et al., 2002). This observation prompted us to search more carefully for possible emission of floral volatiles. Using highly sensitive collection and detection methods, we were able to show that Arabidopsis flowers emit several monoterpenes (e.g., linalool, myrcene and limonene) and as many as 20 different sesquiterpenes (Figure 12-3A and B) (Chen et al., 2003a). The major floral volatile is β-caryophyllene, a sesquiterpene. The total emission of terpene volatiles is in the range of a few nanogram per hour per gram fresh weight of flowers, which is 2–3 orders of magnitude lower than the emission rate of some highly scented flowers (Raguso and Pichersky, 1995). Moreover, the human nose is not particularly sensitive to sequiterpenes. These two observations explain why humans cannot easily detect the scent of Arabidopsis flowers. Nonetheless, some insects might be able to detect these flowers by olfactory cues, and in fact Arabidopsis

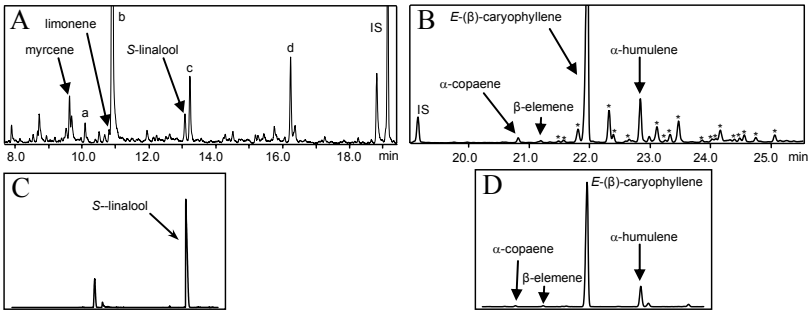

Figure 12-3. Identification of the products of two Arabidopsis terpene synthases. **A.** Gas chromatographic separation of the monoterpenes emitted by Arabidopsis flowers. **B.** Gas chromatographic separation of the sesquiterpenes emitted by Arabidopsis flowers. The amount of the internal standard (IS) is the same in both A and B chromatographs, showing the amount of the monoterpenes is much lower than the amount of sesquiterpenes. The peak labelled "a" in A is octanal, "b" is 2-ethyl-hexanol, "c" is nonanal and "d" is decanal. The peaks labeled with astericks in B are all sesquiterpenes. **C.** Gas chromatographic analysis of the product of the Arabidopsis TPS enzyme encoded by gene At1g61680, indicating that the enzyme catalyses the formation of the monoterpene S-linalool. **D.** Gas chromatographic analysis of the product of the Arabidopsis TPS enzyme encoded by gene At5g23960, indicating that the enzyme catalyses the formation of the four sesquiterpenes α-copaene, α-elemene, β-caryophyllene, and α-humulene. Unlabelled peaks in C and D are not terpenes, and are present in the control reactions as well.

flowers growing in the wild are visited by many types of insects (Hoffman et al., 2003).

Having established that Arabidopsis flowers do synthesize and emit terpenes, we next examined which of the members of the TPS gene family are involved. A set of RT-PCR experiments were carried out on all TPS genes in Arabidopsis and several genes were found to be expressed almost exclusively in the flowers. Complete cDNAs of these genes were isolated, spliced into a bacterial expression vector, and expressed in *E. coli*. The resulting proteins were assayed for activity with the substrate geranyl diphosphate (GPP), the universal precursor of monoterpenes, and farnesyl diphosphate (FPP), the universal precursor of sesquiterpenes (Figure 12-2A). These biochemical experiments identified three monoterpene synthases and two sesquiterpene synthases that are responsible for almost all of the Arabidopsis floral terpene volatiles. That only a small number of Arabidopsis TPS genes account for all the floral terpenes is explained by the observation that while some of these enzymes produced a single product (the linalool synthase is one such enzyme, Figure 12-3C), other enzymes can produce multiple products. For example, one Arabidopsis TPS gene turned out to encode a sesquiterpene synthase that catalyses the formation of four sesquiterpenes (Figure 12-3D), and another florally expressed Arabidopsis sesquiterpene synthase is responsible for the synthesis of as many as 15

compounds (unpublished). That some TPSs produce multiple products had been previously observed (Chen et al., 2003a).

In this investigation, we started with the observation that the coding capacity of the Arabidopsis genome contained potential TPS genes, but no prior information was available about terpene synthesis in Arabidopsis flowers or in any other organs (with the exception of the synthesis of gibberellins, which are diterpenes). The metabolic profiling results indicated that some of the TPS enzymes encoded in the Arabidopsis genome were likely to be active in floral scent biosynthesis. However, a simple comparison of these TPS sequences with functionally defined TPS proteins from other species was not sufficient to identify which Arabidopsis protein corresponds to each volatile (or volatiles), since an extensive body of research has demonstrated that rapid convergent evolution occurs in the terpene synthase gene family in separate plant lineages, so that, for example, the various linalool synthases known from distally related species are not very similar to each other and instead are more similar to other monoterpene synthases from the same lineage. Thus, the linalool synthase from Arabidopsis, for example, could not be identified based simply on sequence similarity to linalool synthases from other species, and the biochemical experiments were therefore crucial in identifying enzymatic functions.

5 IDENTIFICATION OF A BENZOIC ACID/SALICYLIC ACID METHYLTRANSFERASE IN *A. THALIANA*

We investigated a similar situation with regards to the biosynthesis of benzenoid methylesters in Arabidopsis. Methylsalicylate (MeSA) and methylbenzoate (MeBA) are two benzenoid methylesters that are commonly found in floral volatiles of diverse taxa, although not in flowers of Arabidopsis. The genes encoding salicylic acid methyltransferase (SAMT) and benzoic acid methyltransferase (BAMT) were first identified in flowers of *Clarkia breweri* and snapdragons, respectively (Ross et al., 1999; Murfitt et al., 2000), and were recognized to constitute a new type of methyltransferase family, designated the SABATH methyltransferase family (D'Auria et al., 2002). In Arabidopsis, there are 24 related genes (D'Auria et al., 2002; Chen et al., 2003b).

MeSA had been reported to be emitted from vegetative tissues of many plant species, including Arabidopsis, during herbivory (Van Poecke et al., 2001) or viral infection (Shulaev et al., 1997). Given that the Arabidopsis genome has 24 genes with homology to *C. breweri* SAMT, it was likely that at least one of them encodes a SAMT. However, protein sequence comparisons did not identify a single Arabidopsis gene among these 24

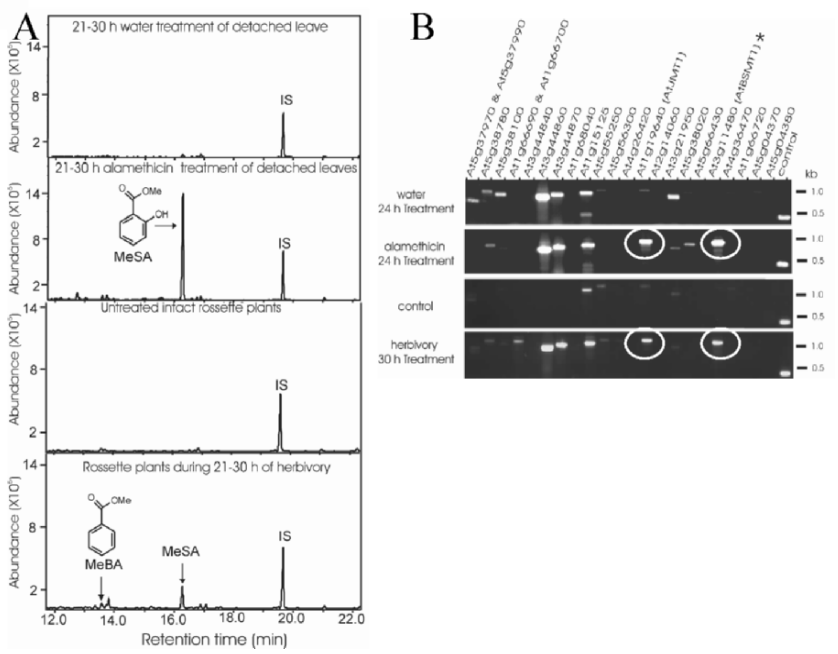

Figure12-4. Metabolic profiling and gene expression profiling to identify the AtSABATH gene involved in benzenoid methyl ester formation. **A**. Gas chromatograph analyses of plant samples under different conditions. **B**. RT-PCR gene expression profiling of all AtSABTH genes under same conditions. Circles show increased transcript levels in conditions eliciting emission of benzenoid methylesters compared with control conditions where no such emission was observed. The gene denoted with an asterisk is the one encoding the enzyme benzoic acid/salicylic acid methyltransferase (BSMT), as proven by subsequent *in vitro* enzyme assays with the purified protein.

SABATH genes which was more similar to *C. breweri* SAMT than to any other Arabidopsis gene in this family, and no Arabidopsis SABATH gene exhibited >50% identity to *C. breweri* SAMT.

Therefore, to identify the Arabidopsis SABATH gene(s) responsible for the synthesis of MeSA, we chose a combined approach of gene expression profiling with metabolic profiling. We first searched for conditions under which MeSA is emitted from Arabidopsis leaves. We examined herbivory in detail, and found out that when Arabidopsis leaves are attacked by the specialized herbivore *Plutella xylostella*, not only is MeSA emitted but also some MeBA is also released (Figure 12-4A). In addition, we established that MeSA (but not MeBA) is emitted from detached Arabidopsis leaves treated

with alamethicin, a fungal elicitor, but not from detached leaves treated with water alone (Figure 12-4A).

We next examined by RT-PCR the expression of the 24 Arabidopsis SABATH genes under these conditions, using specific oligonucleotide primer pairs for each gene. While several SABATH genes were induced during herbivory, and a few other SABATH genes were induced during alamethicin treatment, only two SABATH genes were induced under both of these treatments, and not induced in the controls (Figure 12-4B). One of these genes had previously been identified as jasmonic acid methyltransferase (JMT) (Seo et al., 2001). A full-length cDNA of the other gene was obtained, expressed in *E. coli*, and the protein shown to have the ability to catalyse the methylation of both SA and BA.

6 ENZYMES INVOLVED IN THE BIOSYNTHESIS OF PHENYLPROPENES AND TERPENES IN BASIL GLANDS

The two examples above deal with *A. thaliana*, where the task of identifying candidate genes is made easier due to the availability of the full genome sequence. However, the majority of investigations into the biosynthesis of plant volatiles, and plant secondary metabolites in general, are carried out in species for which very little genetic and genomic information is available. In such systems, metabolic profiling is still very important. In fact, metabolic profiling is usually done first as a surveying tool to identify plants of interests – those that may have interesting floral bouquets or desirable herbal spices (which are volatiles that impart distinct flavours to our foods). Once such volatiles are detected, several sets of tools are developed to make the identification, isolation, and characterization of genes possible. An example is illustrated below with our investigation into the biosynthesis of volatile flavour compounds in basil.

Basil plants have been used since antiquity to spice up food. Many cultivars of basil with distinct aromas have been bred. Basil (*Ocimum basilicum*) is in the Lamiaceae family, and is known for containing both terpenes and phenylpropenes (Figure 12-5). We have investigated in depth three such varieties, known as EMX1, SW, and SD. Metabolic profiling, done initially on material extracted from whole leaves, showed that EMX1 is particularly rich in methylchavicol (and has some methyleugenol as well), SW is rich in eugenol and linalool, and in SD the predominant volatile is citral, a mixture of geranial and neral.

Lamiaceae species, including basil, have numerous glandular trichomes, or glands, on the surface of their leaves. A very common type of gland in basil, called a peltate gland, consists of four cells connected to the epidermal

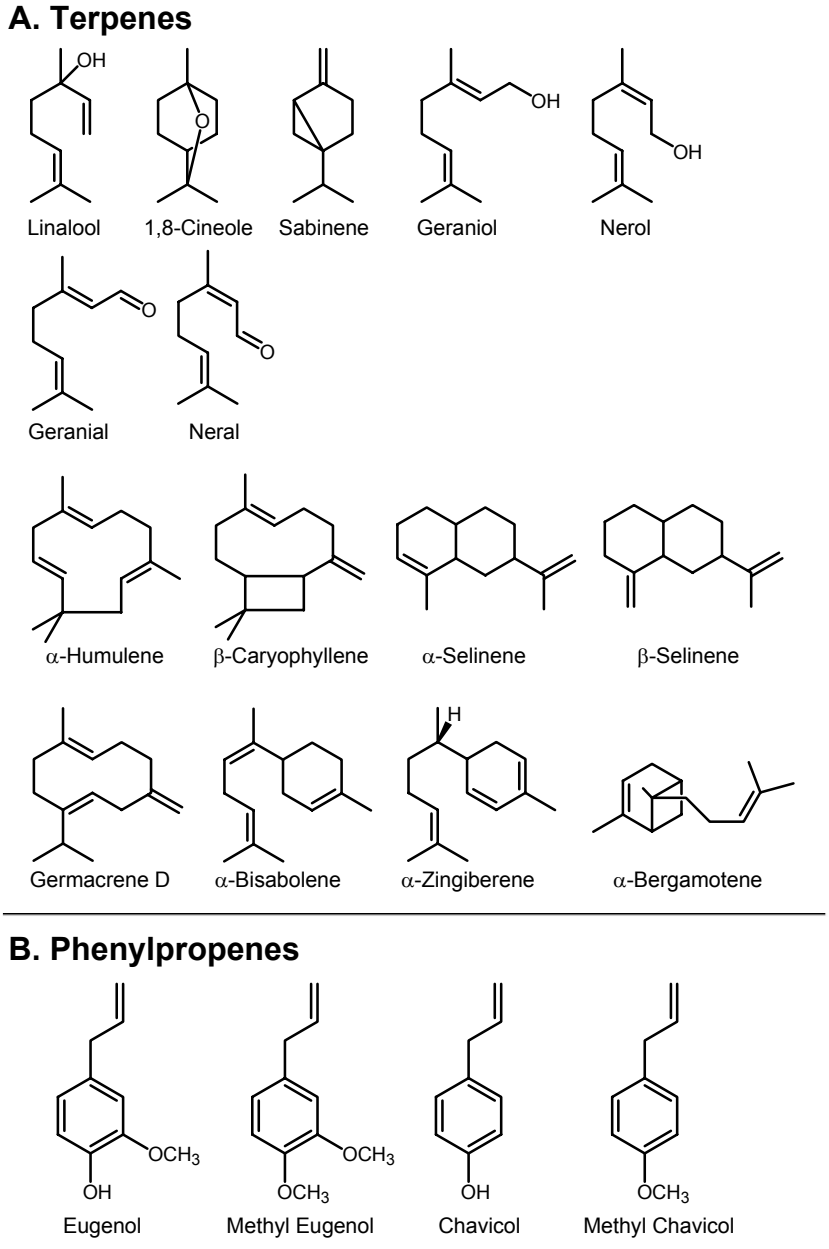

Figure 12-5. Some terpenes (**A**) and phenylpropenes (**B**) found in basil glands.

cell layer by a short-stalk cell. The four cells of the gland are covered by a thick cuticle that can expand into a "sac" to contain material secreted from the gland cells (Figure 12-6). Since previous reports showed that the volatile terpenes found in the mint plant, also in the Lamiaceae family, are synthesized and stored in the leaf peltate glands (Gershenzon et al., 2000), we examined the volatile contents of basil peltate glands by directly extracting material from individual sacs with a micropipette and analysing the material by gas chromatography-mass spectrometry (GC-MS) (Gang et al., 2001). This analysis showed that the volatiles stored in the glands were the same as those detected from whole leaf, whereas leaves devoid of peltate glands did not contain these volatiles, indicating that basil peltate glands, like mint glands, were the site of storage, and possibly synthesis, of these volatiles.

As outlined above, our basic goal is to identify the enzymes and genes responsible for the volatile biosynthesis in plants. But since no gene sequence information was available from basil plant, such information had to be obtained first. However, a genome sequencing approach is currently not feasible for every plant. To circumvent this problem, we chose to obtain sequence information from basil in a way that maximized the information on the desired genes and minimized the investment in obtaining sequence information that was not relevant to our particular interest. This was done by obtaining sequence information on genes that are specifically expressed in

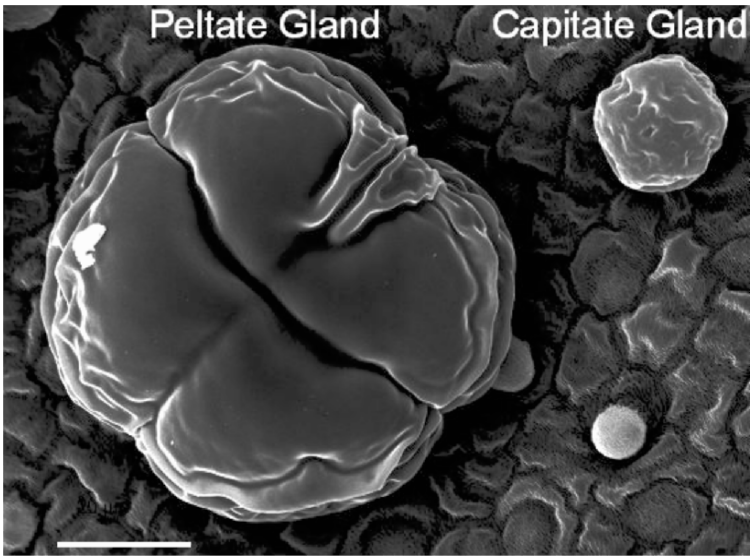

Figure 12-6. Scanning electron micrograph of a 4-celled peltate gland and a 2-celled capitate gland from the surface of a basil leaf.

the glands. To do so, we first adopted a procedure originally developed for isolating mint peltate glands to isolate basil peltate glands. Our procedure yielded intact 4-cell basil peltate glands that were completely separated from the rest of the leaf and from other types of glands, as well as devoid of gland stalks. RNA was extracted from peltate gland cells of each of the three basil cultivars, and cDNA libraries were constructed. Then, the DNA sequences of several thousand cDNAs from each library were determined and analysed for protein coding information, creating expressed sequence tag (EST) databases (Gang et al., 2001; Iijima et al., 2004).

Basil cultivar SD is rich in citral, which is the product of the oxidation of geraniol (Figure 12-2) (Iijima et al., 2004). Geraniol itself is a monoterpene alcohol, an isomer of linalool, and like linalool was believed to be synthesized from GPP, although no geraniol synthase had been identified. Examination of the EST databases of the three cultivars identified many potential terpene synthases, including several sequences that were uniquely found in the SD database. Each of the unique SD TPS sequences was expressed in *E. coli* and tested for enzymatic activity. One of them was found to be geraniol synthase, catalysing the exclusive formation of geraniol from GPP (Iijima et al., 2004).

To examine the synthesis of methylchavicol and methyleugenol in EMX1, the EST database of EMX1 was examined (Gang et al., 2002) for sequences with homology to known methyltransferases, including the enzyme that can methylate eugenol and isoeugenol in *C. breweri* flowers (Wang et al., 1997). Several sequences were identified based on this criterion, and in addition these sequences were not prevalent in the other two EST databases. Enzymatic assays of *E. coli*-produced proteins showed one of them to be eugenol methyltransferase, and another to be chavicol methyltransferase. Both proteins were highly similar to each other (>90% identical), but while they had some sequence similarity to *C. breweri* isoeugenol/eugenol methyltransferase (and no similarity at all to SAMT), they were more similar to other methyltransferases such as isoflavone methyltransferase. This observation indicates that the enzymes that methylate phenylpropenes in basil and *C. breweri* must have evolved their substrate specificity independently.

7 CONCLUSIONS

Metabolic profiling of whole plants or selected tissues and even cell types, combined with detailed information on the sequence of specific genes whose expression is correlated with the production of specific metabolites, is a powerful approach to identifying candidate genes involved in the biosynthesis of such metabolites. However, final identification of enzymatic

function must be achieved by biochemical experiments in which the candidate proteins are shown to possess the postulated catalytic activities.

REFERENCES

Aubourg, S., Lecharny, A., and Bohlmann, J., 2002, Genomic analysis of the terpenoid synthase (AtTPS) gene family of Arabidopsis thaliana, *Mol. Genet. Genomics* **267**:730–745.

Chen, F., Tholl, D., D'Auria, J.C., Farouk, A., Pichersky, E., and Gershenzon, J., 2003a, Biosynthesis and emission of terpenoid volatiles from Arabidopsis flowers, *Plant Cell* **15**:481–494.

Chen, F., D'Auria, J.C., Tholl, D., Ross, J.R., Gershenzon, J., Noel, J.P., and Pichersky, E., 2003b, An Arabidopsis thaliana gene for methylsalicylate biosynthesis, identified by a biochemical genomics approach, has a role in defense, *Plant J.* **36**:577–588.

D'Auria, J.C., Chen, F., and Pichersky, E., 2002, The SABATH family of methyltransferases in Arabidopsis thaliana and other plant species, in: *Recent Advances in Phytochemistry*, Elsevier Science Ltd, Oxford, vol. 37. pp. 253–283.

Dudareva, N., Pichersky, E., and Gershenzon, J., 2004, Biochemistry of plant volatiles, *Plant Physiol* **135**:1893–1902.

Fiehn, O. and Weckwerth, W., 2003, Deciphering metabolic networks, *Eur. J. Biochem.* **270**:579–588.

Hoffmann, M.H., Bremer, M., Schneider, K., Burger, F., Stolle, E., and Moritz, G., 2003, Flower visitors in a natural population of Arabidopsis thaliana, *Plant Biology* **5**:491–494.

Gang, D.R., Wang, J.H., Dudareva, N., Nam, K.H., Simon, J.E., Lewinsohn, E., and Pichersky, E., 2001, An investigation of the storage and biosynthesis of phenylpropenes in sweet basil, *Plant Physiol.* **125**:539–555.

Gang, D.R., Lavid, N., Zubieta, C., Chen, F., Beuerle, T., Lewinsohn, E., Noel, J.P., and Pichersky, E., 2002, Characterization of phenylpropene O-methyltransferases from sweet basil: Facile change of substrate specificity and convergent evolution within a plant OMT family, *Plant Cell* **14**:505–519.

Gershenzon, J., McConkey, M.E., and Croteau, R.B., 2000, Regulation of monoterpene accumulation in leaves of peppermint, *Plant Physiol.* **122**: 205–213.

Iijima, Y., Gang, D.R., Fridman, R., Lewinsohn, E., and Pichersky, E., 2004, Characterization of geraniol synthase from the peltate glands of sweet basil (Ocimum basilicum), *Plant Physiol.* **134**:370–379.

Murfitt, L.M., Kolosova, N., Mann, C.J., and Dudareva, N., 2000, Purification and characterization of S-adenosyl-L-methionine: Benzoic acid carboxyl methyltransferase, the enzyme responsible for biosynthesis of the volatile ester methyl benzoate in flowers of Antirrhinum majus, *Arch. Biochem. Biophys.* **382**:145–151.

Pichersky, E. and Gershenzon, J., 2002, The formation and function of plant volatiles: perfumes for pollinator attraction and defense, *Curr. Op. Plant Biol.* **5**:237–243.

Raguso, R.A. and Pichersky, E., 1995, Floral volatiles from Clarkia breweri and C. concinna (Onagraceae): recent evolution of floral scent and moth pollination, *Plant Sys. Evol.* **194**:55–67.

Ross, J.R., Nam, K.H., D'Auria, J.C., and Pichersky, E., 1999, S-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase, an enzyme involved in floral scent production and plant defense, represents a new class of plant methyltransferases, *Arch. Biochem. Biophys.* **367**:9–16.

Seo, H.S., Song, J.T., Cheong, J.J., Lee, Y.H., Lee, Y.W., Hwang, I., Lee, J.S., and Choi, Y.D., 2001, Jasmonic acid carboxyl methyltransferase: A key enzyme for jasmonate-regulated plant responses, *Proc. Natl. Acad. Sci. USA* **98**:4788–4793.

Shulaev, V., Silverman, P., and Raskin, I., 1997, Airborne signalling by methyl salicylate in plant pathogen resistance, *Nature* **386**:738–738.

Trethewey, R.N., 2004, Metabolite profiling as an aid to metabolic engineering in plants, *Curr. Op. Plant Biol.* **7**:196–201.

Van Poecke, R.M.P., Posthumus, M.A., and Dicke, M., 2001, Herbivore-induced volatile production by Arabidopsis thaliana leads to attraction of the parasitoid Cotesia rubecula: Chemical, behavioral, and gene-expression analysis, *J. Chem. Ecol.* **27**:1911–1928.

Wang, J., Dudareva, N., Bhakta, S., Raguso, R.A., and Pichersky, E., 1997, Floral scent Production in Clarkia breweri (Onagraceae). II. Localization and developmental modulation of the enzyme SAM:(Iso)Eugenol-O-methyltransferase and phenylpropanoid emission, *Plant Physiol.* **114**:213–221.