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Wissen zu erwerben, ohne über das Erlernte nachzudenken, ist sinnlos; nur nachzudenken, ohne zu lernen, führt zu gefährlichen Überlegungen.

(Konfuzius)

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This work is dedicated to my parents
Maria and Alois
and I feel somewhat ashamed that I write it in English
which both do not read or understand.

1. Summary and Rationale

Plant natural product biosynthesis is probably the most versatile and flexible biochemical system on earth. Glycosylation and methylation significantly contribute to the large diversity of the abundance of natural products. This project was initiated to understand and characterize the accumulation of a unique class of glycosylated plant compounds, the betacyanins, which have replaced the anthocyanins as fruit and flower pigments in the majority of the Caryophyllales. Two model plants, *Dorotheanthus bellidiformis* (livingstone daisy) and the halophyte *Mesembryanthemum crystallinum* (ice plant) were chosen due to the unique features of betacyanin accumulation in cell cultures and epidermal bladder cells, respectively. During these investigations not only the accumulation of these and accompanying flavonoid pigments was investigated, but purification techniques for several regio- and substrate specific enzymes involved in the glucosylation or methylation of betacyanin and flavonoids were established, the corresponding genes were cloned and the respective recombinant enzymes characterized. Substrate specificity and sequence alignments of these enzymes provided evidence that betacyanin biosynthesis was likely developed after the structures of flavonoids, including anthocyanins had already been established. The observed parallel accumulation of betacyanin and flavonoid conjugates in the bladder cells of the ice plant led to the identification of a novel subset of cation-dependent *O*-methyltransferases with a broad substrate specificity including glucose esters. This observation contradicts the current perception that these enzymes in plants are only involved in the methylation of the lignin monomers caffeoyl- and 5-hydroxy feruloyl coenzyme A, respectively.

To understand the substrate specificity and the properties of both types of enzymes it was of utmost importance to obtain information on the enzyme structures and reaction mechanisms. Initial attempts to crystallise a betanidin 5-*O*-glucosyltransferase, were hampered by its low abundance in heterologous systems and the resulting multistep purification procedure. Therefore, an alternative approach was used with a combination of sequence similarity search, site-directed mutagenesis, and computer-assisted molecular modelling to resolve the first 3-D structure of a plant natural product glucosyltransferase. A plausible reaction mechanism for this enzyme could be established to explain the inversion of configuration of the attached sugar from α-linked in the donor UDP-glucose to a β-linkage in the glucosylated plant natural product. In case of PFOMT, the novel cation-dependent *O*-methyltransferase discovered during this work from the ice plant, the high yields of recombinant protein combined with the rapid success in crystallisation facilitated our efforts to establish a crystal structure for this subcluster of methyltransferases.

2. Introduction

Sessile plants were forced during evolution to develop effective mechanisms to detoxify all kinds of reactive and toxic compounds within their cells and to repulse possible predators by endogenous chemicals, rather than rely on kinetic and mechanical strength. They have achieved both goals by synthesis, modification and storage of a wide array of natural compounds including alkaloids, cyanogenic glucosides, glucosinolates, phenolics and terpenoids, to name only the most prominent groups. Research in plant natural product biosynthesis has been focussed on the identification and synthesis of these compounds for more than a century. The isolation of morphine by Wilhelm Sertürner as early as 1806, and the chemical synthesis of indigo by Adolf von Baeyer in 1878 are hallmarks of plant derived biochemistry with a tremendous scientific and socio-economic impact to the 21st century.

The parallel development of biochemical and molecular tools during the last 20 years has allowed an in-depth characterisation of plant natural product biosynthetic enzymes, the corresponding genes and an array of regulatory elements organizing the biosynthesis and storage of metabolites (Dixon, 1999). Although much of the research on plant natural product biosynthesis is still at the level of gene discovery, future steps are already taken combining experimental and computer-based analysis of model plants, like *Arabidopsis thaliana* (http://www.arabidopsis.org/tools/aracyc). The abundance of plant natural products can be reduced to only a few biosynthetic pathways and to a few simple starter-molecules originating from primary metabolism. The polyketide- and the shikimate-pathway initiate a variety of target structures from activated acetate units and from the two aromatic amino acids phenylalanine and tyrosine, respectively. In addition, acetate/mevalonate and glycerin-aldehyde 3-phosphate/pyruvate are considered as precursors of terpenoids (Gershenzon and Kreis, 1999; Lichtenthaler, 1999).

The first step to achieve this diversity is generated by ligations, cyclisations and oxidations and already results in arrays of core structures. These are further diversified by a second armada of enzymes, mostly members of the transferase families, including glycosyl- (and glucuronosyl-), methyl-, acyl-, sulfo-, or prenyltransferases. Single or successive modifications by enzymes of these superfamilies result in the observed myriads of natural products, which are not only characteristic for the plant, the fungal, or the microbial kingdom, but even enable the identification of individual species and subspecies by a quantitative or qualitative metabolite pattern (Harborne and Turner, 1984; Watermann and Gray, 1988).

The array of plant natural products is the result of evolutionary diversification of genes and the corresponding enzymes. Changing environmental conditions with a tremendous array

of potential substrates provide the most challenging tests for any enzymatic system. The impact of evolutionary and environmental factors are reflected in the development of large gene families or gene clusters from a single or a few hypothetical ancestors (Moore and Purugganan, 2005). In the genome of *A. thaliana* more than 100 glycosyltransferase (GT) like sequences have been identified (Li et al., 2001) and similar numbers of these enzymes are expected for other plant species. These and other modifying enzymes may hold essential keys for the survival of plants under changing environmental conditions (Lu and Rausher, 2003). Their natural variation and functional redundancy may be compared to the large diversity of antibodies in the vertebrate immune system (Cannon et al., 2004), although immunological reaction cascades appear more complex and target whole cells or proteins rather than small molecules.

Our research is focused on two superfamilies of these enzymes, the plant glycosyl- and methyltransferases (referred to as GTs and MTs). With an emphasis on *O*-glucosylation and *O*-methylation the progress on structural and functional aspects our and other laboratories have made throughout the last decade will be discussed, despite initial difficulties associated with the isolation and characterization of both types of enzymes. Similar enzymes exist in animals, fungi and microbial systems and are referenced sporadically throughout this report (Axelrod and Vesell, 1970; Lampe et al., 1999).

3. Identification and Purification of Transferases

The properties of natural compounds are in part the result of hydrophilic and hydrophobic modifications governed by glycosylation or methylation, respectively. In addition, sulfatation, acylation, and prenylation further enhance the structural complexity and influence the properties and location of the corresponding conjugates.

Glycosylation is considered as one of the final modifications in the biosynthesis of many compounds. Glycosides of all major classes of secondary metabolites, i.e. phenylpropanoids, terpenoids, alkaloids, thiocyanides, cyanohydrins, and steroids have been identified (Figure 1). The large number of naturally occurring glycosides does not correlate with a detailed knowledge of the corresponding enzymes leading to their formation, the glycosyltransferases. Except for their well-documented role in detoxification of xenobiotics and their ability to increase the hydrophilicity of hydrophobic or amphiphilic aglycones, the function of the variety of glycosyltransferases in plant secondary metabolism remained poorly characterized (Jones and Vogt, 2001).

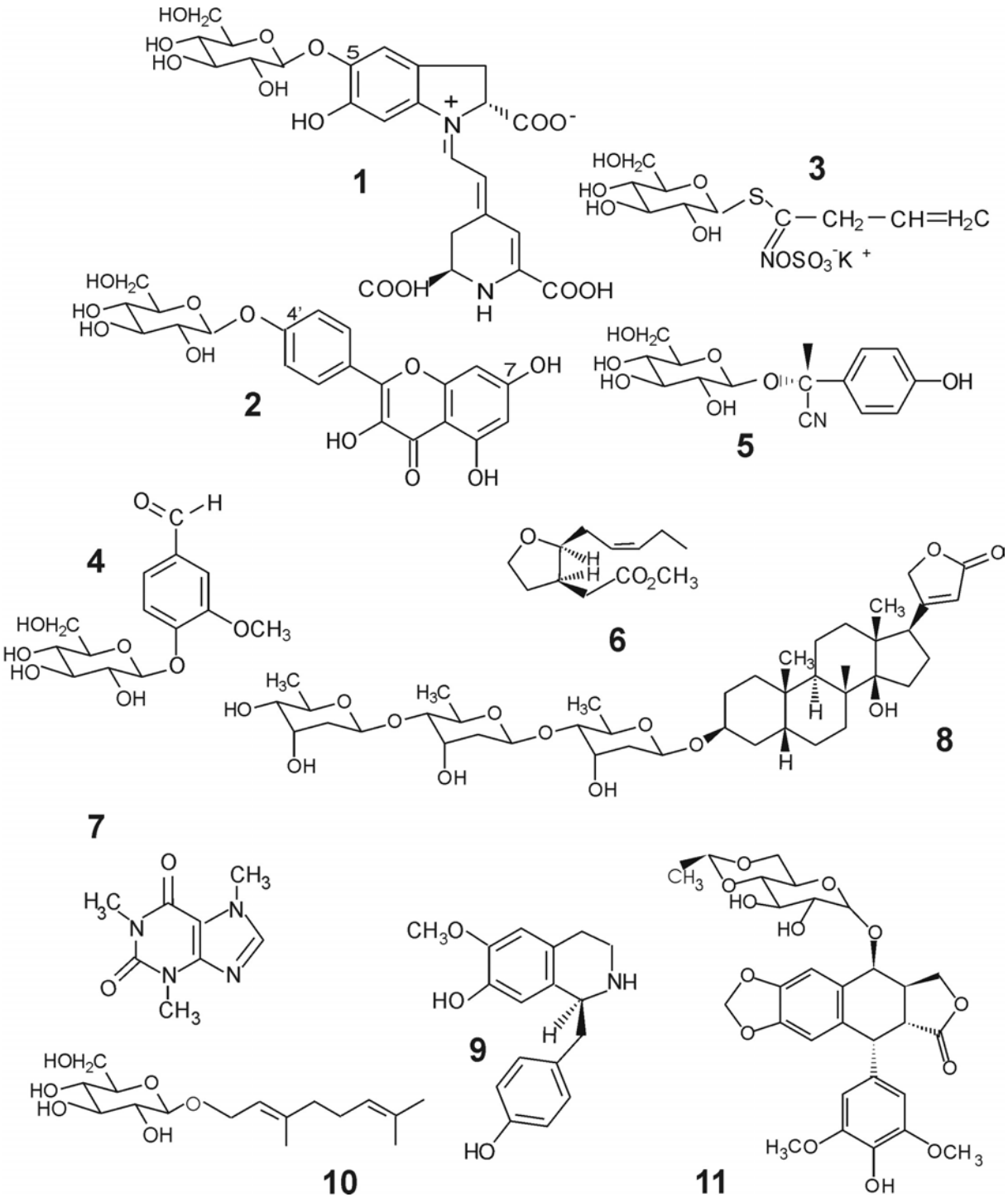

Figure 1 Structures of selected glycosylated and methylated natural products. Betacyanins such as betanin (**1**), flavonols such as quercetin 4′-*O*-glucoside (**2**), glucosinolates such as sinigrin (**3**), phenolics such as vanillin glucoside (**4**), cyanogenic glucosides such as dhurrin (**5**), fatty acid derivatives such as methyl jasmonate (**6**), purine alkaloids such as caffeine (**7**), cardiac glycosides such as digitoxin (**8**), tetrahydrobenzylisoquinoline alkaloids such as coclaurine (**9**), monoterpenes such as geraniol glucoside (**10**), lignans such as etoposide (**11**).

Only in the last decade many GTs have been characterized and associated with individual pathways based on functional expression, substrate specificity, and plant model systems (summarized in several reviews, e.g. Vogt, 2000; Lim and Bowles, 2004).

Methylation of natural compounds in plants is performed by *S*-adenosyl-L-methionine (AdoMet) dependent methyltransferases (MTs) and is a characteristic feature of most secondary metabolites like phenylpropanoids, alkaloids or terpenoids (Figure 1). Plant MTs are able to methylate four different polarized nucleophiles (*O*, *N*, and *S*) or activated C-atoms (carbanions). *O*-Methyltransferases (OMTs) act on a wide variety of target structures and can be classified as either cation-dependent (class I) or cation-independent (class II) proteins (Joshi and Chiang, 1998). Irrespectively of their substrates and specificity, class II enzymes comprise a family of dimeric proteins with an average molecular mass of 40-60 kDa per monomer (Ibrahim and Muzac, 2000). A third class of *O*-methylating enzymes without any cation-dependence has recently been indroduced and termed *SABATH*-enzymes (based on the first two initials of the plants they were found in). These are involved in the formation of volatile aromatic carboxymethylated compounds, like methyl salicylate or methyl benzoate (Ross et al., 1999; Pott et al., 2004). These OMTs share more sequence identities to some *N*-methyltransferases, like theobromine synthase than to any other class II OMTs and therefore, were positioned into a separate subcluster.

In contrast to the universal array of substrates of cation-indepedent enzymes, class I OMTs apparently serve only one function in plants and animals: the methylation of vicinal dihydroxy systems. In animals, catechol *O*-methyltransferase (catechol OMT) inactivates aromatic neurotransmitters in the brain and mutagenic phenolics in the liver and kidney (Mannisto and Kaakkola, 1999; Zhu et al., 1994). In plants, the corresponding enzymes, due to their preferred substrate, are referred to as caffeoyl coenzyme A OMTs (CCoAOMT) and are part of a complex grid of enzymatic reactions to build the structure of plant lignin, besides cellulose the most prominent polymer on earth (Humphreys and Chapple, 2002). Their involvement in the methylation of complex pigment conjugates has recently been established by our results and suggests additional roles of these class I OMTs in plants (Ibdah et al., 2003). Whereas the animal enzymes are considered monomeric, the plant enzymes have a dimeric structure with a monomeric molecular weight between 25 and 30 kDa (Schmitt et al., 1991; Ferrer et al., 2005).

Methylation and glycosylation in plants are usually associated with inactivation or detoxification of natural compounds rather than promoting their biological activities. This is illustrated by a most intriguing example, the cyanogenic glucosides (Conn, 1980), where only

the association of the bound sugar precludes the liberation of the toxic cyanide. A recent report emphasizes the stabilization of cytokinins by glucosylation to prevent these hormones from degradation by oxidases/dehydrogenases (Mok et al., 2005). On the other hand, modification of physical properties by methylation can result in the release of biologically active scents and flavours (Dudareva et al., 2004) or enable the free diffusion of plant hormones, like jasmonic acid (Reinbothe et al., 1994). In coffee beans and in many other plants, several *N*-methylations of xanthosine essentially lead to the formation of the purine alkaloid caffeine, the most prominent legal, bioactive drug (Uefuji et al., 2003; Misako and Kouichi, 2004). In some cases, glycosylation or methylation may have no effect on the properties of biologically active compounds but can be used *in vitro* to specifically label active molecules and decipher their biological function (Vogt et al., 1995; Xu et al., 1997).

The first reports describing the transfer of a sugar moiety from UDP-glucose to natural products were already published in the late 50´s (Hutchinson et al., 1958; Cardini and Yamaha, 1958; Yamaha and Cardini, 1960). A few years later, *S*-adenosyl-L-methionine dependent *O*-methylation of caffeic acid was discovered in plant tissues (Finkle and Nelson, 1963; Legrand et al., 1976). In subsequent reports, the characterisation of purified transferase activities was rendered quite difficult due to the low abundance of these enzymes in plant tissues and the restraint availability of reference compounds to characterize the substrate specificity. Partly purified proteins were characterized based on enzyme properties or, if purified further, to obtain antibodies for cellular localisation (Latchinian-Sadek and Ibrahim, 1991; Hrazdina, 1992).

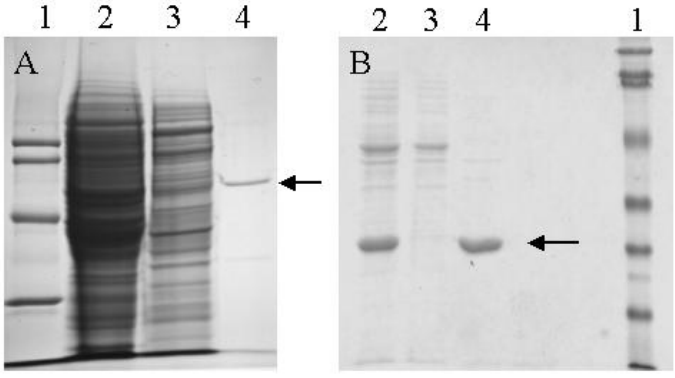

Figure 2 Efficient use of affinity matrices: **A** Purification of native betanidin 6-*O*-glucosyltransferase by dye ligand chromatography on Reactive Yellow 3 (from Vogt et al., 1997). **B** Heterologously expressed CCoAOMT from *Ammi majus* purified by metal affinity chromatography on Talon (from Lukačin et al., 2004). Purification was achieved although the protein does not contain any HisTag. 1, molecular weight markers; 2, crude extracts; 3A, after ion-exchange chromatography; 3B, 10 mM imidazole wash; 4A, 1 M UDP-glucose eluate; 4B, 30 mM imidazole eluate. Arrows indicate the positions of purified proteins.

The purification and unequivocal identification of many transferases was facilitated by the use of affinity or pseudo-affinity matrices, i.e. glucosyltransferases can be sometimes purified in a single step by dye-ligand chromatography (Vogt et al., 1997; Jones et al., 1999; Figure 2), or metal affinity chromatography (Marcinek et al., 2000). Methyltransferase purification was simplified by *S*-adenosyl-L-homocysteine agarose or by adenosine agarose (Sharma and Brown, 1979; Cacace et al., 2003). Recently, our lab developed a purification for cation-dependent OMTs based on immobilized-metal affinity chromatography which was originally established to capture and purify recombinant His-tagged proteins (Lukačin et al., 2004; Figure 2).

DNA-sequencing, combined with rapid developments in mutational analysis and molecular cloning lead to the identification and functional characterisation of several transferases, e.g. the annotation of the *bronze-1* locus in *Zea mays* as a glucosyltransferase encoding gene (Fedoroff et al., 1984) and to the first description and cloning of a plant class I CCoAOMT from tobacco (*Nicotiana tabacum*) (Schmitt et al., 1991). In parallel, improved recovery of proteins combined with enhanced sensitivity to obtain amino acid sequence information enabled the identification of elusive plant GTs and other rare proteins (Ziegler et al., 1997; Matsudaira, 1991; Grimm and Eckerskorn, 1996). Only recent developments in plant biochemistry begin to stress the important role of these modifying enzymes compared to the so-called "key enzymes" of plant natural product biosynthesis, e.g. phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), or hydroxymethylglutaryl (HMG)-CoA reductase.

4. Substrate Specificity

The most obvious questions to be asked about any transferase are two very simple ones: What are their substrates? How specific are the individual enzymes? Earlier reports were often forced to work with only partly purified proteins or crude cell extracts, which were not suited to unequivocally correlate one individual enzyme with the observed specificity. Only by recombinant techniques developed throughout the last decade a more thorough investigation of the specificities was accessible.

To answer the second question first, plant GTs and MTs are quite specific for the individual sugar or methyl group donor, respectively. Especially OMTs use exclusively AdoMet as the substrate donor, whereas in case of the plant GTs an apparent specificity for UDP-glucose is observed. In a few reports, UDP-glucose was only the preferred rather than

the solely accepted sugar donor (Taguchi et al., 2001; Sasaki et al., 2004). In several cases rhamnosyl-, galactosyl-, xylosyl- or even glucuronosyltransferases have also been described (Brugliera et al., 1994; Frydman et al. 2004; Miller et al., 1999; Martin et al., 1999; Schulz and Weissenböck, 1988). The imbalance in favour of the GTs is only in part due to the preferred modification of natural compounds by glucose, but also resides in the limited experimental access of available activated sugars other than glucose, galactose, rhamnose, or xylose. This is evident in case of the pharmaceutically important foxglove (*Digitalis*) species (Figure 3), where only the final glucosylation step in the formation of bioactive cardiac glycosides has been characterized. In contrast, the attachment of the pharmacologically important dideoxy sugars, like digitoxose, has only been described yet for micro-organisms (Albrecht, 1999; Trefzer et al., 1999; Thorson and Vogt, 2003).

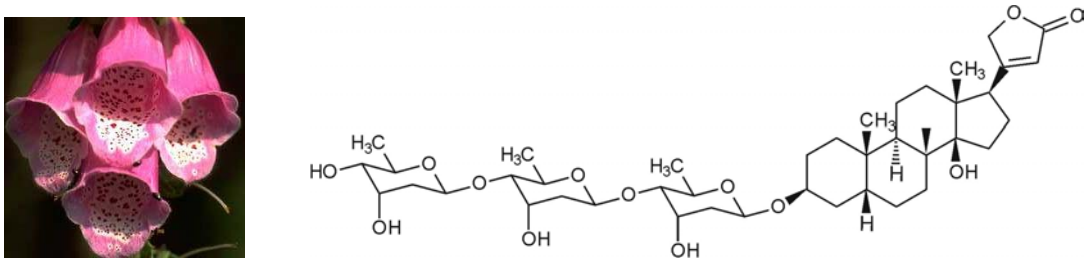

Figure 3 The pharmacologically active cardiac glycoside digitoxin, characteristic for several *Digitalis* species, like *Digitalis purpurea* (**left**).

In contrast to plants, microbial GTs are often observed to be less specific for the individual sugar (Blanco et al., 2001). This is in part compensated by their strong preference for the sugar acceptor, which is usually not observed for plant GTs (Jones and Vogt, 2001). If this specificity has somehow evolved together with the potent antibiotic properties of many microbial products, is an intriguing question (Trefzner et al., 1999; Thorson and Vogt, 2003). In plants, glycosylation like methylation is seen as a "tranquilliser" with a lower bioactivity of the glycosylated product compared to the glycoside. From the few known glycosylated plant-derived and biologically active compounds like the saponins, the attachment of several hydrophilic sugars, rather than of one specific sugar is responsible for the observed detrimental effect on membranes (Osbourn, 1996; Kasai et al., 1999). Apparently, procaryotes and eucaryotes have evolved two different strategies to detoxify or inactivate harmful compounds.

In the last decade it became increasingly evident that functional characterisation of either GTs or MTs should strictly differentiate between *in vitro* and *in vivo* properties of

characterized proteins. *In vitro* specificities are only limited by the supply of possible substrates. True *in vivo* functions of these proteins, however, are much more difficult to access. We and other have repeatedly emphasized this important point.

Cloning and functional expression of the *p*-hydroxymandelonitrile GT leading to the formation of dhurrin *in vivo* proved the remarkable specificity of this enzyme towards the *p*-hydroxylated mandelonitrile as compared to the similar *p*-hydroxymandelic acid and similar structures *in vitro* (Jones et al., 1999). In contrast, the same enzyme *in vitro* also converts structurally very different hydroxylated terpenoids, like geraniol, to the corresponding glycoside, although with a lower catalytic efficiency (Jones et al., 1999; Hansen et al., 2003). Although the observed kinetic properties suggest that the *in vitro* and *in vivo* substrates are identical, which was proven using *A. thaliana* as a host plant for the entire dhurrin biosynthetic pathway (Tattersall et al., 2001), the flexibility of these enzymes *in vitro* is striking.

The red-violet betacyanins have replaced the anthocyanins as pigments in the majority of the Caryophyllales (Strack et al., 2003), but only recently the biosynthetic pathway based on the presence of a novel 4,5-extradiol cleaving enzyme, the DOPA 4,5-dioxygenase has been resolved (Christinet et al., 2004). The question wether glycosylation takes part before or after the spontaneous condensation of betalamic acid with *cyclo*-DOPA in the biosynthetic pathway stimulated our own interest in this pathway and still is an ongoing debate (Vogt, 2002; Sasaki et al., 2004). Two alternative routes have been proposed and *in vitro* experimental data for two distinct, heterologously expressed and functionally characterized subgroups of enzymes were presented (Heuer et al., 1996; Vogt et al., 1999a; Sasaki et al., 2004, 2005). In livingstone daisy (*Dorotheanthus bellidiformis*), two enzymes specific for the aglycone betanidin have been described, which also glucosylate a variety of flavonoids with similar catalytic efficiency as the substrate betanidin (Vogt et al., 1997; Vogt, 2002). A homologous set of enzymes was observed in red beet (*Beta vulgaris*), with identical properties towards the tested flavonoids, but with a strong preference for flavonols as compared to betanidin (Isayenkova J, Wray V, Strack D, Vogt T, in preparation). In contrast, two distantly related enzymes described from two related species *Mirabilis jalapa* and *Celosia argentea* (Sasaki et al., 2004, 2005), promote the glucosylation of *cyclo*-DOPA. The problem therefore persists, whether glucosylation *in vivo* is performed at the betanidin or at the *cyclo*-DOPA level or two parallel routes exist. Unless knockout mutations or *in vivo* functional complementation do not oppose the observed specificities, it seems plausible, that in this

monophyletic pathway, present in only 2% of all vascular plants, specificity may have evolved two times independently from an existing pool of transferases.

The specificities of class II OMTs of *Thalictrum tuberosum* associated with isoquinoline alkaloid biosynthesis *in vivo*, are also not restricted to the corresponding substrates. Methylation of a variety of phenolic substrates *in vitro* was observed (Frick and Kutchan, 1999). A total of six different alleles may have arisen by gene duplication and the specificity of two of those enzymes investigated resides in the change of a single amino acid, tyrosine instead of cysteine. In their search for 16-hydroxy tabersonine MT of *Catharanthus roseus* Schröder and co-workers purified four class II OMTs with activities towards this alkaloid. Unexpectedly, with the recombinant enzymes no activity towards the alkaloid was observed, but two sequential methylations of the common flavonol myricetin (Cacace et al., 2003). Either the specificity of the heterologously expressed enzymes has changed or, if both activities belong to different enzymes which co-eluted in all chromatographic steps, the biochemical similarity between alkaloid and flavonol methylating OMTs in this plant is remarkable. That this similarity may sometimes be misleading can be exemplified for a pair of regiospecific isoflavone daidzein 7- and 4´-OMTs. The 7-OMT was originally proposed to catalyse the methyl transfer also to the 4´- group of daidzein *in vivo* (Liu and Dixon, 2001). Only by cloning of the "correct" enzyme it was proven that two separate enzymes exist and daidzein biosynthesis requires a specific 4´-OMT *in vitro* and *in vivo* (Akashi et al., 2003).

The 5-hydroxyferulate/caffeic acid OMT from alfalfa (*Medicago sativa*) involved primarily in the formation of syringyl lignin has a much broader specificity than initially anticipated. This multifunctional enzyme methylates the hydroxyl group in meta position of small benzaldehyde like aromatics more efficiently than caffeic acid, although the position specificity remains the same (Kota et al., 2004). In contrast, other class II enzymes may retain their high specificity *in vitro* and *in vivo*. Eugenol and chavicol OMT show a conserved high substrate specificity and are both expressed in the same tissue, in basil peltate glands (Gang et al., 2002).

These few examples emphasize that care should be taken to predict the substrate specificity of any GT and MT in question. Knockout mutations combined with complementation studies, RNAi-mediated gene silencing and metabolite profiling may be required to elucidate the true *in vivo* role of a given enzyme. A final example, selected from our own work on class I OMTs, illustrates several additional problems associated with *in vitro* versus *in vivo* specificity. These include redundancy of similar proteins with different

specificities, posttranslational *in vivo* modifications and artificially high *in vitro* ratios of substrates compared to enzyme quantities.

In addition to betacyanins, the ice plant (*Mesembryanthemum crystallinum*) accumulates large amounts of glycosylated and methylated flavonoid conjugates in epidermal tissues (Vogt et al., 1999b; Ibdah et al., 2002). Methylation is performed by a unique class I cation-dependent CCoAOMT (termed PFOMT), which displayed a broad substrate specificity towards various flavonoids as well as caffeic acid and its conjugates (Ibdah et al., 2003). This broad specificity was in contrast to previously reported caffeoyl CoA-specific properties of these highly conserved enzymes involved in lignin modification (Pakusch et al., 1989; Maury et al., 1999). At least two subsets of functionally active enzymes of the same subgroup exist in many species (Ibdah et al., 2003). In the databases all of these enzymes are classified as CCoAOMTs involved in lignin biosynthesis. Therefore, it is not only difficult to predict the specificity based on sequence data, but to compare the observed substrate promiscuity *in vitro* with the *in vivo* situation. Are there any developmental or spatial separations of these activities *in vivo*? PFOMT has the unique property to methylate caffeoylglucose *in vitro* and could be involved in the methylation of ester *in vivo*. The product feruloylglucose could then be the potential acyl donor for the abundant feruloylated betacyanin conjugates in epidermal bladder cells (Bokern et al., 1991; Vogt et al., 1999). A second, substrate-specific CCoAOMT already cloned from the ice plant may then only be involved in caffeoyl- and 5-hydroxy feruloyl CoA methylation in vascular tissues, resulting in a strict tissue specificity. However, the situation is further complicated, since in *A. thaliana* tissues (and most likely in the ice plant) one member of the class II OMTs is described with the same overlapping substrate preferences as both class I enzymes (Muzac et al., 2000, T. Vogt, unpublished). The corresponding transcripts of all these class I and class II OMT-genes could already be detected in *A. thaliana* plants (Vogt, unpublished). Whether the corresponding enzymes are active in the same tissues or are spatially separated remains to be proven by further transcript analysis and immunolocalization studies.

A further level of complexity can be reached by post-translational modifications. Upon sequencing of the native PFOMT, an N-terminal truncation of this protein has been observed (Ibdah et al., 2003) which may lead to an altered substrate specificity (Vogt, 2004). Consistent with the subsequent methylation properties of the native enzyme, only 6- and 3′-*O*-methyl derivatives of the endogenous quercetagetin were found in epidermal tissues (Vogt et al., 1999b). In contrast, the "full-length" recombinant enzyme also methylates the 5-hydroxy group of quercetagetin (Ibdah et al., 2003) resulting in two strikingly yellow fluorescent

compounds. No trace of any yellow fluorescent compound was ever observed in the ice plant. Therefore, a full-length PFOMT with the *in vitro* properties is unlikely to exist *in vivo*. The observed truncation of the PFOMT *in planta* could be experimentally mimicked and a comparable *in vivo* specificity could be reproduced with *in vitro* designed proteins (Vogt et al., 2004; Figure 4) confirming the data obtained with the native plant enzyme.

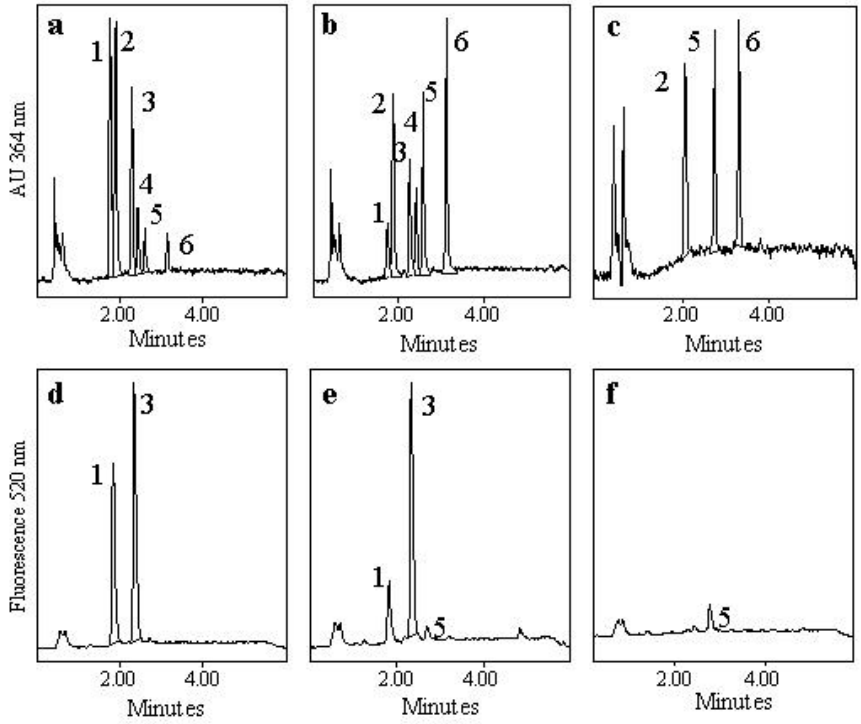

Figure 4 Variation in reaction products of recombinant PFOMT isoforms. HPLC run of full length (a,d) (N0), N-terminally five amino acids shorter (N-5) (b,e), and N-terminally eleven amino acids shorter (N-11) (c,f), respectively. Endogenous quercetagetin (queg) was used as the substrate. UV-detection at 364 nm (a-c); Fluorescence detection (d-e), excitation at 370 nm and emission at 520 nm. Peak identification: **1**, queg-5-OMe; **2**, queg (substrate); **3** queg-5,3´-diOMe; **4**, queg-3´-OMe; **5**, queg-6-OMe, **6**, queg-6,3´-diOMe. Substrate **2**, and products **5**, **6**, and traces of **4** are also observed with the native enzyme. Yellow fluorescing peaks **1** and **3** are products of N0 and N-5 recombinant enzymes only (from Vogt, 2004).

Cellular *in vivo* ratios of enzyme and substrate might also influence the specificity even of *in vitro* "absolute specific" enzymes. When highly concentrated solutions of the full length PFOMT were incubated with the flavonol quercetin at a 1:1 enzyme to substrate ratio, very rapid methylation of the substrate was accompanied by the formation of a new yellow fluorescent product. This product was never observed in the usual *in vitro* assay, with saturating substrate to enzyme ratios (T. Vogt, unpublished). HPLC-analysis indicated that about 1% of the predicted product, quercetin 3´-*O*Me, was also methylated at the 5-hydroxy position which is not part of a required vicinal dihydroxy system. The new product gives the

observed bluish/yellow fluorescence of que-5,3´-diOMe under UV-light (Figure 5) comparable to that of the quercetagetin 5-OMe derivatives (Ibdah et al., 2003). This phenomenon could result in products, not observed under standard experimental conditions. If only a few molecules of these compounds provide an advantage for the species, they may contribute to natural selection, a key element in molecular evolution.

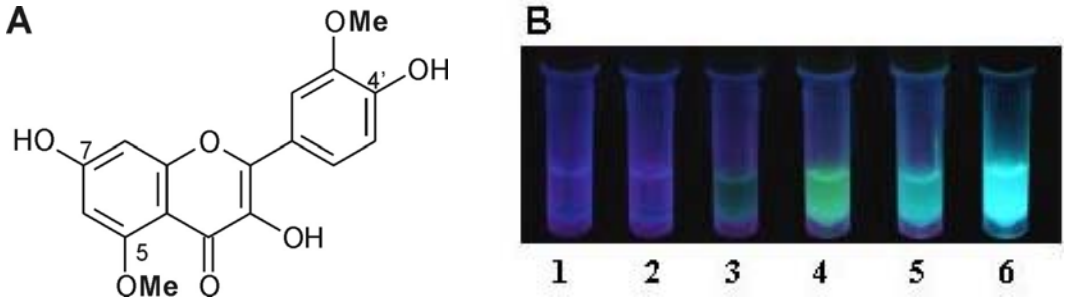

Figure 5 A. Structure of fluorescent quercetin-5,3´-diOMe and **B.** enzyme assays with concentrated PFOMT (N0) at 10 mg/ml. **1**, enzyme only; **2**, enzyme + 1 mM Mg^{2+}; **3**, enzyme + 200 µM quercetin; **4**, enzyme + 200 µM quercetin + 1 mM Mg^{2+}; **5**, enzyme + 200 µM quercetin + 1 mM AdoMet; **6**, enzyme + 1 mM Mg^{2+} + 200 µM quercetin + 1 mM AdoMet. Fluorescence under UV light (λ_{max} 365 nm). Traces of bivalent cations in the double destilled H_2O cause the observed slight yellow/blue fluorescence in vial **5**.

Enantioselective properties may further enhance the specificity of some GTs and probably also of some MTs. An *A. thaliana* GT was reported to glucosylate selectively the plant hormone (+)-abscisic acid, whereas some enantioselectivity was also reported earlier for a similar abscisic acid-specific GT from adzuki bean (*Vigna angularis*) (Lim et al., 2005; Xu et al., 2002). The enantioselective properties may become more prominent, once further enzymes have been analysed which modify optically active aliphatic and cyclic structures.

In summary, these examples raise doubt, how accurate substrate and regiospecificity observed *in vitro* can be correlated with the corresponding activity *in vivo,* unless an enzyme is characterized on the biochemical level and mutants with the expected, altered chemotype are observed.

5. Structural Characterisation and Catalytic Mechanisms

In the new millenium, considerable progress has been made on the structural elucidation of plant methyltransferases, more than on the glucosyl- or glucuronosyltransferases. In 2001 and 2002, Zubieta et al. published the first crystal structures of several natural product class II

OMTs from plants, a chalcone, an isoflavone, and a caffeic acid/5-OH-ferulic acid *O*-methyltransferase from alfalfa, termed ChOMT, IOMT, and COMT respectively (pdb signatures: 1FP1, 1FP2, 1KYZ). This breakthrough enabled for the first time an in-depth look at the organisation of the dimeric structure and the possibility to critically examine the plausibility of motif predictions based on sequence similarities (Ibrahim, 1997; Joshi and Chiang, 1998). The X-ray data also paved the way to understand the catalytic mechanism, correlating substrate specificity with the active site topology, and served as a template to model similar enzymes with different specificities (Gang et al., 2002; Yang et al., 2004).

All crystallized AdoMet-dependent MTs, including the class II plant enzymes, share a common core structure, the "AdoMet-dependent MT fold". Central to this shared core structure is a mixed seven-stranded β-sheet, with strand number seven inserted antiparallel between strands five and six (Cheng and Roberts, 2001). The C-terminal catalytic domains in the COMT, ChOMT, IOMT maintain a highly conserved binding pocket for *S*-adenosyl-L-homocysteine (SAH) fixed through a network of hydrogen bonds as well as van-der-Waals interactions (Figure 6). Methylation proceeds via a typical S_N-2-mechanism, a base-assisted deprotonation of the hydroxyl group by a histidine residue, followed by a nucleophilic attack at the reactive methyl group in AdoMet (Zubieta et al., 2001, 2002).

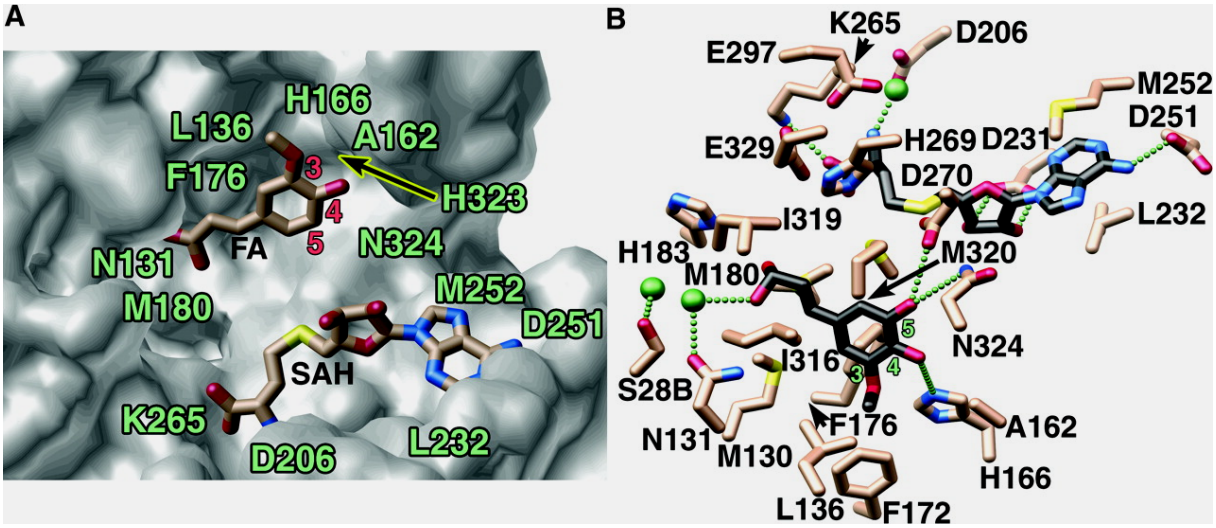

Figure 6 Schematic close-ups of the active site cavity of COMT. **A**. Connolly surface of the protein illustrating the perfect match and complementary shape of bound ferulic acid (FA) and SAH. **B**. Active site arrangement for the COMT–SAH–5-hydroxyconiferaldehyde complex. Bonds of the COMT active site residues are colour coded. Carbon atoms of SAH and 5-hydroxyconiferaldehyde are shown in black to distinguish them from the active site residues. His 269 acts as the catalytic base. (from Zubieta et al., 2002).

Mutation of the catalytic base histidine completely inhibits the methyl transfer. Once AdoMet is bound, specificity is governed by single amino acids, which more or less facilitate the binding of the different methyl acceptors by unique arrangements of spatial, hydrophobic, or hydrophylic constraints.

In class I metal dependent *O*-methylation reactions, as performed by the mammalian catechol OMT, deprotonation is facilitated by a bivalent cation, usually Mg^{2+}. This is bound after AdoMet-binding and converts the vicinal dihydroxy groups of the acceptor to be more easily ionisable (Vidgren et al., 1994). Again by an S_N-2-type reaction, a lysine residue acts as the catalytic base and accepts the proton specifically from the hydroxyl group of the catechol like structure in the 3´-position, accompanied by a nucleophilic attack of the methyl group of AdoMet. Interestingly, and in contrast to the corresponding plant proteins, the catechol OMT exists in a soluble (liver, kidney) and a membrane-bound (brain) form with a 20 amino acid extended N-terminal anchor. In both cases these enzymes are monomeric. There is no dramatic effect on catalytic efficiency, however, the association with the hydrophobic environment slightly promotes substrate binding and a lower apparent K_m for the catechol substrates is observed (Rivett and Roth, 1982; Mannisto and Kaakkola, 1999). This indicates that also the physico-chemical environment of enzyme withotherwise identical amino acid sequences can be important to correctly assess the substrate specificity and kinetic parameters.

Most recently, the structure of the class I OMTs from alfalfa (a substrate specific CCoAOMT) and from the ice plant (the promiscuous PFOMT) were resolved (Ferrer et al., 2005; D. Rauh, T. Vogt, J. Kopycki, M.T. Stubbs, in preparation; Figure 7).

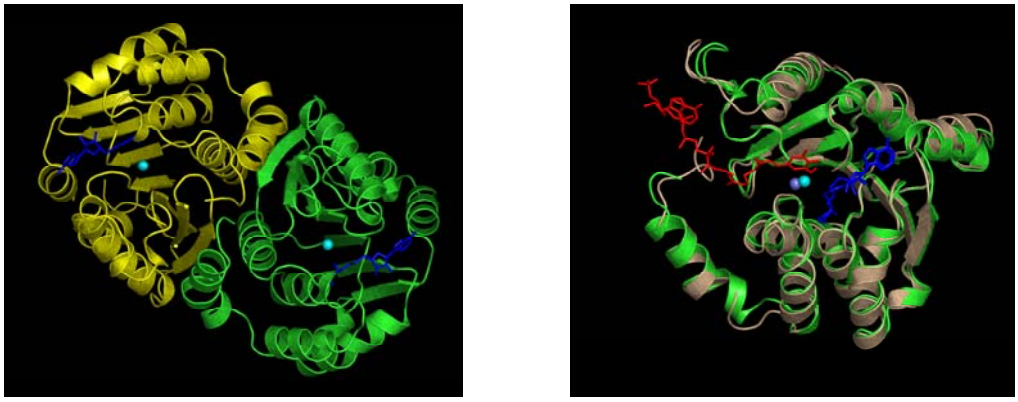

Figure 7 Dimeric 3D-structure of PFOMT from the iced plant (**left**). Superimposed 3D-structures of the CCoAOMT from alfalfa in grey (from Ferrer et al., 2005) and PFOMT from the ice plant in green illustrate the structural similarity of both enzymes (**right**). 5-OH feruloylCoA (alfalfa) and SAH (PFOMT) are illustrated in red and blue, respectively. Ca^{2+} (CCoAOMT) and Mg^{2+} (PFOMT) are marked as purple and blue dots, respectively. The design and overlay of both structures is a courtesy of Jacub Kopycki, MLU Halle-Wittenberg, Germany using PyMol (DeLano Scientific, San Carlos, CA, USA).

Despite the low sequence identities between the rat catechol, the alfalfa, and ice plant OMTs, the metal dependence and the requirements for vicinal dihydroxy groups already suggests similar structural motifs and catalytic centers of both sets of enzymes (Vidgren et al., 1994; Ferrer et al., 2005). Each monomer of the CCoAOMT and the PFOMT form a catalytic domain with a core α/β/α Rossman fold that provides the binding site for SAH (Rossman et al., 1974). The catalytic S_N-2-like mechanism was described for the alfalfa CCoAOMT, mediated by a stabilized and chelated cation (Mg^{2+}, Ca^{2+}, Mn^{2+} or Zn^{2+}), abstraction of a proton by a catalytic lysine residue, and subsequent nucleophilic attack of the methyl group (Ferrer et al., 2005). The dimeric structure of both plant enzymes are not critical for substrate recognition like in the case of class II OMTs, and each substrate and cofactor only interacts with one monomer. However, the first 20 amino acids of the CCoAOMT which up to now could not be resolved by electronic density maps, appear to be critical for substrate specificity, at least in case of the PFOMT (Vogt, 2004). The structure of this enzyme is very similar to the alfalfa enzyme, except for a C-terminal loop and this flexible N-terminal domain likely related to the profound differences in the substrate specificity. The observed drastic changes in catalytic efficiencies of different truncated isoforms obtained from heterologously expressed PFOMT (Vogt, 2004) should reward a thorough examination of this part of the protein and may lead to the identification of catalytically important amino acids in this extremely variable domain of class I OMTs.

In contrast to the microbial, animal, or plant MTs, much less is known about the glycosyltransferase/glucuronosyltransferase structures and catalytic mechanisms. This is in part due to low overexpression yields of these enzymes in pro- and eucaryotic systems, combined with problems to crystallize the proteins in sufficient quality to obtain high resolution electron density maps (Gustafsson et al., 2004; Wang, X, Samual Roberts Nobel Foundation Inc., Ardmore, OK, USA, pers. communication). In addition, the mammalian enzymes may be glycosylated and are usually associated with the endoplasmatic reticulum membranes, enhancing the problems of purification and expression (Radominska-Pandya et al., 1999). From the array of more than 70 GT families described in the carbohydrate active enzyme database (http://afmb.cnrs-mrs.fr/CAZY/; Coutinho and Henrissat, 1999), only a few structures are solved and none of these are related to plant Family 1 enzymes. This family displays the observed activity towards small natural products, and its members are characterized by a nucleotide recognition domain and a mechanism leading to the inversion of the sugar configuration (NRD1β-GTs).

The solved crystal structures can be divided into two distinct structural types, the GT-A and the GT-B fold (Hu and Walker, 2002), with the structure of the vancomycin glucosyltransferase from *Amycolatopsis orientalis*, recently crystallized as the first member of the GT-B family, to which the plant natural product GTs belong (Mulichak et al., 2001). They both show the inversion of configuration and belong to the NRD1β-GTs. The overall sequence similarities of the plant Family 1 proteins with this microbial sequence is less than 20%. But the secondary structure predictions for one member of the Family I enzymes, the betanidin 5-GT from livingstone daisy, UGT73A5 (nomenclature by MacKenzie et al., 1997), was sufficiently similar to consider building of a model structure. Similar molecular modelling studies were applied previously to P450 monooxygenases with sequence identities as low as 13%, again based on common motifs and very similar secondary structure predictions (Rupasinghe et al., 2003). Our efforts finally succeeded to build the first 3-D model of a plant natural product GT. The model structure of UGT73A5 was simultaneously supported by site-directed mutagenesis of the enzyme (Hans et al., 2004). The proposed structure for UGT73A5 not only showed the expected two Rossman-fold-like domains with a deep cleft already known from the *A. orientalis* structure. The model also explained the loss of enzymatic activity after mutations of the conserved glutamate to alanine in the sugar binding domain, the so-called PSPG-box (Hughes and Hughes, 1994; Vogt and Jones, 2000), and of the N-terminally conserved histidine 22 to leucine, respectively.

Based on this model a catalytic mechanism was proposed, which explains the observed inversion of the bound sugar, by an S_N-1-type mechanism, contrary to the scenario with the S_N-2-type mechanism proposed for the OMTs, but with similar amino acid residues involved (Figure 8). Histidine 22 acts as the catalytic base, abstracting a proton from the substrate betanidin with subsequent transfer of the proton to the conserved glutamate. In a second step, a proton transfer from the charged lysine 31 to the oxygen atom of the ester bond in the activated sugar is proposed. After cleavage of this bond, the resulting sugar carbocation forms a glycosidic bond with the nucleophile, in this case the deprotonated betanidin. The inversion of the configuration is the result of a fixed orientation of the substrates and the preferred direction of the attack of the nucleophile at the Cα-sugar carbon. The alternative S_N-2-reaction mechanism, previously proposed to explain the inversion of conformation for NRD1β-types of enzymes (Kapitonov and Yu, 1999), is kinetically less favoured. The activation enthalpy for the direct attack of the nucleophile betanidin at the Cα-atom of the bound sugar (30.4 kcal/mol) as compared to 11 kcal/mol observed for cleavage of the Cα-O(P) bond is significantly higher (Figure 8c). The proposed glucosyltransferase model has recently been

confirmed by modelling of the dhurrin GT from millet (*Sorghum bicolor*) (BL Møller, Kopenhagen, DK, pers. communication).

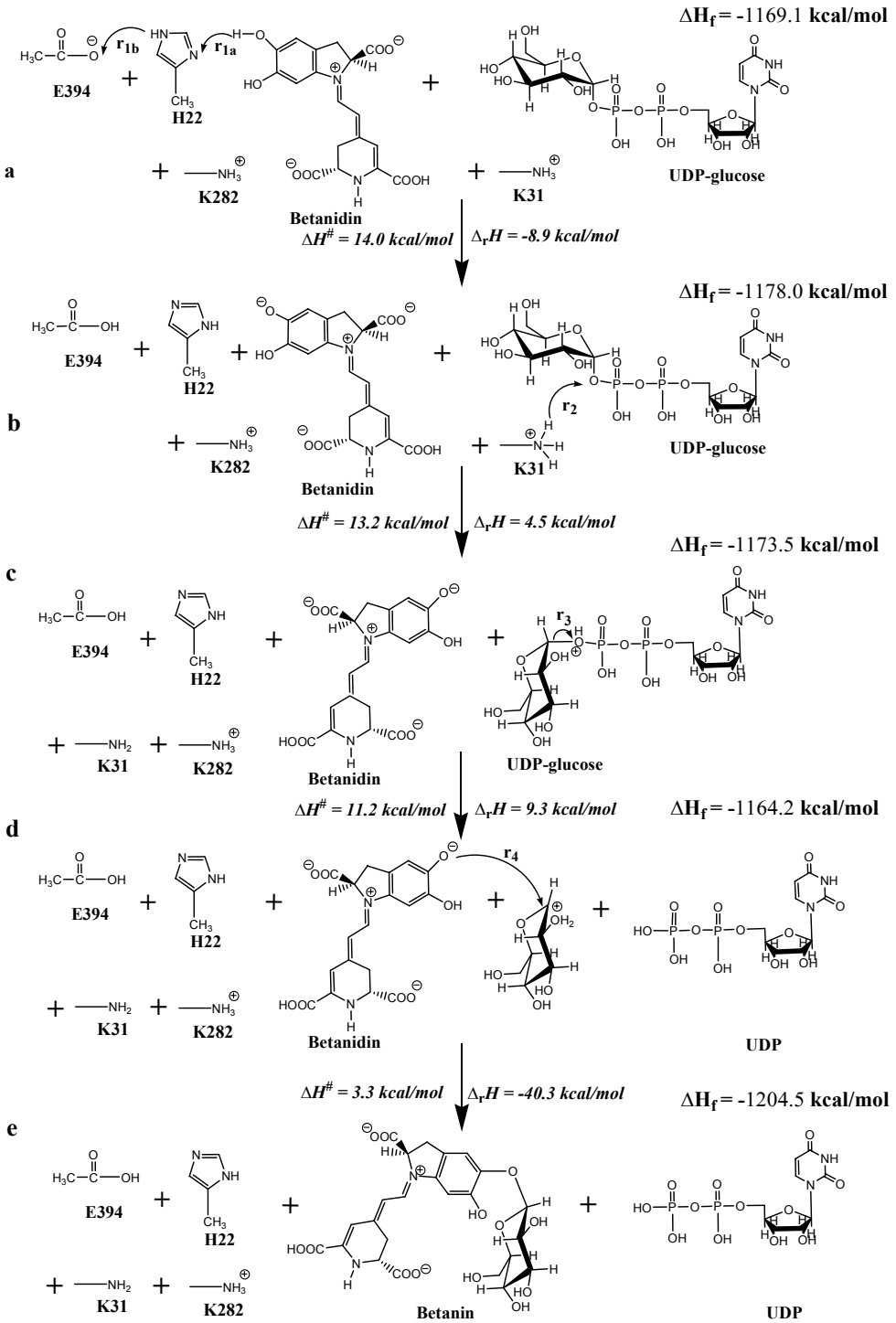

Figure 8 Proposed reaction scheme to explain the S_N-1-type mechanism and the inversion of sugar configuration for the NRD1β-GT, UGT73A5 from *D. bellidiformis.* (from Hans et al., 2004). Successive catalytic steps are indicated (a-e).

While a model structure combined with site-directed mutagenesis may explain the observed reaction mechanism and subsequent docking studies confirm that betanidin is the best substrate, these algorithms are less capable to describe the constraints of substrate binding and specificity as detailed as a high resolution crystal structure. In fact, as seen from an early proposed model of the CCoAOMT, these may be incorrect in predicting the involvement of certain amino acids in the catalysis (Hoffmann et al., 2001; Ferrer et al., 2005), and they are not well suited to describe the flexible loops of the enzyme with high accuracy. In case of the plant Family I GTs, the involvement of the conserved PSPG-box as a scaffold for UDP-glucose binding was predicted, but the involvement of a histidine from the N-terminal domains of the protein was unexpected. Analogous to the plant OMTs or the mammalian glucuronosyltransferases, some amino acids of the N-terminal domain are not only involved in substrate binding (Moehs et al., 1997), but also play an important role in catalytic activity (Hans et al., 2004; Radominska-Pandya et al., 1999).

Still, the overall heterogeneity of the N-terminal domains of the GTs is striking. Currently, it is just a more or less educated guess that this heterogeneity could be essential for other tasks. It may be essential for individual GTs to recognize or interact with other associated proteins or protein complexes, already suggested for the N-terminal domain of glycoprotein glucosyltransferases (Guerin and Parodi, 2003). Gel filtration and analytical ultrafiltration performed with concentrated, recombinant UGT73A4 from red beet did not raise any doubts on the monomeric nature of this protein. On the other hand, low angle X-ray scattering on the same solution obtained from recombinant protein suggested a tendency to form dimeric units *in vitro* (H. Lilie and S. König, Martin-Luther-University Halle-Wittenberg, Germany, pers. communications). Whether plant GTs *in vivo* are soluble and monomeric or, similar to class II OMTs (Zubieta et al., 2001) require association to another GT or a cellular proteins for full enzymatic activity remains to be solved and may be directly correlated with the question of their intracellular or compartmental localisation.

6. Cellular Localisation

The most challenging questions are concerned with cellular or compartmental localisation of individual GTs and MTs and their interaction with other proteins. At a first glance this problem is simple: The corresponding plant proteins are soluble and their presence may be expected therefore in the cytosol. However, nobody seriously expects a free floating movement of the NRD1β-GTs in search for their substrates. Based on immunolocalisation

studies Latchinian-Sadek and Ibrahim (1991) proposed a vesicle or a cytoplasmatic membrane association of flavonoid B-ring specific GTs. Is there at least some similarity to the membrane association of mammalian UGTs? Membrane association of pollen-specific GTs of conditionally male fertile *Petunia hybrida* was suggested based on the requirements for detergents to solubilize the corresponding activities (Vogt and Taylor, 1995). At the same time Warnecke and Heinz (1994) published the successful solubilisation and purification of a truly membrane bound NRD1β-GT from oat (*Avena sativa*). Therefore, weak or strong membrane association should be considered, even when soluble enzymes are observed *in vitro*. When tobacco plants were transformed with a construct of the betanidin and flavonoid specific enzyme UGT73A5 under the constitutive 35S-promotor, transcript accumulation and enzyme activity were observed, but no changes in product formation between WT and transformed tobacco plants were detected (S. Ebert, S. Rosahl, T. Vogt, unpublished). One obvious explanation could be that the enzyme does not have any access to the appropriate target substrates quercetin or kaempferol, or affinity to the presumed flavonoid multi-enzyme complex present in tobacco and other plants (Winkel, 2004) and therefore, is excluded from product formation. Protein-protein interactions, specifically with other glucosyltransferases or methyltransferases are likely required in the formation of complex acylated, methylated, and glycosylated conjugates (Sawada et al., 2005). Modulation and interaction of a mammalian morphine glucuronosyltransferase (UGT2B7) with the cytochrome CYP3A4 was already described for cultered avian COS-1 cells by co-immunoprecipitation and kinetic studies (Takeda et al., 2005). Similar interactions should be expected for plant GTs and MTs in several biosynthetic pathways, like anthocyanin, glucosinolate or cyanogenic glucoside biosynthesis.

Apart from the cellular localization, it is obvious from many studies that GTs as well as MTs are regulated in a tissue- and developmentally specific manner. One example for a highly tissue-specific GT is the strictly gametophyte associated, reversible flavonol 3-*O*-galactosyltransferase of the Solanaceae (Vogt and Taylor, 1995; Taylor and Miller, 2002). In this case reversibility of this enzyme is of central importance, since it is one factor likely regulating the release of free flavonol aglycones, essential for pollen germination. Tissue specific accumulation of anthocyanidin-GT transcripts has been observed in grapevine (*Vitis vinifera*) skins and *Perilla frutescens* red grape skins or leaves, respectively. In both cases expression of transcripts correlates with anthocyanin formation (Gong et al., 1997; Kobayashi, 2001). Accumulation of the glycosylated products into plant vacuoles is mediated by two different transport mechanisms, a proton/antiporter system for endogenously supplied

substrates or a typical ATP-binding cassette type of transporters for exogenously applied xenobiotica (Frangne et al., 2002). Interactions of GTs with glutathione *S*-transferases or vacuolar membranes may occur, but remain to be established.

The localization of MTs in plants has been the focus of several recent investigations. In general, the transcripts are usually tissue and developmentally specific distributed. Two OMTs, the class II COMT and the class I CCoAOMT, both involved in lignin formation, are strictly separated in *Zinnia elegans* (Ye and Varner, 1995). COMT is mainly found in the phloem tissue whereas higher signal intensities of CCoAOMT at both, the mRNA and the protein level were localized in developing xylem elements. Consistent with this observation, both enzymes are associated with developing vascular tissue in alfalfa (Inoue et al., 1998).

The association of modifying enzymes in the same tissues than the corresponding products is expected and plausible. Expression of (iso)eugenol OMT (IEMT), associated with fragrance production in *Clarkia breweri*, is associated with petal tissues, the site of volatile production, but also with stamens (Dudareva and Pichersky, 2000; Figure 9).

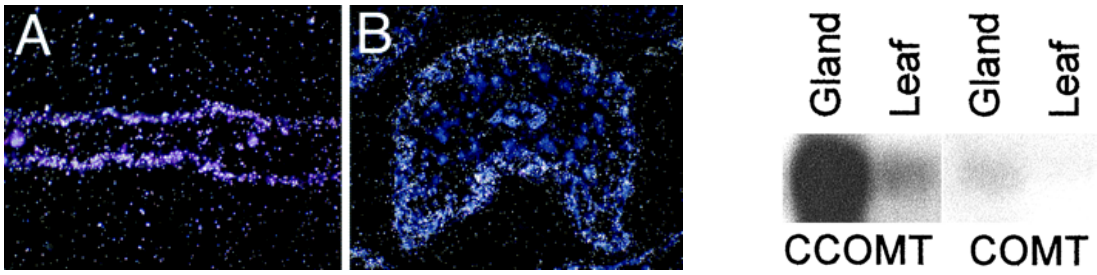

Figure 9: Tissue specific accumulation of OMT transcripts. *In situ* localization of IEMT in cross-sections of a petal (A) and stamen (B) (From Dudareva and Pichersky, 2000) (**left**). CCoAOMT (CCOMT) and caffeic acid OMT (COMT) signals in RNA from peltate glands and leaves of basil (from Gang et al., 2001) (**right**).

A similar situation was observed for peltate glands of *Ocimum basilicum* (basil), where the high abundance of transcripts in a gland-specific EST-database for eight marginally different CCoAOMT isoforms perfectly matched the peltate specific high expression of this transcript in Northern blot analyses, as compared to the caffeic acid OMT (Gang et al., 2001; Figure 9). Immunolocalization of benzoic acid OMT in epidermal tissues of snapdragon (*Anthirrhinum majus*) flowers, is also consistent with these observations (Kolosova et al., 2001).

Immunolocalization of two class II OMTs involved in alkaloid biosynthesis in opium poppy (*Papaper somniferum*), the (*R,S*)-3'-hydroxy-*N*-methylcoclaurine 4'- and reticuline 7-OMTs is associated with parenchyma cells of vascular bundles, adjacent to, but not within

laticifers as well as in the pericyle of the stele in root tips, concomitant with their early to intermediate role in the biosynthetic pathway (Weid et al., 2004; Kutchan, 2005; Figure 10).

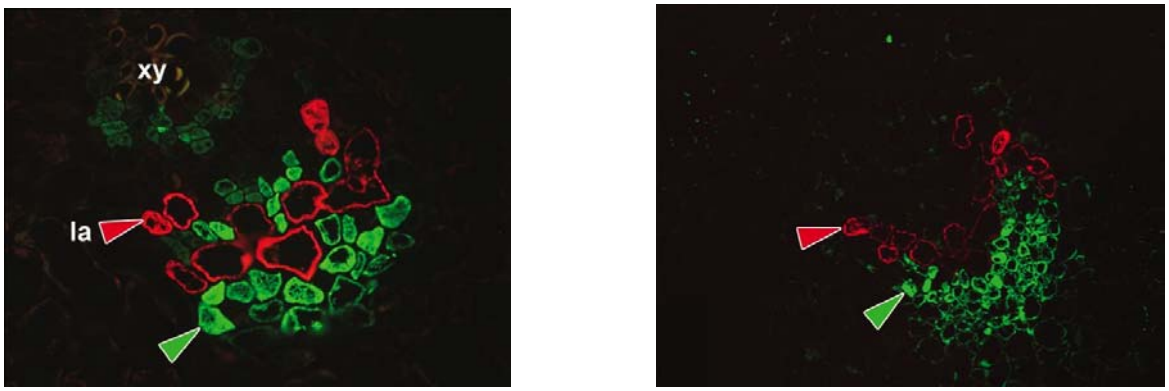

Figure 10 Fluorescence immunocytochemical labelling of (*R*,*S*)-3'-hydroxy-*N*-methylcoclaurine 4'- (**left**) and reticuline-7-OMTs (**right**) (green arrowheads) in cross sections of opium poppy stems. Red arrowheads mark the major latex protein in laticifers (from Kutchan, 2005). Xylem tissue is also marked (xy).

Another interesting and functionally rational localization of a MT has been described in chlorophyll biosynthesis. Mg-protoporphyrin IX methyltransferase, methylating one of the carboxyl groups of chlorophylls a and b is an N-terminally membrane-associated enzyme and could be localized in both the thylakoid membranes as well as in the chloroplast envelope, with a suggested but still hypothetical role not only in biosynthesis, but also in signalling between the chloroplast and the nucleus (Block et al., 2002).

Tissue and cell specific localization, either bound or loosely linked to biosynthetic metabolons seems likely for many MTs and GTs. Identification of these protein-protein interactions either by immunolocalization, use of the yeast two-hybrid system or by microscopic techniques like fluorescence resonance energy transfer (FRET) and fluorescence correlation spectroscopy (FLS) (Hink et al., 2002), combined with transcriptional profiling should facilitate efforts to establish the localization and association of these enzymes within the cells. It may likewise contribute to understand their observed natural redundancy.

7. Biological significance

The activity of the glycosyl- and methyltransferase superfamilies, in addition to generating a diversity of metabolites, is crucial for the survival and cellular homeostasis of plants. A few examples may highlight their important contributions.

Attraction of pollinators is of utmost importance to all flowering plants. Glycosylation and methylation play pivotal, yet contrary roles, especially B-ring-specific glucosylation and subsequent acylation promote intra- and intermolecular stacking, essential for enhanced colour formation by flavonoids and anthocyanins (Goto and Kondo, 1991). Although glycosylation is not the reason for the enhanced colour by itself, B-ring attached sugars form bridges between the coloured flavylium cation of anthocyanins and the esterified colourless phenylpropanoid residues, like caffeic acid, which then stabilize the chromophore by intramolecular co-pigmentation. This leads to a very intensive colour appearance as shown for several *Gentiana* species (Yoshida et al., 2000; Fukuchi-Mizutani et al., 2003). In addition to hydroxylation and glycosylation, different methylation patterns of anthocyanins from simple pelargonidin to highly methylated capensinidin, have a potent effect on flower colour (Strack and Wray, 1989). In contrast to floral colours, floral scent is the dominant means of long distance attraction, independent of daylight, and therefore indispensable to attract night-active insects (Dudareva and Pichersky, 2000; Guterman et al., 2002). Methylation, resulting in aromatic ester and methyl ether formation strongly enhances floral scent volatility. In several cases emission of methylated fragrances and volatiles is accompanied by resin formation. Leaf resin is a complex mixture of terpenoids, waxes, and usually methylated flavonoids (Dell and McComb, 1978). The resultant exudate protects young developing flower and leaf buds from mechanical stress, damaging UV-radiation, and serves as an antimicrobial protectant (Rhoades, 1977; Vogt et al., 1991).

Key players in response to stress are jasmonic acid and salicylic acid carboxyl OMTs, (Seo et al., 2001; Ross et al., 1999). Airborne methyl jasmonate and methyl salicylate, the products of these enzymes, play a pivotal role in many plant-insect interactions (Miller et al., 2005; Shulaev et al., 1997; Chen et al., 2003). Methyl jasmonate can then further activate specific pathways and enzymes involved in herbivore or pathogen defense (Martin, 2003). Methyl jasmonate formation may be regarded as the general defence response and can also induce other MTs, like those involved in methoxylated indol glucosinolate or those of methyl salicylate biosynthesis in *Arabidopsis* (Hudgins and Franceschi, 2004; Mikkelsen et al., 2003; Chen et al., 2003).

Cellular homeostasis is maintained and regulated by various hormones, like auxins, cytokinins, and abscisic acid. In this case, several suitable candidate GTs have been identified and functionally characterized, to modify the structure, polarity and action of these hormones e.g. UGT84B1 from *Arabidopsis* (Figure 11), glucosylating the auxin 3-indol acetic acid

(Jackson et al., 2001), a cytokinin GT from maize (Mok et al., 2005), and the abscisic acid-specific GT from adzuki bean (Xu et al., 2002).

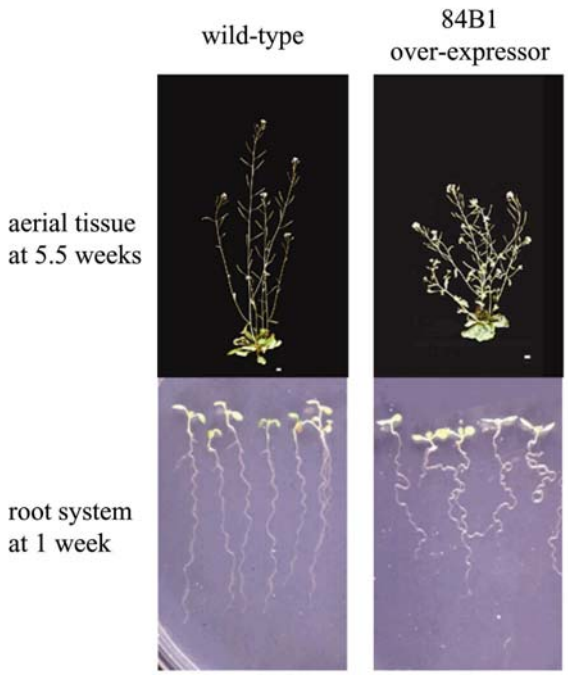

Figure 11 Phenotype of wildtype *A. thaliana* (**left**) and the constitutive transgenic over-expressor UGT84B1 (**right**) encoding a GT of the auxin indole-3-acetic acid. Note especially the impaired gravitropism of the root system. (from Lim and Bowles, 2004).

A variety of GTs and MTs are transcriptionally up-regulated upon biotic or abiotic stresses (Horvath and Chua; 1996; Schmitt et al., 1991; Ibdah et al., 2003; Sachan and Falcone, 2002). Oxidative stress and reduced virus resistance could be correlated with antisense-mediated down-regulation of the scopoletin- and phenylpropanoid GT (TOGT) from tobacco (Chong et al., 2002). Whether up-regulation of transcripts, combined with an observed accumulation of these potentially active phytoalexins indeed has any effect on viral replication, is still controversial (Gachon et al. 2004; Matros and Mock, 2004), and may likely be dependent on the virus strain.

Induction of naringenin 7-OMT was reported from rice plants upon radiation with UV-light (Rakwal et al., 1996). The specific accumulation of the product sakuranetin might point at an important function of this enzyme in protecting rice plants from oxidative stress. This protection may be dependent on external factors, endogenous substrate concentrations, or even on the virus strains. Detoxification of exogenous toxic compounds, like the mycotoxin deoxynivalenol, a sesquiterpenoid produced by several *Fusarium* species, by UGT73C5 from *A. thaliana* stresses the importance of a promiscuous specificity of many GTs (Poppenberger

et al., 2003). On the other hand fungi also fight back with similar "weapons" as shown for the glucosyltransferase mediated detoxification of the cruciferous phytoalexin brassinin by the stem rot fungus *Scelotinia sclerotiorum* (Pedras et al., 2004). Due to the ongoing application of herbicides, pesticides, and fungicides which are targets for the reservoir of *O*- and *N*-glycosyltransferases, promoting endogenous, inducible detoxification and defence responses will be of immediate relevance and economically rewarding (Loutre et al., 2003; Messner et al., 2003).

In mammals, the methylation of reactive mutagenic flavonoids by class I COMT has been mainly attributed to their lack of mutagenic activity *in vivo* as compared to the *in vitro* situation (Zhu et al., 1994). Deglycosylation, followed by glucuronylation and methylation of natural products from food and beverages by the intestine or the liver, is of ongoing debate. Specific modifications are reported to promote the uptake of "healthy" metabolites into the bloodstream and thereby increase or decrease their health beneficial properties, like protection from atherosclerosis and congestive heart failure (Reed, 2002; Nielsen et al., 2003).

8. Molecular Evolution

All superfamilies of plant enzymes, either the P450s, the glycosyl-, the methyltransferases, and many others essentially derive from one or a few remnant ancestors. As we did not witness the diversification, we are faced with the tremendous challenge to describe the observed diversity and analyze the organizing principles while simultaneously explaining the genomic and evolutionary constraints leading to this diversification.

The first comprehensive summary of plant Family 1 GTs established 107 putative glycosyltransferase genes from *A. thaliana* (Li et al., 2001) and was later complemented by a second analysis (Paquette et al., 2003). Both studies essentially grouped this allelic diversity into 14 phylogenetic subclusters, strongly supported by bootstrap analysis. Most of the *A. thaliana* glycosyltransferases have a monoexonic coding sequence, and neither has a signal sequence nor any membrane-spanning domains (Lim and Bowles, 2004). This is in contrast to many animal glucuronosyltransferases, where complex architectures with up to six exons spanning about 25 kb have been described (Turgeon et al., 2000). The observed clustering of many GT-genes in plants is consistent with at least three whole genome duplications in *A. thaliana* (Maere et al., 2005). On the other hand, specific GTs e.g. those involved in the biosynthesis of the triterpenoid avenacin in oat (*Avena sativa*), are clustered with several other genes of the avenacin biosynthetic pathway, which is non-consistent with a gene duplication

event for these GTs (Qi et al., 2004). Still, the presence of large gene families is the result of an expanding genome with a gain of function surpassing loss of genes and gene function.

Let us take a look at the organising principles in GT-cluster formation. Is it specificity for the donor or the acceptor? Is it regio- or position specific? From a detailed investigation of 50 functionally expressed GTs from *Arabidopsis* with the model substrate esculetin, it was evident that several members of different subclusters were able to glucosylate this coumarin *in vitro*, although with different regiospecificities (Lim et al., 2003). Other subclusters were highly specific for the substrates and the positions.

Among all plants investigated up to now the members of anthocyanidin 3-GTs and anthocyanin 5-GTs are highly homologous, up to 60% identity even when enzymes from different species are compared. This is significantly higher than the less than 20% homology over the whole natural product GT-superfamily (Vogt and Jones, 2000). In addition they display a uniform and strict specificity for the individual hydroxyl groups (See also Figure 12). This specificity compared with sequence data can then be used to annotate other unknown sequences from the databases. An *A. thaliana* GT-sequence with the database accession number CAC01717 (Figure 12) can therefore easily be correlated with a flavonoid 3-GT (Figure 12). Conservation of sequence identities and functional specificity represent the importance of the conserved flavonoid and anthocyanin biosynthetic pathway.

Other subclusters are far less specific for a single substrate, not even for the glucose donor. Figure 12 illustrates various other protein sequences which *in vitro* are considered to glycosylate flavonoids and anthocyani(di)ns. Although the two large clusters to the right contain a variety of, at a first glance, different enzymes from taxonomically unrelated species, a close-up view reveals striking similarities within one subcluster. All members specified as "Flavonoid A- and B-Ring specific GTs" marked in blue (Figure 12), transfer glucose preferentially to the 3′- or 4′-hydroxyl group of the flavonoid B-ring or the 7-OH group of the A-ring, but not to the 3-hydroxy position of the heterocycle. In case of the enzyme from scullap (*Scutellaria baicalensis)* (BAA83484, Figure 12) glucose is transferred exclusively to the 7-OH of the A-ring, since no hydroxylated B-ring is present in substrates baicalein or scutellarein (Hirotani et al., 2000).

This specificity for oligohydroxylated structural motifs is crucial to the whole cluster to which flavonoid 7-GTs from *A. thaliana* (Figure 12, AAR01231) and rice (*Oryza sativa*), UGT73A5 from livingstone daisy (Figure 12, Y18871), UGT73A4 from red beet (Figure 12, AY526080), a 2′-chalconaringenin GT from *Dianthus caryophyllus* (Fig. 12, BAD52007) (Ogata et al., 2004), sequences from tomato (*Lycopersicon esculentum*) (Figure 12, X85138),

tobacco (Fig. 12, U32644), gentian (Figure 12, BAC54092) and scullap (Figure 12, BAA83484) belong to.

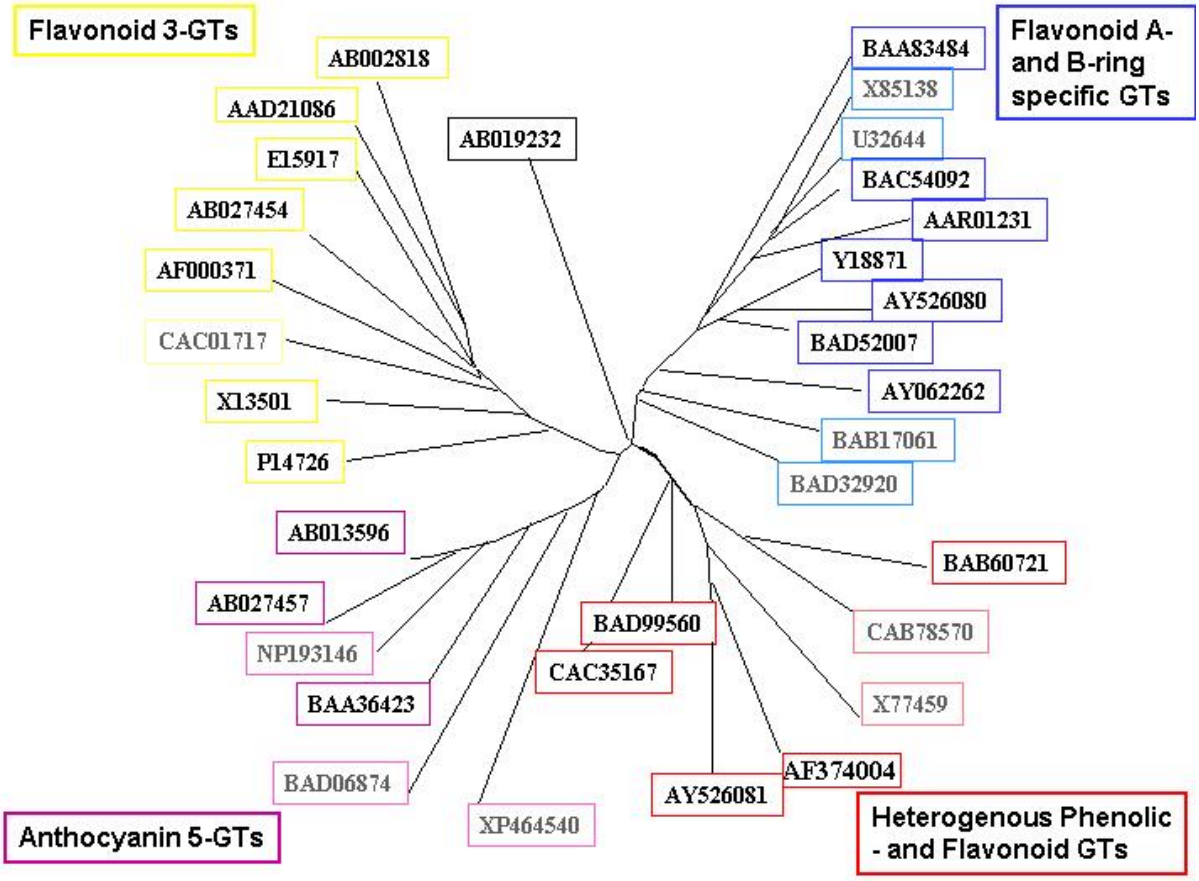

Figure 12 Various flavonoid and anthocyanidin GTs (indicated by their NCBI-database accession numbers) cluster according to position specificity towards individual hydroxyl groups. *A. thaliana* sinapic acid GT (AB019232) was used as an outlier. Solid numbers and colours indicate that the corresponding proteins were functionally characterized, whereas soft numbers and colours indicate proteins with poorly or uncharacterized functions (Cladogram created with Clustal W, PAM 250 matrix, Thompson et al., 1994)

This subclass UGT73 (MacKenzie et al., 1997) together with the second very heterogenous subcluster of "phenolic and flavonoid GTs referenced by a naphtol GT from tobacco (Fig. 12, BAB60721), by arbutin synthase (Figure 12, CAC35167.1), UGT71F2 characterized as a betanidin 6-GT ((Figure 12, AF374004), a red beet flavonoid and betanidin GT, UGT71F1 (Figure 12, AY536081) and other GTs with a broad acceptor specificity, including flavonoids and anthocyanidins is clearly separated from the conserved clusters of flavonoid and anthocyanin 3- and 5-GTs, respectively (Figure 12). Due to this heterogeneity it will be difficult to predict the substrate profile of new members of both subclusters. This

profile is likely species specific and a detailed analyses of the aglycone pattern of the plant may be required to suggest potential candidates.

Diversification into the individual specificities, as we observe them today has occurred by gene duplication and subsequent loss and gain of functions (Moore and Purugganan, 2005). Genomic clustering as observed for some *A. thaliana* GTs may be regarded as a proof for gene duplications, which have occurred during evolution of vascular plants. Clusters like UGT73 and UGT71 may have developed at some time from one or several ancestors and due to their promiscuity they "aquired" or new functions. One excellent example to illustrate this hypothesis may be mentioned briefly. Ogata et al. (2005), most recently described an anthocyanidin GT from *Rosa hybrida cultivar* (rose) with the unusual property to simultaneously glucosylate anthocyanidins at the 5-OH and the 3-OH position (Figure 12, BAD99560) combining the specificities of an anthocyanidin 3-GT and the anthocyanin 5-GT. Apparently, plants have maintained these sets of promiscuous and "flexible" enzymes, different from the conserved clusters of specific enzymes, with the obvious evolutionary advantage to adjust to changing or potentially lethal conditions. It is noteworthy that several putative "Flavonoid A- and B-Ring specific GTs" (Figure 12) were reported to be transcriptionally up-regulated during stress-responses, like the wound-inducible *Is5A* and *Is10A* genes from tomato or the *twi1* gene from tomato (Horvath and Chua, 1996; O´Donnell et al., 1999). Again, in response to environmental or endogenous stimuli a pool of enzymes with high promiscuity combines the required, overlapping specificity to modify a variety of compounds with the precise position selectivity to ensure high affinity.

An overlapping substrate specificity but precise position specificity of clusters UGT71 and UGT73 should again be illustrated for betanidin 5- and 6-GTs from *D. bellidiformis,* which share less than 20% amino acid sequence identity, yet glucosylate the same substrate betanidin and flavonoids with a different regiospecificity (Vogt et al., 1999a; Vogt, 2002). This polyphyletic origin of two enzymes accepting identical substrates, yet show no significant sequence identity is also nicely illustrated by the set of scutellarein glucosyl- and glucuronosyltransferases, which share the same acceptor and even identical position specificities, but use different sugars (Hirotani et al., 2000; Nagashima et al., 2000). Both sets of enzymes therefore, have evolved from at least two different ancestors, presumably both involved in the modification of unknown, extinct or remnant substrates and at some point have "gained" the properties to modify novel endogenous metabolites, like betanidin or scutellarein, respectively.

MTs are classified based on their dependence on a metal cofactor and the class I and the class II nomenclature (Joshi and Chiang, 1998). Although all AdoMet-dependent enzymes share identical structural features (Cheng and Roberts, 2001), a single ancient separation of both classes of enzymes has occurred to explain the astonishing 35% amino acid sequence identities between class I OMTs from plants, cyano-, and myxobacteria (Ibdah et al., 2003). Dimerization apparently is restricted to the plant class I OMTs, since the mammal and also the functionally not characterized cyanobacterial enzymes are monomers. Plant class I OMTs can be further divided into at least two subclasses based on substrate and regiospecificity (Ibdah et al., 2003; Vogt, 2004). Exceptionally high sequence conservation (up to 90%) points to an ancient, important, and conserved function of one subset of the class I OMTs, the CCoAOMTs involved in lignin monomer formation. A less rigid conservation of amino acid sequences is observed for those few promiscuous enzymes detected recently (Ibdah et al., 2003). Diversification and specialization of class I OMTs resulted in at least six different genes in *Arabidopsis*, of which only two have been functionally characterized yet (Vogt, unpublished). A similar situation is observed in other plant species, e.g. in sweet basil, where several slightly different CCoAOMT isoforms were observed in peltate glands (Gang et al., 2001). Probably only one is specifically involved in the methylation of guaiacyl lignin monomers, whereas the substrates of the other members, all targeted to vicinal dihydroxy functions, remain elusive. The precursors of the basil peltate phenylpropenes chavicol or eugenol (Gang et al., 2002), which are modified by two distinct class II enzymes chavicol OMT and eugenol OMT could be targeted initially by one or two different class I isoenzymes.

The diversification of plant species and the rapid evolution of substrate diversity apparently is concomitant with the development of class II OMTs, where sequence similarities are much less conserved (Ibrahim and Muzac, 2000). Predicting their *in vivo* substrates based on sequence identities is a tremendous challenge (Schröder et al., 2002). Flexible and overlapping substrate specificities are described for class II OMTs, e.g. the caffeic acid OMT methylates also caffeoyl CoA, the sole substrate of the lignin specific class I cluster enzymes. The lignin specific class I enzyme, however, does not or very poorly methylate caffeic acid (at least *in vitro*). OMTs from *T. tuberosum* which do not distinguish between a benzylisoquinoline alkaloid or a simple phenylpropanoid have already been mentioned (Frick and Kutchan, 1999). Seoighe and Gehring (2004) explain that gene duplication is a selective process and expansion of a gene family is dependent on a series of evolutionary constraints. If this is the case, the multiplicity of class II OMTs may be the result of more flexible structural motifs of these enzymes as compared to a rigid, cation-dependent

structure of the class I OMTs. The structurally very similar subcluster of promiscuous class I OMTs, as compared to the highly conserved and stringent CCoAOMTs (Ibdah et al., 2003), was possibly established later in evolution to fill a niche, not immediately accessible to the class II enzymes for yet unknown reasons. Similar principles may also hold true for the evolution of *N*-, *S*-, and *C*-methyltransferases.

9. Economical Relevance

Glycosylation and methylation of endogenous unstable precursors of cyanogenic glycosides, glucosinolates, or anthocyanidins are of major ecochemical importance for the plants (Zagrobelny et al., 2004; Wittstock et al., 2004). Designing natural products by genetic engineering of the corresponding enzymes may not only protect plants from pathogens and herbivores (von Rad et al., 2001; Tattersall et al., 2001), but could also lead to desired modifications of colour, taste, or fragrance of flowers, nutraceuticals, and beverages with a promise for potential economical benefits (Dudareva et al., 2004; Marillia, 2001; Kazuma et al., 2003).

Beside the expected *in vivo* use of these enzymes, their *in vitro* application as potential biocatalysts is already established (Arend et al., 2001). By mixing recombinant *E. coli* strains, which over-expressed a mixture of one *A. thaliana* GT and a set of peppermint MTs, Willits et al. (2004) produced glucosylated and methylated flavonol conjugates *in vitro*. In principle, both types of enzymes, can therefore be used in biofermentation or by combinatorial enzymology to produce a desired mixture of compounds *in vitro* without the necessity to use organic solvents or toxic catalysts.

Stevia rebaudiana contains a complex mixture of intensely sweet terpenoid glycosides, the steviosides, which have been proposed to, but finally rejected by the European Commission as natural, non toxic sugar replacements (CS/ADD/EDUL167final, 17/06/1999). The rejection was in part based on the poorly defined formulation of the drug. Nevertheless, steviol glycoside preparations are legally sold and used as food additives in Asia and the US. Successful cloning and functional expression of several glucosyltransferases involved in rebaudioside A biosynthesis may now promote further *in vitro* studies to critically examine the potentially carcinogenic nature of these low calorie sweeteners and circumvent the risk of toxic plant contaminants (Richman et al., 2005). The market for low calorie sweenteners from plants is up to US$ 1.5 billion just in the US is governing the search for plants and enzymes with desired properties to result in healthy natural sweeteners (Kinghorn and Soejarto, 2002).

The monoglucuronide of the similar terpenoid sweetener glycyrrhizin from liquorice roots (*Glycyrrhiza glabra*) is already commercially produced using a β-glucosidase which cleaves selectively the 1,2-β linkage of the two glucuronic acid residues (Tanaka, 1997).

TABLE 1 Plant glycoside sweeteners and bitter glycosides

Name of the Compound	Plant source	Structure	(+) fold sweeter than sucrose (-) bitter, threshold
Stevioside	*Stevia rebaudiana*	Diterpene glycoside	+ 210
Rebaudioside A	*Stevia rebaudiana*	Diterpene glycoside	+ 240
Rubososide	*Rubus suavissimus*	Diterpene glycoside	+ 110
Glycyrrhizin	*Glycyrrhiza glabra*	Triterpene glycoside	+ 170
Neohesperidin dihydrochalcone	*Citrus spec.*	Hydrogenated flavanone glycoside	+ 1000
Naringin	*Citrus pummelo*	Flavanone diglycoside	(-)
Limonoid glycoside	*Citrus unshiu*	Diterpenoid glycosdide	(-)
Gentiopikrin	*Gentiana lutea*	Lactone glycoside	(-)

The importance of a position-specific transfer, combined with the high position specificity of some GTs should be emphasized again as essential for sensoric properties. The successful cloning and expression of a position-specific naringenin 7-*O*-glucoside-1,2 rhamnosyltransferase from *Citrus* fruits resulted in the production of the bitter neohesperidoside (Bar-Peled et al., 1991; Frydman et al., 2004). In contrast, the 1,6-linked disaccharid is a tasteless compound. Like rebaudioside A and the Solanaceae pollen flavonoids, neohesperidoside contains a 1,2-α-glycosidic bound between two sugars, which may be a characteristic feature for the interaction and perception with unknown receptors (Taylor and Miller, 2002). Irrespectively of the unknown function in plants, cloning and characterization of the newly discovered *Citrus* GT will now likely result in fruits and vegetables with altered aromatic properties.

Glycosylation is a process difficult to be controlled organo-chemically *in vitro*. Therefore, isolation of GTs with desired specificities are also of major interest for organic synthesis. Arendt et al. (2001), by heterologous expression of arbutin synthase from *Rauvolfia serpentina*, produced arbutin, a potent inhibitor of human melanin biosynthesis, which was directly released into the culture medium. The extremely promiscuous specificity of this

enzyme promises rewarding discoveries within the GT or MT superfamily in the search for specific targets (Hefner et al., 2002).

Production of pharmacologically important compounds is often hampered by their low water solubility, like in case of the yellow coloured curcumin (diferuloylmethane), used as food additive and in traditional folk medicine for its anti-inflammatory properties, its anti-cancer properties and recently, to reduce alcohol induced liver disease. Two glucosyltransferases from *Catharanthus roseus* (whatever their physiological function might be) with broad substrate specificities have been functionally expressed and displayed activity towards the diferuloyl conjugate curcumin and its monoglucoside, to give curcumin diglycoside (Kaminaga et al., 2004). Strongly increased water solubility of the resulting glycosides may promote their further use as potent antioxidants. Increased solubility and stabilization was also achieved for the bitter apocarotinoid aglycone crocetin by a GT-activity isolated from the stigmas of *Crocus sativus* (Moraga et al., 2004). Crocetin, and the corresponding glycosides crocin and picrocrocin are responsible for the yellow colour of saffron, the dried stigmas of this plant, the world´s most expensive spice. These examples not only illustrate the power of plant biosynthetic systems, but may also encourage researchers to screen exotic plant species for activities and properties, which are otherwise difficult to achieve by synthetic chemistry.

Health promoting *in vitro* antioxidative effects of a variety of anthocyanins and vitamins in fruits and vegetables are often scavenged by the rapid oxidation, degradation, and poor uptake of these compounds *in vivo* (Rice-Evans et al., 1997; Arts et al., 2004). Rational design and production of compounds with enhanced bioavailability either by transgenic approaches *in vivo* or by combinatorial enzymology *in vitro* could facilitate the production of non-toxic antioxidants and vitamins for human consumption (Křen and Martinková, 2001).

Today the market for ornamental plants and cut flowers totals over US$ 90 billion. Therefore, variation of flower colour is not only a matter of plant reproduction, but promises huge economical benefits for the cut-flower industry. The search for a blue or a black rose, is an ongoing, and money consuming vision for years (Mol et al., 1998). Recently, after the commercial release of the first bluish carnation “Moondust” in 2004 the Japanese company Suntory Ltd. successfully created the first blue rose by genetically engineering the presence of the highly substituted anthocyanidin delphinidin, which does not naturally occur in roses. To match the demands and needs of the market, successful introduction of the anthocyanidin core structure, will likely be followed by efforts to enhance colour formation e.g. by genetically engineering regiospecific GTs and MTs which might promote intra- and intermolecular

stacking (Markham et al., 2004; Mori et al., 2005). This will not only result in a set of transgenic cut-flowers, but pave the way to understand the molecular principles of flower colouration.

What holds true for flower colour, definitely is the case for volatiles, where the complexity of genes and enzymes involved in the emission of floral scents is just emerging (Schieberle and Hofmann, 2003; Dudareva et al., 2004). Genetically engineered design of methylated or esterified aroma compounds *in planta* or *in vitro*, should result in a stable bouquet of flavours with a potential use in health promoting issues like aroma therapy or the control of plant-insect interactions (Pare and Tumlinson, 1999). In addition, controlling the specificity and selectivity of the sugar transfer could facilitate the trapping and release of aromatic compounds, with the aim to produce nutrients with desired aromatic properties (Schieberle and Hofmann, 2003).

Class I OMTs involved in lignin modification are key players in various efforts to modify lignin contents of plants (e.g. Zeneca, British patent 9119279.9). A decrease in the lignin content of the raw material or a shift of the ratio of guaicyl/syringyl lignin could increase pulp yield and aid delignification. For forage crops this would increase the digestibility and energy value of animal food. In contrast, production of woody materials with a greater percentage of lignin than normal should result in stronger and more resistant wood products, as well as improve the fuel value.

10. Summary and Outlook

The development of both molecular and biochemical tools have greatly accelerated the research on plant natural product glycosyl- and methyltransferases. We and others have shown that regio- and position specificity are more consistent with amino acid sequence based cluster formation than substrate specificities. Although it may not yet be possible to predict precisely the specificity of a newly discovered enzyme, progress in functional characterisation of both superfamilies of enzymes have greatly facilitated the annotation of newly described sequences. Difficulties to unequivocally correlate the *in vivo* specificity with the observed *in vitro* properties are largely due to the promiscuous nature of both classes of enzymes. But this promiscuity may be an important strategy in evolution to guarantee that local and global environmental changes, accompanied by ecochemical challenges, are not necessarily perceived as a risk, but as the driving force to promote new properties and developments.

Two key issues may influence research on GTs and MTs in the future. One will be concerned with the elucidation of specific protein structures to describe and understand how enzyme specificity is governed by individual protein domains. A solid basis has already been generated by our lab and the work of others using molecular modelling and crystallography to understand the principles of sugar and methyl group transfer to plant products. Progress to determine protein structures in solution by NMR spectroscopy could help to solve currently difficult or impossible to crystallize enzymes, like the soluble or membrane-bound glycosyl- and glucuronosyltransferases. Visions to create new enzymes with desired properties *in silico* or by rational experimental design may not be too far fetched. The second, even more ambitious task will be concerned with the function, regulation and integration of these enzymes into complex metabolic systems and requires support by genomics, proteomics, metabolomics, and bioinformatics. Based on the wealth of information and knowledge currently generated it will be only be a question of time that this research will result not only in scientific but also in economical benefits.

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Notes added in proof:

The following important references with emphasis on the structural biochemistry of glycosyltransferases appeared after submission of thís thesis:

Thorsøe K, Bak S, Olson CE, Breton A, Imberty C, Møller BL (2005) Determination of catalytic key amino acids and UDP sugar donor specificity of the cyanohydrin glycosyltransferase UGT85B1 from *Sorghum bicolor*. Molecular modeling substantiated by site-specific mutagenesis and biochemical analyses. Plant Physiol. 139, 664-673.

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Declaration

With this statement I confirm that this Habilitation has been performed without any additional help or resources from outside. All sources which have been used are cited in the text or are marked as such.

Halle, (Dr. Thomas Vogt)

Curriculum vitae

Name: Thomas Vogt
Date of birth: August, 6th, 1960
Place of birth: St. Goar, Germany
Citizenship: German
Family status: single

Educational degrees:

High School degree: 1979 German Abitur

University degrees: 1980 to 1985, Cologne, Germany
1986 Diploma thesis in Biology at the University of Cologne
1989 PhD thesis at the Department of Botany, University of Cologne with Prof. Hans Reznik on "Extracellular and intracellular flavonoids in the genus *Cistus*, L., Cistaceae"
PhD "Dr. rer. nat." (magna cum laude)

Positions held:

1990-1992 Postdoctoral Feodor-Lynen-Fellowship at the Dept. Plant Science, University of British Columbia, Vancouver, B.C. Canada.with Prof. Brian Ellis. Project: Purification of sinapine synthase in *Brassica napus*

1992-1993 Post-doctoral fellowship at the Dept. of Horticulture, Washington State University, Pullman WA, U.S.A. with Prof. Loverine Taylor. Project: Flavonols and pollen development in *Petunia hybrida*

From 1993 Research Scientist at the Dept. of Secondary Metabolism, Leibniz-Institute of Plant Biochemistry, Halle (Saale), Germany with Prof. Dieter Strack.
Projects: Biochemistry and molecular biology of natural product glucosyl- and *O*-methyltransferases.

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Statement on personal contributions to the work listed in the publications

The work on pollen germination and tissue specific flavonol glucosyltransferases was performed at Washinton State University, Pullman WA, USA under the guidance of Prof. L. Taylor. As the principle investigator, discovery of the enzymes, all experiments, characterisation of suitable substrates and finally, preparing the radioactive labelled derivatives for my successor (Xu et al., 1995) were performed by myself.

In continuation of the work on glucosyltransferases, starting with Heuer et al. (1996) the forthcoming purification and characterization of the betacyanin glucosyltransferases was performed by myself in the Dept. of Secondary Metabolism at the Leibniz-Institute of Plant Biochemistry (IPB), Halle (Head: Prof. Strack). If not stated otherwise my contribution to individual publications include the enzymatic work, the cloning and characterisation of recombinant proteins, and the purification or biosynthesis of secondary metabolites. The novel approach to concentrate highly dilute proteins (Ziegler et al., 1997) was found by myself for glucosyltransferases and further developed for general use in a collaboration with Dr. Jörg Ziegler (IPB, Halle). Dr. Michael Meyer performed the computerised modelling of flavonoids (Vogt et al., 1997). The computerized modelling of UGT73A5 (Hans et al., 2004) was performed by my former PhD student, Dr. Judith Isayenkova (at that time Judith Hans) under guidance of Dr. Wolfgang Brandt (IPB, Halle). Site-directed mutagenesis and protein purification was done by myself.

The second project on class I plant natural product OMTs was initiated by myself based on observations made during a macroarray-scan of the halophyte *Mesembryanthemum crystallinum*. UV-induced accumulation of betacyanins was initiated at the IPB Halle (Vogt et al., 1999; Ibdah et al., 2002) und further improved and mathematically quantified by Dr. Andreas Krins and Dr. Harald Seidlitz (GSF, Forschungszentrum für Umwelt und Gesundheit, Munich). The enzyme was purified, cloned, and functionally expressed by my former PhD student Dr. Ibdah. Recombinant OMT-proteins were generated and characterized by myself and extended toward homologous OMTs from other species (*Ammi majus*) with the focus on kation-dependence (Vogt, 2004; Lukačin et al., 2004). Crystallisation of the recombinant proteins was done by myself with guidance and support from Dr. Daniel Rauh and Prof. Milton T. Stubbs (Dept. Biochemistry/Biotechnology, Martin-Luther-University Halle-Wittenberg). OMT crystal structure(s) were resolved by Dr. Rauh and Prof. Stubbs.

All MS- and NMR-data of isolated and purified natural products were recorded and interpreted by Dr. Jürgen Schmidt (IPB, Halle), Dr. Manfred Nimtz and Dr. Victor Wray (Gesellschaft für Biotechnologische Forschung, Braunschweig), respectively. All protein sequencing data were recorded and interpreted by Dr. Rudi Grimm (Agilent Technologies, Waldbronn).

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