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Phytochemistry 69 (2008) 88–98
PHYTOCHEMISTRY
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Metabolon formation in dhurrin biosynthesis

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Received 10 April 2007; received in revised form 26 June 2007; accepted 27 June 2007
Available online 15 August 2007

Abstract

Synthesis of the tyrosine derived cyanogenic glucoside dhurrin in *Sorghum bicolor* is catalyzed by two multifunctional, membrane bound cytochromes P450, CYP79A1 and CYP71E1, and a soluble UDPG-glucosyltransferase, UGT85B1 (Tattersall, D.B., Bak, S., Jones, P.R., Olsen, C.E., Nielsen, J.K., Hansen, M.L., Høj, P.B., Møller, B.L., 2001. Resistance to an herbivore through engineered cyanogenic glucoside synthesis. Science 293, 1826–1828). All three enzymes retained enzymatic activity when expressed as fluorescent fusion proteins *in planta*. Transgenic *Arabidopsis thaliana* plants that produced dhurrin were obtained by co-expression of CYP79A1/CYP71E1-CFP/UGT85B1-YFP and of CYP79A1/CYP71E1/UGT85B1-YFP but not by co-expression of CYP79A1-YFP/CYP71E-CFP/UGT85B1. The lack of dhurrin formation upon co-expression of the two cytochromes P450 as fusion proteins indicated that tight interaction was necessary for efficient substrate channelling. Transient expression in *S. bicolor* epidermal cells as monitored by confocal laser scanning microscopy showed that UGT85B1-YFP accumulated in the cytoplasm in the absence of CYP79A1 or CYP71E1. In the presence of CYP79A1 and CYP71E1, the localization of UGT85B1 shifted towards the surface of the ER membrane in the periphery of biosynthetic active cells, demonstrating *in planta* dhurrin metabolon formation.

Keywords: Cytochrome P450; Glucosyltransferases; Multienzyme complexes; Green fluorescent protein; Dhurrin; Cyanogenic glucosides; CYP79A1; CYP71E1; UGT85B1

1. Introduction

Cyanogenic glucosides are amino acid-derived natural products (Conn, 1981; Zagrobelny et al., 2004; Bak et al., 2006; Morant et al., 2007). The ability to synthesize these glucosides is common across many plant genera, including several plant species that are important crop plants like sorghum (*Sorghum bicolor*), cassava (*Manihot esculenta*), flax (*Linum usitatissimum*) and almonds (*Prunus dulcis*). Degradation of cyanogenic glucosides is catalyzed by β-glucosidases and α-hydroxynitrilases and results in release of hydrogen cyanide. Accordingly, cyanogenic glucosides are classified as phytoanticipins that may play a role in plant defense (Tattersall et al., 2001). The biosynthetic pathway for the cyanogenic glucoside dhurrin has been

Abbreviations: CaMV, cauliflower mosaic virus; CFP, cyano fluorescent protein; CLSM, confocal laser scanning microscopy; YFP, yellow fluorescent protein; 3D, three dimensional.

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doi:10.1016/j.phytochem.2007.06.033

elucidated using a microsomal system from sorghum that catalyses the conversion of the parent amino acid L-tyrosine to the aglycone *p*-hydroxymandelonitrile (Morant et al., 2003; Møller and Seigler, 1999). The pathway involves a number of unusual and labile intermediates like an *N*-hydroxyamino acid, an *N,N*-dihydroxyamino acid, *E*- and *Z*-oximes and a cyanohydrin (Fig. 1). Except for the *Z*-oxime, these intermediates are efficiently channelled for dhurrin production and difficult to trap during biosynthesis (Møller and Conn, 1980; Kahn et al., 1997; Sibbesen et al., 1995; Kristensen et al., 2005). At the genetic level, the pathway is surprisingly simple because the conversion from tyrosine to the aglycone is catalysed by two multifunctional cytochromes P450 (CYPs) each encoded by a single structural gene. CYP79A1 catalyzes conversion of L-tyrosine into *Z*-*p*-hydroxyphenylacetaldoxime (Koch et al., 1995; Sibbesen et al., 1995) which is subsequently converted by CYP71E1 to the cyanohydrin *p*-hydroxymandelonitrile (Bak et al., 1998; Kahn et al., 1997). Finally, the labile *p*-hydroxymandelonitrile is stabilized by glucosylation *via* a soluble UDP-Glc glucosyltransferase UGT85B1 to produce dhurrin (Jones et al., 1999; Kahn et al., 1999; Thorsøe et al., 2005). The entire pathway for dhurrin synthesis has been transferred to *Arabidopsis thaliana* and *Lotus japonicus* using genetic engineering (Tattersall et al., 2001; Morant et al., 2003,2007; Kristensen et al., 2005). The dhurrin content of these transgenic *A. thaliana* plants is high and accounts for 4% (w/w) of leaf dry-weight. As with *S. bicolor*, pathway intermediates were hardly detectable.

Glycosyltransferases involved in plant natural product synthesis primarily belong to family 1 and are designated UDP-glycose-glycosyltransferases (UGTs) (Mackenzie et al., 1997). The fully sequenced *A. thaliana* genome (The Arabidopsis Genome Initiative, 2000) has provided annotation of 112 predicted full-length family 1 UGTs and eight apparent pseudo-genes (Paquette et al., 2003). All three biosynthetic enzymes including sorghum UGT85B1 are required for dhurrin production in transgenic *A. thaliana* plants (Tattersall et al., 2001). Thus none of the endogenous *A. thaliana* glucosyltransferases available are able to mediate α-carbon specific glucosylation of *p*-hydroxymandelonitrile. Like other cyanohydrins, *p*-hydroxymandelonitrile is labile at physiological pH dissociating into *p*-hydroxybenzaldehyde and hydrogen cyanide (Fig. 1). To avoid constant generation of toxic hydrogen cyanide in those parts of the sorghum plant where dhurrin synthesis takes place, UGT85B1 is envisioned to glucosylate the labile cyanohydrin before it dissociates. The early

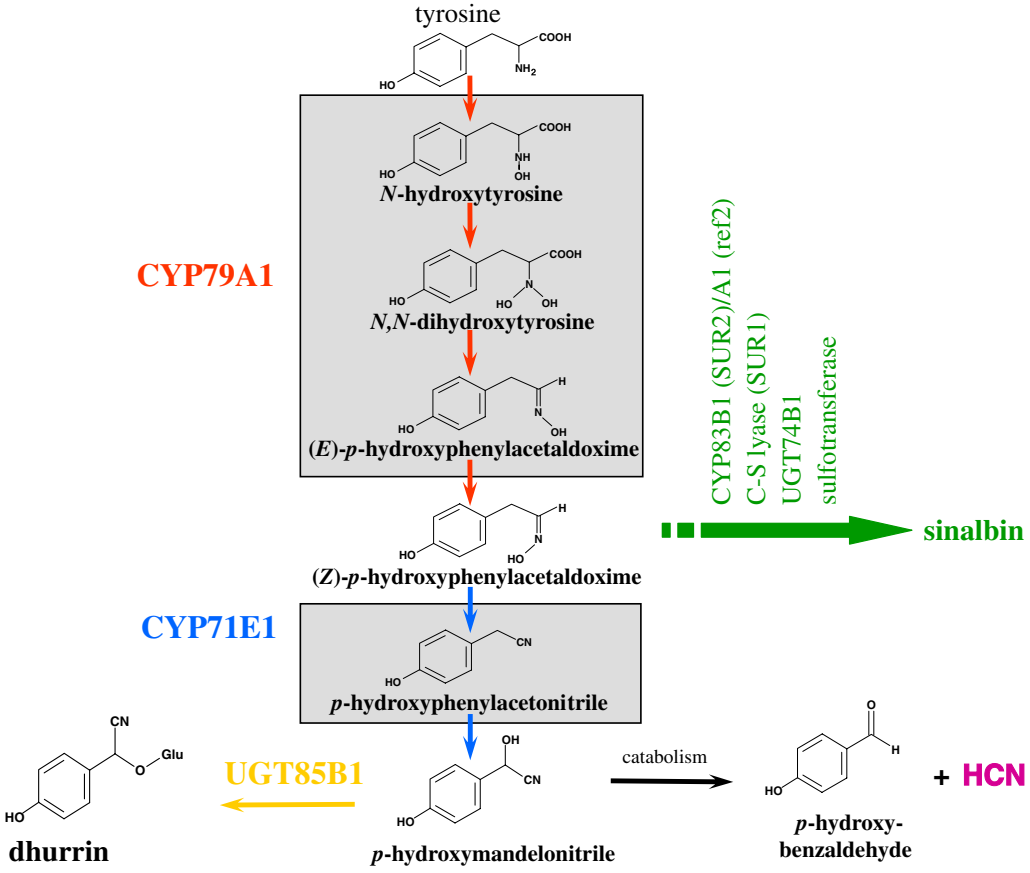

Fig. 1. Biosynthetic pathway for the cyanogenic glucoside dhurrin. The scheme shows the enzymatic reactions catalysed by each of the three biosynthetic enzymes CYP79A1, CYP71E1 and UGT85B1 isolated from *Sorghum bicolor*. Intermediates entrapped in the active sites of the two multifunctional cytochrome P450s are shown on a gray background.

intermediates in dhurrin biosynthesis are also labile. Entrapment of the majority of these early intermediates is secured by their formation and further conversion within the active sites of the multifunctional and membrane-bound CYP79A1 and CYP71E1 (Fig. 1) (Sibbesen et al., 1995; Bak et al., 1998). A mechanism to ensure efficient transfer of the labile *Z*-aldoxime that is the product of CYP79A1 and the substrate for CYP71E1 must also be envisioned. UGT85B1 is isolated as a soluble enzyme (Jones et al., 1999; Thorsøe et al., 2005). Efficient *in vivo* entrapment of the *Z*-aldoxime and of the cyanohydrin could be mediated by assembly of a metabolon in which UGT85B1 associates tightly to a CYP71E1-CYP79A1 enzyme complex (Figs. 1 and 2).

To investigate the possibility of metabolon formation directly at the molecular level, the two membrane bound CYPs as well as the cytosolic UGT85B1 were stably and transiently expressed as fluorescent fusion proteins in *A. thaliana* and *S. bicolor*, respectively. Analyses of biosynthetic activity supported by determination of the subcellular localization of UGT85B1 in the presence or absence of CYP79A1 and CYP71E1 by the use of confocal laser scanning microscopy (CLSM) provided further evidence for metabolon involvement in dhurrin synthesis.

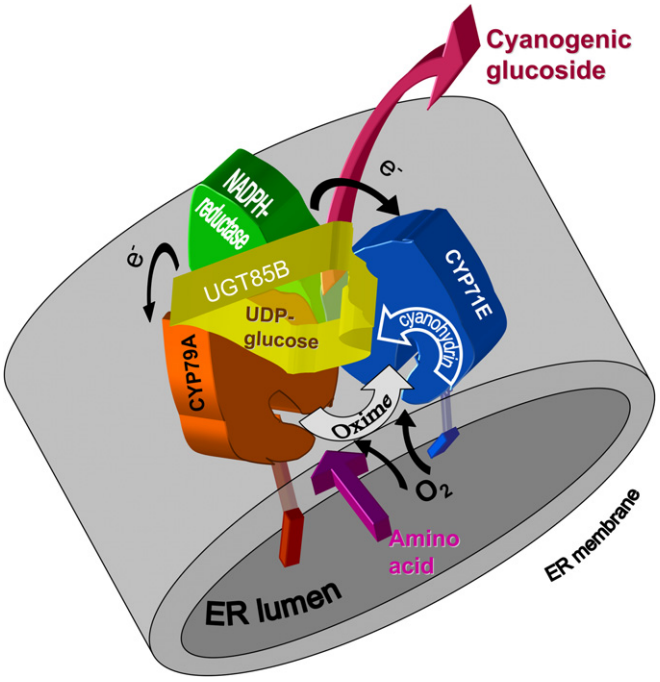

Fig. 2. Metabolon composed of the two membrane bound cytochromes P450 CYP79A1 and CYP71E1 and the cytosolic UGT85B1 localized at the cytosolic surface of the endoplasmic reticulum. An NADPH dependent membrane bound NADPH-cytochrome P450 oxidoreductase provides reducing power in the form of single electrons. The substrate L-tyrosine enters the catalytic site of CYP79A1 together with molecular oxygen O_2. Two molecules of O_2 are used by CYP79A1 to form *Z*-*p*-hydroxyphenylacetaldoxime. CYP71E1 converts *Z*-*p*-hydroxyphenylacetaldoxime to the cyanohydrin *p*-hydroxymandelonitrile with the consumption of a single molecule of oxygen. The aglycon is glycosylated by the UDPG dependent glycosyltransferase, UGT85B1, and released as dhurrin.

2. Results

2.1. Functional characterization of the fusion proteins

Fusion proteins of CYP79A1, CYP71E1 and UGT85B1 were designed with the attachment of YFP or CFP as a fluorescent marker. CYP79A1 and CYP71E1 contain N-terminal sequences buried in the ER membrane as a membrane anchor (Bak et al., 1998; Koch et al., 1995) (Fig. 2). Accordingly, to avoid interference with membrane insertion, the CYP-fusion proteins were designed with the fluorescent proteins attached to the C-terminus (Fig. 3). For detailed analyses of subcellular localization, combinations of YFP and CFP markers were co-integrated into plant cells by use of the binary vector pPZP221.101ab, a plasmid allowing cloning and expression of two individual genes each under the control of the CaMV35S promoter for example one gene fused to a YFP coding sequence together with a second gene encoding cytosolic, free CFP such as pPZP221.UGT85B1-YFP.CFP (Fig. 3). This design allowed screening for strong, fluorescent signals originating from cytosolic accumulation of marker protein to ease and validate detection of weaker fluorescence from fusion proteins *in planta*. Sequential scans of the two markers when subjected to CLSM enabled a thorough analysis in 3D reconstructions of cells to distinguish relative fluorescence intensities and differences in distribution of fluorescence even when both fluorescent markers accumulated in the same subcellular compartment.

The *in planta* activity of the heterologously expressed native proteins or fusion proteins was determined by LC/MS metabolite profiling of transgenic *A. thaliana* plant lines harbouring different protein constructs with special emphasis on dhurrin and sinalbin (*p*-hydroxybenzylglucosinolate) formation (Fig. 1, Table 1). In transgenic *A. thaliana* plants expressing CYP79A1 as the only transgene, metabolite profiling demonstrated that the *p*-hydroxyphenylacetaldoxime produced from tyrosine in these plants was effectively used by the endogenous enzymes in the glucosinolate pathway (CYP83B1/CYP83A1, C-S Lyase (SUR1), UGT74B1, sulfotransferase), to produce the glucosinolate sinalbin (*p*-hydroxybenzyl glucosinolate) (Fig. 1, Table 1). This was easily demonstrated experimentally, because wild-type *A. thaliana* does not contain this tyrosine derived glucosinolate. The amount of sinalbin produced corresponded to ~3% of leaf dry weight. This is in close agreement with data previously obtained (Bak et al., 2000; Kristensen et al., 2005) and stoichiometrically corresponds to the amount of dhurrin produced when the entire dhurrin pathway was introduced into *A. thaliana* by co-expression of *CYP79A1*, *CYP71E1* and *UGT85B1* (Bak et al., 2000; Tattersall et al., 2001; Kristensen et al., 2005).

A similar amount of dhurrin as observed upon co-expression of *CYP79A1*, *CYP71E1* and *UGT85B1* was also formed in transgenic plants expressing *CYP79A1*, *CYP71E1* and *UGT85B1-YFP i.e.* when UGT85B1 was

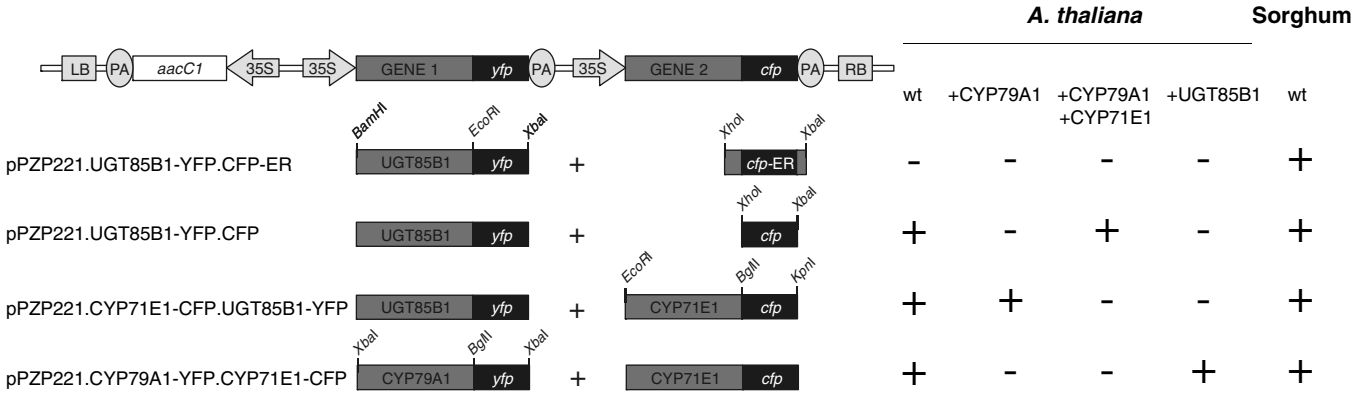

Fig. 3. Expression constructs used in the transformation of *A. thaliana* and *S. bicolor*. (A) A schematic illustration of each vector construct showing the integrated DNA region between the left border (LB) and right border (RB) of pPZP221, which was used for transformation. Each chimera gene was cloned between the CaMV 35S promoter (35S) and a polyadenylation signal (PA). The pPZP221 plasmid contains the *aacC1* gene, which confers resistance to gentamycin. (B) A table summarising the different genetic backgrounds of *A. thaliana* into which constructs shown in A were stably expressed and the constructs used for transient expression in sorghum.

Table 1
Relative content of the cyanogenic glucoside dhurrin and of the glucosinolate sinalbin in wild-type and in transgenic Arabidopsis plants expressing different gene constructs

Transgene inserted to express	Relative content (%)	
	Sinalbin	Dhurrin
None (wild-type)	0	0
CYP79A1	100*	0
CYP79A1/CYP71E1	14	0
CYP79A1/CYP71E1/UGT85B1	0	100**
CYP79A1/CYP71E1/UGT85B1-YFP	6	110
CYP79A1-YFP/CYP71E1-CFP	0	0
CYP79A1/CYP71E1-CFP/UGT85B1-YFP	1	6

* The sinalbin content of the plants transformed to express CYP79A1 constituted 3% of leaf dry weight and is set as 100%.
** The dhurrin content of the plants transformed to express CYP79A1/CYP71E1/UGT85B1 constituted 4% of leaf dry weight and is set as 100%.

replaced by the fusion protein *UGT85B1-YFP* (Table 1). Because no dhurrin production is observed in *CYP79A1* and *CYP71E1* expressing *A. thaliana* plants (Tattersall et al., 2001; Kristensen et al., 2005), this unambiguously demonstrated that UGT85B1-YFP was enzymatically active in the transgenic plants and able to ensure fast and efficient conversion of the labile *p*-hydroxymandelonitrile into dhurrin (Table 1). Plants expressing *CYP79A1* and *CYP71E1* appeared stressed and displayed a stunted morphological phenotype (Kristensen et al., 2005). Upon co-expression of *UGT85B1-YFP*, the wild-type phenotype was recovered. Thus, the UGT85B1-YFP fusion protein retained the structural integrity and catalytic activity of the unmodified UGT85B1 protein.

Dhurrin was also formed in transgenic *A. thaliana* plants harbouring constructs for *CYP79A1*, *CYP71E1-CFP* and *UGT85B1-YFP* (Table 1). This demonstrated that the CYP71E1-CFP fusion protein was also enzymatically active in plants. The dhurrin content in these plants was about one tenth the level obtained in transgenic *A. thaliana* plants expressing *CYP79A1*, *CYP71E1* and *UGT85B1* (Bak et al., 2000; Tattersall et al., 2001; Kristensen et al., 2005) or expressing *CYP79A1*, *CYP71E1* and *UGT85B1-YFP* (Table 1). This may indicate that simultaneous presence of the fusion proteins CYP71E1-CFP and UGT85B1-YFP abolished proper interaction. The transgenic *A. thaliana* plants harbouring constructs for *CYP79A1*, *CYP71E1-CFP* and *UGT85B1-YFP* also accumulated sinalbin but at a much lower level corresponding to 0.002% of plant dry weight or ~0.6% of the level found in the *CYP79A1* expressing plants discussed above. This demonstrated that *in planta*, the CYP71E1-YFP fusion protein was able to prevent the endogenous glucosinolate producing enzymes from converting *p*-hydroxyphenylacetaldoxime produced by CYP79A1 into sinalbin. This may either reflect effective CYP71E1-CFP catalyzed conversion of *p*-hydroxyphenylacetaldoxime into *p*-hydroxymandelonitrile or direct physical association between CYP79A1 and CYP71E1-YFP that abolished access and binding of the endogenous *p*-hydroxyphenylacetaldoxime metabolizing enzymes CYP83B1 and CYP83A1 in glucosinolate synthesis (Fig. 1). Because little dhurrin was produced, it can not be excluded that the CYP71E1-CFP fusion protein was less active than the native enzyme. The *CYP79A1*, *CYP71E1-CFP* and *UGT85B1-YFP* expressing plants exhibited no morphological phenotype.

2.2. Localization of UGT85B1 in transgenic *A. thaliana* plants

The subcellular localization of the UGT85B1-YFP fusion protein in *A. thaliana* was studied by CLSM using transgenic *A. thaliana* plants obtained by pollination of *CYP79A1* plus *CYP71E1* expressing plants with plants expressing *UGT85B1-YFP* plus free *CFP* (Fig. 4A–F). In root cells of the homozygotic progeny, extension of the entire cytosolic space in the root cells was visualized by accumulation of a cytosolic version of CFP (Fig. 4A and D). The accumulation of UGT85B1-YFP in the presence

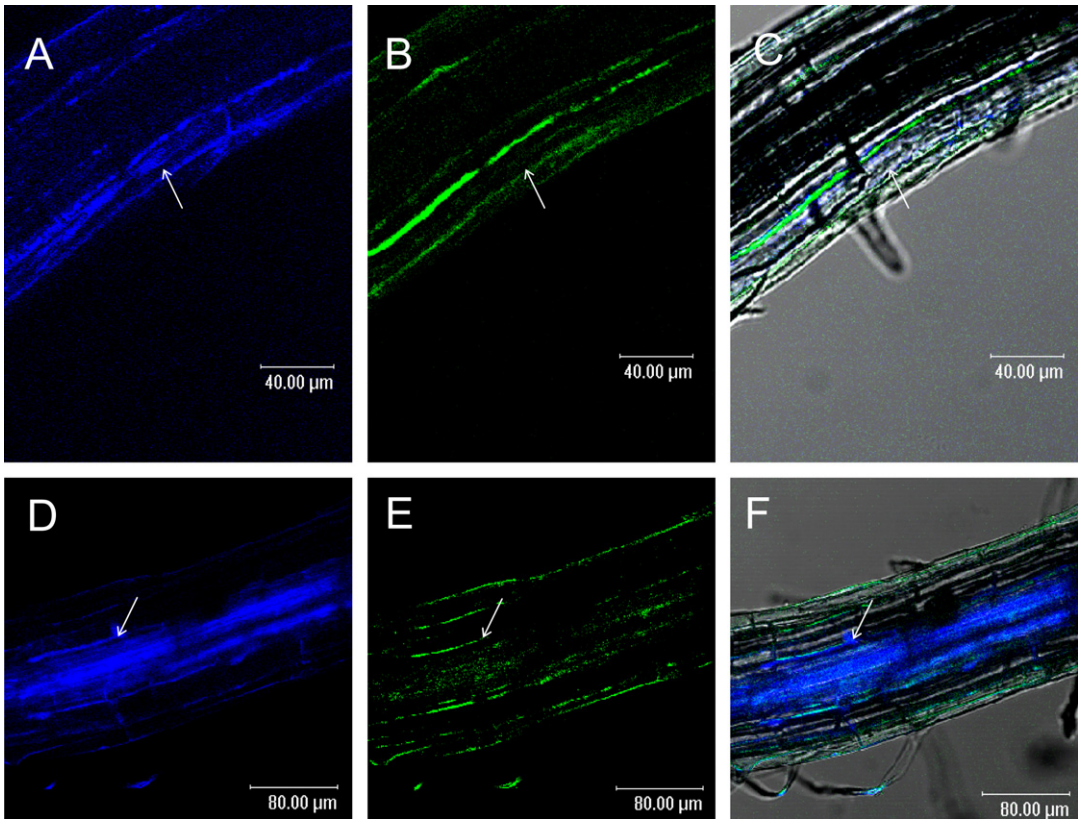

Fig. 4. Location of UGT85B1-YFP fusion protein in transgenic *A. thaliana* root cells from plants obtained by crossing of plants expressing *UGT85B1-YFP* plus *CFP* with plants expressing *CYP79A1* and *CYP71E1*. Panel A: The distribution of the entire cytosol as visualized by fluorescence from free CFP. Panel B: Distribution of the UGT85B1-YFP fusion protein is not uniform throughout the cytosol with an enhanced accumulation of UGT85B1-YFP towards the plasmamembrane. Panel C: Corresponding overlay presentation on transmitted light image to visualize the enhanced accumulation of green fluorescence from UGT85B1-YFP towards the plasma membrane of the cells. Panels D-F: Similar images as presented in Panels A–C but obtained with a different transgenic line. In all panels, 3D reconstructions are presented using blue color code for CFP and green for YFP. Arrows point towards cytosolic space with preferential CFP accumulation.

of CYP79A1 and CYP71E1 (Fig. 4B and E) did not match the cytosolic distribution of free CFP (Fig. 4A and D) as evident from the overlay projections on transmitted light images (Fig. 4C and F) of 3D reconstructions of the images obtained with the two different fluorescent markers. These differences with respect to the cellular localization of CFP and UGT85B1-YFP in the presence of CYP79A1 and CYP71E1 indicated the possibility of *in planta* formation of a specific endoplasmatic reticulum localized dhurrin metabolon comprised of CYP79A1, CYP71E1 and UGT85B1. Data obtained from roots were presented to avoid autofluorescence from chloroplasts and natural products present in leaves. As expected from the literature (Chytilova et al., 1999), the CaMV 35S promoter directed strong expression in the root tip and root pericycle cells.

2.3. Localization of UGT85B1 in S. bicolor

The studies in transgenic *A. thaliana* were based on expression of sorghum-derived genes. These studies were extended to the homologous *S. bicolor* plant using transient expression of the fusion constructs in epidermal cells from leaves of 2-week-old light-grown seedlings bombarded with gold particles to introduce different gene fusions. Cells expressing high levels of fluorescent cytosolic marker protein (CFP or YFP) were easily identified and a number of these cells were subjected to CLSM to localize co-expressed fusion protein(s). Accumulation of cytosolic YFP and CFP demonstrated networks of intracellular cytoplasmic bridges traversing large central vacuoles with the major volume of the cytosol located around the nucleus and at the outer sphere of the epidermal cells (Fig. 5A). The large central vacuole of each epidermal cell is visualized as dark areas by Neutral Red staining (Fig. 5D). When expressed in a wild-type background, the UGT85B1-YFP fusion protein was found to be localized throughout the cytosol with a relatively high fluorescence signal obtained from the periphery of the cells and from trans-vacuolar cytoplasmic bridges (Fig. 5B). Co-expression of UGT85B1-YFP with CYP79A1 and CYP71E1 resulted in a highly uneven and distinct localization of UGT85B1-YFP with the main part being located in the cytosol in selected regions at the outer sphere of the cell (Fig. 5C). Like most other plant cytochrome P450s, CYP79A1 and CYP71E1 are anchored at the cytoplasmic surface of the ER through a short hydrophobic membrane spanning segment near the N-terminal

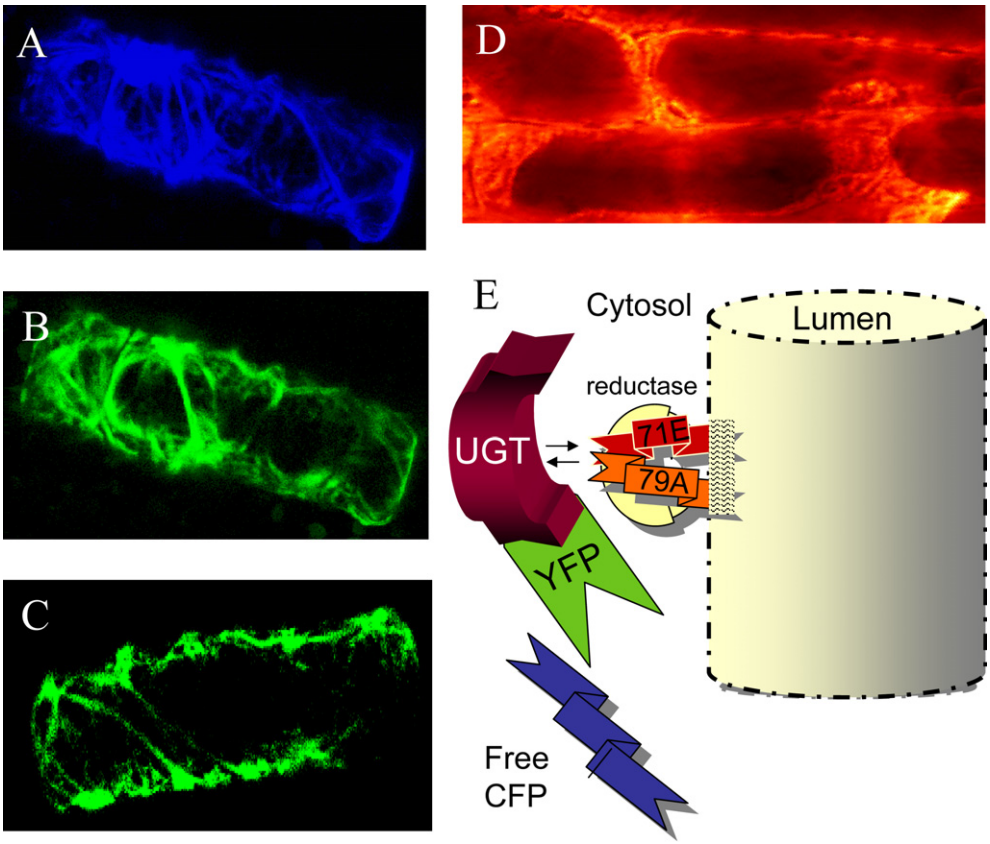

Fig. 5. Formation of the dhurrin metabolon as studied by transient expression in sorghum epidermal cells. 3D reconstructions are presented using blue color code for CFP and green for YFP. Panel A and B: Expression of *CFP* and *UGT85B1-YFP*, respectively, in the same cell. CFP and the UGT85B1-YGP fusion protein co-localize except that some cytosolic strands do not appear to contain UGT85B1. Panels C: Cellular localization of UGT85B1-YFP upon co-expression of *UGT85B1-YFP*, *CYP79A1* and *CYP71E1*. The UGT85B1-YFP fusion protein is essentially absent from the trans-vacuolar cytoplasmic bridges and the main part located in the cytosol in selected regions at the outer sphere of the cell. Panel D: Visualization of the large central vacuole of the epidermal cells as dark areas in transformed as well as non-transformed cells following incubation with Neutral Red. Panel E: Schematic presentation of the association of the UGT85B1-YFP fusion protein with CYP79A1 and CYP71E1 to form a dhurrin metabolon at the surface of the ER network.

end with the major portion of the protein including the catalytic site protruding into the cytosol (Fig. 2) (Werck-Reichhart et al., 2002). Accordingly, metabolon formation would direct the UGT85B1 fusion protein to the ER as shown on the model depicted in Fig. 5E. Due to the large central vacuole present in the epidermal cells, the major portion of the ER is appressed towards the cell membrane (Fig. 5D). Accordingly, in a traverse cell section, the metabolons would be positioned at the interface between the cytosol and ER pressed against the outer periphery of the cells by the large central vacuole.

To study this possibility in more detail, the extension of the ER lumen space was visualized through the construction of an ER-targeted and -retained version of CFP (Haseloff et al., 1997) designated CFP-ER. Expression of *CFP-ER* enabled a 3D reconstruction of the extension of the ER lumen to directly visualize the intricate tubular network present throughout the cytoplasm at the outer sphere of the epidermal cells (Fig. 6A). The position of the nucleus was evident from a strong perinuclear localized fluorescence derived from CFP-ER present in the lumen of the ER surrounding the nucleus (Fig. 6B) and was contrasted by the signal from free YFP that accumulated in the nucleus except at the position occupied by the nucleolus (Fig. 6C). Strong labelling of the nucleus by free CFP was also observed in Fig. 5.

To investigate the subcellular localization of UGT85B1 in relation to the ER, *UGT85B1-YFP*, *CYP79A1*, *CYP71E1* and *CFP-ER* were transiently co-expressed. The fluorescence from CFP-ER and hence the distribution of the ER network lumen as well as the fluorescence from UGT85B1-YFP are presented in Fig. 6D and E, respectively. A preferential accumulation of the UGT85B1-YFP signal was observed in the cytosolic space that surrounded or was in close proximity to the ER as expected if UGT85B1-YFP was preferentially localized in a metabolon linked to ER segments containing CYP79A1 and CYP71E1. The transmitted light image of the same cell revealed the presence of gold particles from the cell bombardment with the highest concentration in the upper part of the image (Fig. 6F). The presence of gold particles gave rise to non-specific reflection of fluorescence apparent as bright spots at both wavelengths in Fig. 6D and E.

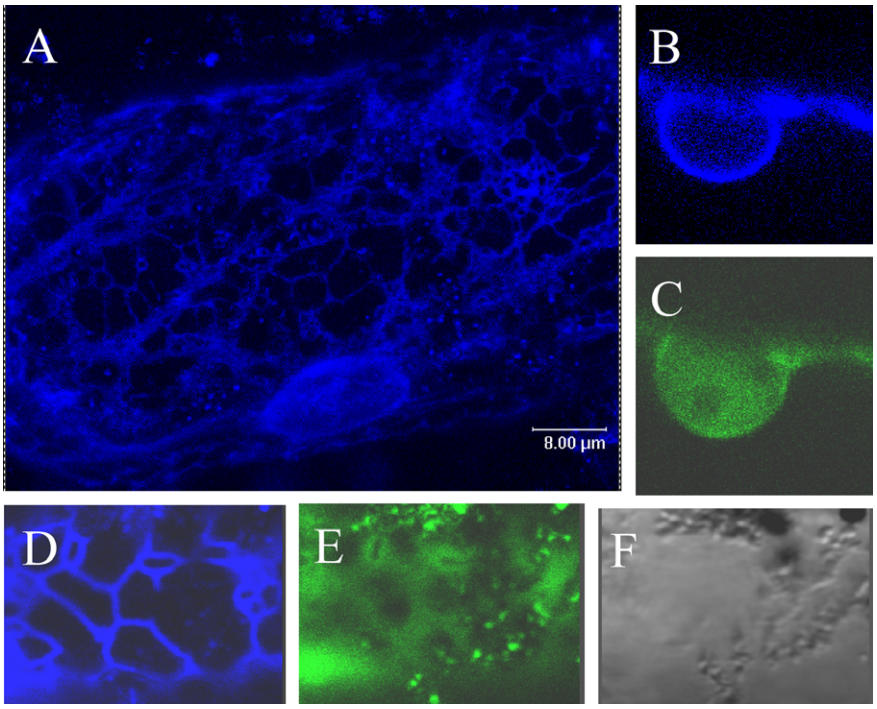

Fig. 6. Formation of the dhurrin metabolon as studied by transient expression of gene fusion constructs in sorghum epidermal cells. Panel A: Expression of *CFP-ER* to monitor the extension of the ER lumen. Panel B: ER surrounding the perinuclear membrane fluoresces due to accumulation of the CFP-ER fusion protein. Panel C: Expression of *YFP* in the nucleus except for the region harbouring the nucleolus. Panels D–F: Images within a single focal plane of the same cell to monitor extension of the ER-lumen by the fluorescence of CFP-ER (Panel D), preferential location of UGT85B1-YFP at or near the surface of segments of the ER in the cell periphery when co-expressed with CYP79A1 and CYP71E1 (Panel E) and corresponding transmitted light image (Panel F).

3. Discussion

In recent years, the importance of metabolons in plant natural product synthesis has gained new momentum (Winkel, 2004; Jørgensen et al., 2005). In the biosynthesis of flavonoids, studies using the yeast two hybrid system, affinity chromatography, co-immuno-precipitation and immuno-localization strongly support specific interactions among at least one cytochrome P450 enzyme and several soluble enzymes (Burbulis and Winkel-Shirley, 1999; Saslowsky and Winkel-Shirley, 2001). Epitope-tagging of cinnamate 4-hydroxylase and L-phenylalanine ammonia lyase (isoform 1) demonstrated that these two enzymes co-localized on the ER and that cinnamate 4-hydroxylase might serve to organize the complex for membrane association and channelling (Achnine et al., 2004). GFP imaging of single enzymes has also provided some evidence for existence of protein–protein interactions (Ro et al., 2001) and represents a powerful, non-invasive technique for the investigation of intracellular protein localization (Hanson and Köhler, 2001). In general, evidence for metabolon formation is difficult to obtain and a prerequisite is a detailed molecular, biochemical and genetic knowledge of the pathway of interest.

Early studies on the biosynthesis of the cyanogenic glucoside dhurrin in which two substrates labelled with different radioisotopes were simultaneously administered to the enzyme system had shown that the biosynthetic pathway proceeded in a highly organized enzyme system where all internally produced intermediates except (*Z*)-*p*-hydroxyphenylacetaldoxime were utilized 25–115 times more efficiently in the subsequent step compared to externally added intermediates (Møller and Conn, 1980). The first two enzymes in the dhurrin pathway, CYP79A1 and CYP71E1, are membrane bound multi-functional cytochrome P450s and carry out six of seven steps in dhurrin synthesis with (*Z*)-*p*-hydroxyphenylacetaldoxime as the inter-enzymatic intermediate (Morant et al., 2003). The last step involves glycosylation of the labile cyanohydrin *p*-hydroxymandelonitrile by soluble UGT85B1. The ability to engineer high amounts of dhurrin synthesis in transgenic *A. thaliana* plants without inadvertent effects on the transcriptome and metabolome further underpinned the significance of efficient substrate channelling (Kristensen et al., 2005). In the present work, we have gained further knowledge on the subcellular localization and interaction of the three biosynthetic enzymes using CLSM and spectral variants of GFP as tags.

Using various *A. thaliana* transformants, we were able to show that chimeric fluorescent fusion proteins of CYP79A1, CYP71E1 and UGT85B1 retained biochemical function *in planta*. Two lines of evidence can be used to support metabolon formation. Firstly, plants containing CYP79A1-YFP, CYP71E1-CFP and native UGT85B1

did not accumulate detectable amounts of dhurrin. This indicated that CYP79A1-CFP and CYP71E1-YFP were unable to interact in a multi-enzyme complex, when both were fused to fluorescent proteins although each separate fusion protein retained its catalytic activity. Secondly, in *A. thaliana* plants containing UGT85B1-YFP and free CFP, the two respective fluorescence signals were obtained from the cytoplasmic space. In contrast, the YFP derived fluorescence signal in plants containing UGT85B1-YFP in the presence of CYP79A1 and CYP71E1, was not uniformly distributed within the cytosol. The shifted subcellular position of the fluorescence signal from UGT85B1-YFP, and the ability of these transgenic *A. thaliana* plants to accumulate dhurrin indicated that the fusion protein of UGT85B1 was able to interact with the membrane bound partner enzymes and participate in assembly of a functional metabolon. This is in spite of the attachment of YFP to the C-terminus of UGT85B1. Importantly, co-introduced, cytosolic CFP did not show distinct subcompartmental accumulation in response to presence/absence of the cytochrome P450 proteins.

In the transient expression studies in *S. bicolor*, fluorescent signals obtained from epidermal cells containing UGT85B1-YFP and CFP or CFP-ER were strong and indicated that UGT85B1-YFP localized to regions associated with the endomembrane complex when the epidermal cells were co-transformed with genes encoding native versions of CYP79A1 and CYP71E1. This provided an independent verification of metabolon formation supporting the results obtained in stably transformed *A. thaliana* (Fig. 4). In all experiments, co-expression of genes encoding free CFP, which located to the entire cytoplasmic space and the nucleus, or of CFP-ER, which located throughout the lumen of the endomembrane complex, including the perinuclear membrane, served as controls.

Specific ER compartmentalization with the formation of distinct ER bodies has been demonstrated in *A. thaliana* (Matsushima et al., 2003). These ER bodies were shown to contain a β-glucosidase that was completely absent from cisternal ER. As in the present study, an ER directed GFP protein was shown to accumulate evenly in the entire ER (Matsushima et al., 2003). Likewise, a high-throughput viral expression system exploiting CLSM of unknown expressed plant proteins by monitoring cDNA-GFP protein fusions revealed a number of novel subcompartments within organelles (Escobar et al., 2003). The two ''cyanogenic'' P450s CYP79A1 and CYP71E1 reported in this study may constitute yet another example from plants of such sub-compartmentalization. Envisioned evolutionary advantages of this type of sub-compartmentalization could be to enhance the concentration of the initial substrate (tyrosine), to ensure presence of an optimal isoform of the NADPH-cytochrome P450 oxidoreductase or of certain membrane lipid constituents that may help to channel dhurrin biosynthesis and facilitate post-biosynthetic translocation of the cyanogenic glucoside to the central vacuole. Future studies are needed to identify and define structural features within the enzymes that direct and control protein–protein interactions and hence the assembly of metabolons. A suitable approach might be to identify the interacting protein domains by chemical cross-linking. This approach has been successfully used to functionally characterize the photosystem I complex from barley that contains more than 20 different subunits (Jansson et al., 1996). Detailed biochemical knowledge on the sub-cellular formation and localization of metabolons will provide a vital platform for successful engineering of plant natural product synthesis in the future, particularly with respect to production of novel compounds using pre-existing scaffolds of biosynthetic pathways.

4. Experimental

4.1. Construction of GFP-fusion genes

Two spectral variants of GFP were used. The topaz spectral variant of GFP ($\lambda_{ex} = 514$ nm; $\lambda_{em} = 527$ nm) (Packard Bioscience, Meridan, CT, USA), here referred to as YFP (yellow fluorescent protein), and the enhanced cyan spectral variant of GFP ($\lambda_{ex} = 433$ nm; $\lambda_{em} = 501$ nm) (Clontech, Palo Alto, CA, USA), here referred to as CFP (cyan fluorescent protein).

The entire open reading frames (ORFs) of CYP79A1 (Bak et al., 1999), CYP71E1 (Bak et al., 1998), UGT85B1 (Jones et al., 1999), CFP and YFP were amplified by the polymerase chain reaction (PCR) using *PfuTurbo*® DNA polymerase (Stratagene, La Jolla, CA, USA). Specific primers were used to introduce unique restriction sites immediately preceding the start codons of all fore mentioned ORFs. In addition, unique restriction enzyme sites were incorporated to replace the stop codons of CYP79A1, CYP71E1 and UGT85B1. The resulting PCR products were cloned into pBluescript SK+ (Stratagene) or a into modified version of pThioHisA (Invitrogen, Carlsbad, CA, USA) called pThioHisAΔ, which was constructed by digestion with *Nde*I/*Kpn*I, followed by incubation with T4 DNA polymerase, and ligation. Fusion of CYP79A1, CYP71E1 and UGT85B1 to specific GFP spectral variants was accomplished by excision and ligation of ORFs of CFP or YFP into these plasmids. This resulted in C-termini in-frame CFP or YFP fusion ORFs. A plant ER targeted version of CFP (CFP-ER) was constructed by ligation of synthetic linkers containing the ER targeting and retainment sequences (Haseloff et al., 1997) into pECFP (Clontech) using available unique restriction enzyme sites. DNA sequencing was used to verify the correctness of all cloned products.

4.2. Construction of binary vectors for plant transformation

Initially, pPZP221 (Hajdukiewicz et al., 1994) was digested with *Sal*I/*Eco*RI, incubated with T4 DNA polymerase (New England Biolabs, Beverly, MA, USA), and

ligated to remove undesired restriction sites. The newly formed plasmid was called pPZP221Δ. Two multi-cloning site variants of the plasmid pRT101 (Töpfer et al., 1987) were then made. Firstly, the plasmid pRT101a was constructed by digesting pRT101 with *Xho*I/*Kpn*I, followed by incubation with T4 DNA polymerase and ligation. Secondly, the plasmid pRT101b was constructed by digesting pRT101 with *Sma*I/*Xba*I, followed by incubation with T4 DNA polymerase and ligation. The multi-cloning site, CaMV 35S promoter and polyadenylation signal of pRT101a was excised with *Pst*I and ligated into the *Pst*I site of pPZP221Δ to produce pPZP221Δ.101a. Similarly, the multi-cloning site, CaMV 35S promoter and polyadenylation signal of pRT101b was excised with *HinD*III, and ligated into the *HinD*III site of pPZP221Δ.101a to finally produce pPZP221Δ.101ab.

For expression studies *in planta*, the GFP fusion genes and GFP spectral variants were cloned into pPZP221Δ.101ab. For the construction of the plasmids encoding CYP71E1-CFP plus CYP79A1-YFP or UGT85B1-YFP, CYP71E1-CFP was firstly excised using *EcoR*I/*Kpn*I and ligated into the respective sites of pPZP221Δ.101ab. Secondly, CYP79A1-YFP was excised and introduced into pPZP221Δ.101ab using *Xba*I, whilst UGT85B1-YFP were excised and introduced using both the *BamH*I and *Xba*I restriction sites. This resulted in the plasmids named pPZP221.CYP79A1-YFP.CYP71E1-CFP, pPZP221.CYP71E1-CFP.UGT85B1-YFP. For the construction of the plasmid encoding UGT85B1-YFP plus CFP or CFP-ER, UGT85B1-CFP was firstly excised with *BamH*I/*Xba*I and ligated into the respective sites of pPZP221Δ.101a. Secondly, CFP or CFP-ER were excised and introduced into pRT101b using the *Xho*I and *Xba*I restriction enzyme sites. Lastly, CFP or CFP-ER, and the CaMV 35S promoter and polyadenylation signal were then excised with *HinD*III and introduced into pPZP221. 101a.UGT85B1-YFP. This resulted in the plasmids named pPZP221.UGT85B1-YFP.CFP and pPZP221. UGT85B1-YFP.CFP-ER.

4.3. Agrobacterium-mediated transformants of A. thaliana

A. thaliana (Ecotype Columbia (Col 0)) was transformed, selected and grown as previously reported (Tattersall et al., 2001). Wild-type plants, and plants expressing *CYP79A1* plus *CYP71E1* (Bak et al., 2000), were transformed with the plasmid pPZP221.UGT85B1-YFP.CFP. Plants expressing *UGT85B1* (Tattersall et al., 2001) were transformed with pPZP221.CYP71E1-CFP.CYP79A1-YFP, and plants expressing *CYP79A1* (Bak et al., 1999) were transformed with pPZP221.CYP71E1-CFP. UGT85B1-YFP. A homozygous *A. thaliana* line containing a single copy of UGT85B1-YFP and CFP (see above) was crossed with a homozygous line expressing CYP79A1 plus CYP71E1 (Bak et al., 2000). Progeny was selected that expressed all four transgenes, and subsequently inbred until homozygous plants were obtained.

4.4. Transient expression in S. bicolor

Transient expression in single epidermal sorghum cells was carried out using primary leaves of light-grown sorghum seedlings (*S. bicolor* L. Moench cv Sordan 1000). Detached leaf segments (5 cm in length) were mounted on wetted filter paper in sterile Petri dishes (Nielsen et al., 1999). For bombardment, gold particles (1 μm Bio-Rad) were coated with gene fusion plasmids according to supplier's instructions (Bio-Rad). Co-expression of more genes was accomplished by co-coating with combinations of plasmids of interest like for example pPZP111.79A1 and pPZP111.71E1. Plasmids were introduced for transient expression in sorghum epidermal cells using a Helios Gene gun device (Bio-Rad). To minimize wounding of the tissue, He pressure was reduced to around 90 psi. After bombardment, samples were left overnight at room temperature in the dark.

4.5. Metabolite analysis of transgenic A. thaliana

Five or more plants were combined for analysis and plant tissue was extracted with 85% (v/v) MeOH. After removal of the MeOH by evaporation, 0.5 ml H_2O was added and the aqueous phase extracted with *n*-pentane (Nielsen et al., 2002) and its glucoside content examined by LC/MS (Nielsen et al., 2002; Tattersall et al., 2001; Kristensen et al., 2005).

4.6. Confocal laser scanning microscopy

4.6.1. Preparation of transgenic biological specimens

Intact root, leaf and petiole tissues of *A. thaliana* were studied by mounting whole seedlings (<7-day-old) in water directly on microscopy slides. Sorghum leaf segments were incubated for 24–72 h after bombardment before specimens were screened for fluorescent cells. Samples were studied using a Leica dissection microscope MZ12 equipped with GFP filter settings. Individual leaf segments with high numbers of fluorescent cells were selected and mounted in water directly on microscopy slides. Cover slips and seedlings or leaf segments were fixed by double-sided tape.

4.6.2. Monitoring

CLSM was performed using a Leica TCS SP2 system on a Leica DMRXE upright microscope (Leica Microsystems, Mannheim, Germany). Specimens were studied under HC PL APO 10×/0.40 CS or PL APO 63 × 1.2 water-corrected lenses. The microscope was equipped with optics for concomitant detection of fluorescent and transmitted light digital signals. YFP signals were detected by excitation with a monochromatic Ar/Kr laser beam (λ_{Ex} 488 nm) combined with collection of emission signals from λ 525–540 nm using Vista software (Leica Microsystems). CFP signals were detected using Ar/Kr laser beam (λ_{Ex} 458 nm) and collection of emission signals in the range from

480–520 nm. To visualize the specificity of the emission spectra from YFP and CFP, options in the Vista software to perform spectral scanning were used. The focal plane in the specimen was fixed and emission data were collected in the range from 500–700 nm in 50 steps of 5 nm, overlapping, increments. Images are presented in various ways using graphical tools in the Vista software according to supplier's instructions (Leica Microsystems).

Upon request, materials integral to the findings presented in this publication will be made available in a timely manner to all investigators on similar terms for non-commercial research purposes.

Acknowledgements

The authors thank Søren Bak for providing transgenic *A. thaliana* and for fruitful discussions. The work was partly funded through support from the Danish National Research Foundation to The Center for Molecular Plant Physiology (PlaCe), from The Danish Research Council for Technology and Production (Program for Innovation Post-docs) and by an Australian postgraduate Award to P.R.J.

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