Functional Analysis of the Tandem-Duplicated P450 Genes *SPS/BUS/CYP79F1* and *CYP79F2* in Glucosinolate Biosynthesis and Plant Development by *Ds* Transposition-Generated Double Mutants[1]

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A significant fraction (approximately 17%) of Arabidopsis genes are members of tandemly repeated families and pose a particular challenge for functional studies. We have used the *Ac-Ds* transposition system to generate single- and double-knockout mutants of two tandemly duplicated cytochrome P450 genes, *SPS/BUS/CYP79F1* and *CYP79F2*. We have previously described the Arabidopsis *supershoot* mutants in *CYP79F1* that exhibit massive overproliferation of shoots. Here we use a cytokinin-responsive reporter ARR5::*uidA* and an auxin-responsive reporter DR5::*uidA* in the *sps/cyp79F1* mutant to show that increased levels of cytokinin, but not auxin, correlate well with the expression pattern of the *SPS/CYP79F1* gene, supporting the involvement of this gene in cytokinin homeostasis. Further, we isolated *Ds* gene trap insertions in the *CYP79F2* gene, and find these mutants to be defective mainly in the root system, consistent with a root-specific expression pattern. Finally, we generated double mutants in *CYP79F1* and *CYP79F2* using secondary transpositions, and demonstrate that the phenotypes are additive. Previous biochemical studies have suggested partially redundant functions for *SPS/CYP79F1* and *CYP79F2* in aliphatic glucosinolate synthesis. Our analysis shows that aliphatic glucosinolate biosynthesis is completely abolished in the double-knockout plants, providing genetic proof for the proposed biochemical functions of these genes. This study also provides further demonstration of how gluconisolate biosynthesis, regarded as secondary metabolism, is intricately linked with hormone homeostatis and hence with plant growth and development.

Plant growth and development requires coordination of networks of biological processes within the plant, as well as with responses to external environments. The control of shoot branching by auxin and cytokinin is a well-known example of hormone interactions in controlling plant development. Cytokinin plays a key role in promoting bud growth, whereas auxin has an inhibitory effect. Therefore, the outcome appears to depend on the ratio of the two hormones (for review, see Tamas, 1995; Li and Bangerth, 1992). The two plant hormones not only play opposite roles in controlling plant growth and development, but also influence each other hormone homeostasis (Binns et al., 1987; Palni et al., 1988; Bangerth, 1994; Zhang et al., 1995; Makarova et al., 1996). We and others have described Arabidopsis mutants called *supershoot* (*sps*) or *bushy (bus)*, which are disrupted in the gene encoding the cytochrome P450 CYP79F1 (Reintanz et al., 2001; Tantikanjana et al., 2001). These mutants exhibit massive proliferation of shoots, together with other developmental defects. The quantification of hormone levels in *sps/cyp79F1* mutant plants indicates that both auxin and cytokinins are higher in the mutants, but the phenotypes of the *sps/cyp79F1* plants are consistent with higher levels of cytokinins rather than auxins as the key factor responsible for the change in branching pattern (Tantikanjana et al., 2001).

Despite the fact that *sps/cyp79F1* plants resemble hormone mutants, biochemical studies have shown that *SPS/CYP79F1* gene encodes an enzyme catalyzing metabolism of both short-chain and long-chain elongated Met-derivatives in the biosynthesis of aliphatic glucosinolates (Hansen et al., 2001; Chen et al., 2003). Glucosinolates are a group of secondary plant metabolites known to play a role in plant defense and are sources of flavor compounds, cancer-preventing agents, and bioherbicides. It has recently been shown that blockage of plant secondary metabolic pathways can result in severe repercussion for hormone homeostasis. The high levels of auxin in the *superroot1* (*sur1*) and *superroot2* (*sur2*) are caused by indirect effects due to blockage of the C-S lyase (Mikkelsen et al., 2004) and

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the cytochrome P450 CYP83B1, respectively (Barlier et al., 2000; Bak et al., 2001), in the biosynthetic pathway of the natural plant products glucosinolates. The high-auxin phenotypes of the *sur1* and *sur2* are derived from accumulation of indole-3-acetaldoxime that is channelled into indole-3-acetic acid (IAA), which shows that indole-3-acetaldoxime plays a critical role in auxin homeostasis and functions as a key branching point between primary and secondary metabolism.

In order to elucidate the potential roles of cytokinin and auxin in controlling branching pattern in the *sps/cyp79F1* mutant plants, we have used cytokinin and auxin-responsive reporters to study the changes of hormone levels with greater spatial resolution. For comparison, we have investigated the effect of disruption of the *CYP79F2* gene, which is a tandem duplication of *SPS/CYP79F1,* by the isolation and characterization of *cyp79F2* mutants with transposon insertions. Furthermore, we have generated double mutants of *sps/cyp79F1* and *cyp79F2* to analyze redundant functions of these two closely linked genes using an approach based on multiple transpositions of a *Ds* element. This method could be utilized to generate double-knockout lines of other tandemly repeated genes in the genome. Characterization of the phenotypic changes and glucosinolate profiles in these double-knockout mutants are presented.

RESULTS

Expression Patterns of the Cytokinin and Auxin-Responsive Reporters in the *sps/cyp79F1* Mutants

As previously reported, several physiological changes in the aerial part of the *sps/cyp79F1* mutant plants are strikingly similar to the effects of increased cytokinins levels. These physiological changes include the release of lateral buds from apical dominance and an increase in bud initiation, as well as the delay of senescence. Quantification of the cytokinin levels in the *sps/cyp79F1* mutants yielded results consistent with this prediction, as several types of cytokinins in the *sps/cyp79F1* mutants are present at higher levels than in wild-type plants (Tantikanjana et al., 2001). These mutants also have elevated auxin levels (Reintanz et al., 2001; Tantikanjana et al., 2001), however, the proliferation of shoots observed in the *sps/cyp79F1* mutant is inconsistent with increased auxin levels, which is expected to produce the opposite effect. It has been shown that when cytokinin homeostasis is perturbed, the level of auxin can be affected (Binns et al., 1987; Makarova et al., 1996). Therefore we suggested that the phenotypic changes in *sps/cyp79F1* mutants result primarily from increased cytokinin levels, and that the changes in auxin levels are a consequence of feedback mechanisms involved in hormone interactions (Tantikanjana et al., 2001).

To further investigate the relationship between hormone levels and the *sps/cyp79F1* mutant, we have used a cytokinin-responsive reporter ARR5::*uidA* (D'Agostino et al., 2000) and a synthetic auxin-responsive reporter DR5::*uidA* (Ulmasov et al., 1997) to study developmental changes affected by cytokinin and auxin levels. These reporters are useful to investigate changes in hormone levels within defined regions of wild-type and mutant plants (e.g. Casson et al., 2002; Harrar et al., 2003; Werner et al., 2003 for the cytokinin reporter ARR5; and Blilou et al., 2002; Long et al., 2002; Willemsen et al., 2003 for the auxin reporter DR5), because the hormone measurements on entire aerial parts of the plants may not reflect hormone levels at specific developmental sites, which may be masked by the changes elsewhere in the plant. We selected the *sps1-1* allele for the investigation, as this allele does not exhibit β-glucuronidase (GUS) expression derived from the *Ds* gene trap insertion. The analysis showed that expression levels of both cytokinin and auxin-responsive reporters in the *sps/cyp79f1* mutants are indeed higher than those of wild-type plants, in agreement with the hormone measurements. Importantly, the reporters revealed that the increases in the two hormone levels occur at different sites in the plant. The level of the cytokinin-responsive reporter is increased the most at the axil of the mutant leaf (Fig. 1B), as compared to that of the wild type (Fig. 1A). On the other hand, we could not detect any expression of the auxin-responsive reporter at the leaf axil in neither the mutant nor wild-type plants. Instead, expression of the auxin-responsive reporter was found at higher level in the leaf blade of the mutant (Fig. 1D) than in the wild type (Fig. 1C). These data further support the possible involvement of the *SPS/CYP79F1* gene in modulating cytokinin level at the site of bud initiation and that changes in levels of cytokinin at these sites, rather than auxin, are responsible for the altered shoot branching pattern.

Distinct Roles of *SPS/CYP79F1* and *CYP79F2* in Plant Growth and Development

We have shown that disruption of the *SPS/CYP79F1* gene leads to severe developmental defects in the aerial architecture of the plants. The expression pattern of the *SPS/CYP79F1* gene and analysis of mosaic plants has prompted the suggestion that this gene acts locally in its effects on plant growth and development (Tantikanjana et al., 2001). Immediately upstream of the *SPS/CYP79F1* gene, there is a second closely related gene that has been designated *CYP79F2* (Nelson, 1999; http://biobase.dk/P450), which has also been shown to function in aliphatic glucosinolate metabolism (Chen et al., 2003). The two genes share 89% sequence identity at both nucleotide and amino acid levels. In order to reveal possible physiological functions of the *CYP79F2* gene, a collection of *Ds* insertion lines were used to screen for *cyp79F2* knockout mutants by reverse-genetics approach. A total of three *cyp79F2* alleles were isolated. Locations of the *Ds* elements in different *cyp79F2* alleles are shown in

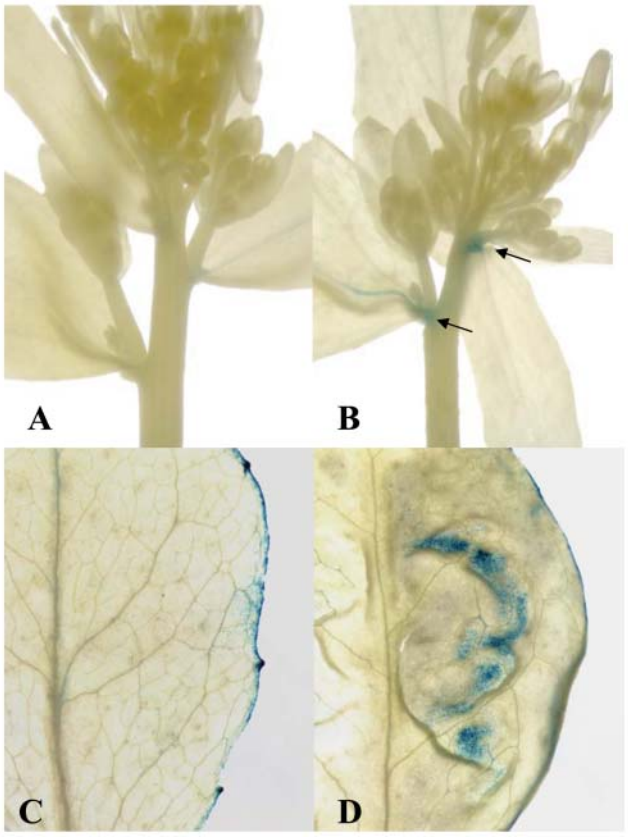

Figure 1. Expression patterns of the cytokinin-responsive reporter ARR5::*uidA* and the synthetic auxin-responsive reporter DR5::*uidA* in *sps/cyp79F1* mutants. A, Expression patterns of the cytokinin-responsive reporter ARR5::*uidA* in young inflorescences of wild type and B, *sps/cyp79F1* mutant plant. Arrows indicate expression of the GUS reporter at the sites of bud initiation in the *sps/cyp79F1* mutant. C, Expression patterns of the synthetic auxin-responsive reporter DR5::*uidA* in the wild type leaf, and D, in the *sps/cyp79F1* mutant leaf.

Figure 2. In all three *cyp79F2* alleles, the mutant plants have slight reduction of aerial growth but otherwise normal aerial structure. Unlike the *sps/cyp79F1* mutant that has severe defects in the aerial parts of the plant, *cyp79F2* mutants are predominantly defective in the root system. When lateral root length of 8-d-old seedlings were measured, *cyp79F2* mutants exhibit only 65% root growth compared to the wild-type starter line that was used to generate *cyp79F2* mutants (see ''Materials and Methods''; Fig. 3), whereas the number of lateral roots in the mutant is unaffected (data not shown). There were no obvious differences in cell size between *cyp79F2* and wild-type (starter line) plants, suggesting that the primary effect of the mutant might be on cell division. In contrast, the lateral root length, as well as the lateral root number, of the *sps/cyp79F1* mutant does not differ from the wild-type starter line (Fig. 3). The observation of root defects only in *cyp79F2* mutants, but not in *cyp79F1* mutants, both of which were derived from the same starter line, strongly suggests that only the *CYP79F2* gene has a role in root growth. The reduction in lateral root length of the *cyp79F2* mutants is observed only when seedlings are grown in soil. This defect is not obvious in mutant seedlings growing on agar plates containing Murashige and Skoog basal medium, suggesting that expression of the phenotypic changes in *cyp79F2* mutants is influenced by growth conditions. We could not detect any obvious changes in the primary root length under the conditions where we observed an effect on lateral root lengths, but we note that it is more difficult to obtain intact main roots as they grow deep into the soil. We have found that the severity of the *sps/cyp79F1* mutant phenotypes is also affected by growth conditions such as growing medium and light condition (data not shown).

To further understand its role in controlling root development, we analyzed expression pattern of the *CYP79F2* gene using the *Ds* gene trap insertions. The *Ds* gene trap insertions in *CYP79F2* should provide an accurate expression profile of the gene, because the *GUS* reporter fusion in the gene trap is in the correct chromosomal context (Sundaresan et al., 1995). Two of the *cyp79F2* alleles confer GUS reporter gene expression. Both *cyp79F2* alleles show *GUS* expression pattern primarily in the root system. Occasionally, GUS staining was detected in the vascular tissue of the cotyledons. However, no expression was observed in the leaf or other parts of the aerial structure. In the root system, *CYP79F2* gene is developmentally regulated. *CYP79F2* is expressed above the root elongation zone and continues for a few millimeters before fading away in the more mature region (Fig. 4). We could not detect any expression in the root primodium. The data suggest that the *CYP79F2* gene also affects plant growth and development locally, as was observed with the *SPS/CYP79F1* gene. The nonoverlapping expression patterns, together with the observed phenotypic changes, indicate that *SPS/CYP79F1* and *CYP79F2*, in addition to being involved in biosynthesis of aliphatic glucosinolates, might function in different parts of the plant with the former primarily in the aerial parts and the latter primarily in the root system.

Generation of *sps/cyp79F1* and *cyp79F2* Double Mutants by Multiple *Ds* Transpositions

Despite high sequence similarity between *SPS/CYP79F1* and *CYP79F2* genes, the analysis of single-knockout mutants and the expression patterns of the genes suggest that the two genes might play different roles, via differences in their spatial regulation. Recently *SPS/CYP79F1* and *CYP79F2* genes have been shown to have distinct but overlapping metabolic functions. SPS/CYP79F1 metabolizes both short-chain and long-chain aliphatic Mets, whereas CYP79F2 exclusively metabolizes the long-chain elongated aliphatic Mets (Hansen et al., 2001; Chen et al., 2003). In order to reveal any possible overlapping physiological roles, as well as to confirm metabolic roles of the *SPS/CYP79F1* and *CYP79F2* genes, we generated *sps/*

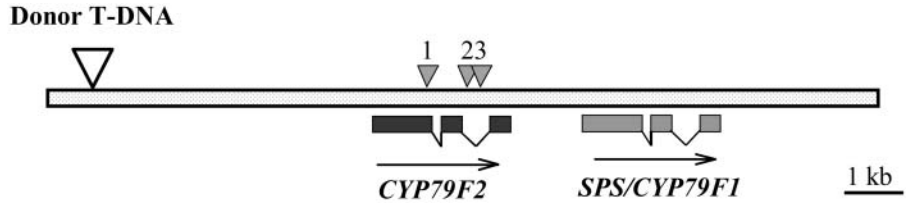

Figure 2. Positions of the donor T-DNA and insertion sites of *Ds* gene trap elements in different *cyp79F2* alleles. Positions of the *SPS/CYP79F1* and *CYP79F2* genes on the bacterial artificial chromosome clone accession number AC006341 are shown. Boxes represent exons. Insertion sites of *Ds* elements in the *CYP79F2* gene are indicated as small arrowheads; position of the donor T-DNA is indicated as big arrowhead. Insertion site of the *cyp79F2-1* is in the first exon, whereas the *cyp79F2-2* and *cyp79F2-3* are in the second intron.

cyp79F1 and *cyp79F2* double mutants by multiple *Ds* transpositions. Transposon mutagenesis using the *Ac-Ds* system has features that are advantageous for generating multiple mutations within closely linked physical distances. First, the *Ac/Ds* elements preferentially transpose to linked genomic sites (for review, see Sundaresan, 1996). Furthermore, the mutation can be retained at the original donor site after remobilization of the transposon, either by the element that is still present at the donor site or by the footprint left behind at the empty donor site after excision of the element, as the *Ac/Ds* element undergoes transposition either during or after chromosome replication (for review, see Fedoroff, 1983).

An experimental procedure was designed so that the screening for double-knockout mutants could be done with ease. Because *cyp79F2* mutants have normal aerial structure and are fertile, *cyp79F2-1* allele containing a *Ds* element inserted into the first exon of the gene was used for the reactivation of *Ds* transposition. Although a PCR screen for the double knockouts could be performed, we preferred a phenotypic screen, reasoning that if the *Ds* element excises from the *CYP79F2* gene and reinserts into the *SPS/CYP79F1*, we would observe families segregating for plants with defects in the aerial structure in the subsequent generation. After reactivation of the *Ds* transposition, a total of 516 families were screened for plants displaying abnormal aerial architecture. Four independent families segregating for plants resembling the *sps/cyp79F1* mutant were isolated. Of these four families, two families still segregating for mutant plants in the next generation were characterized in detail. Positions of the *Ds* elements in the *SPS/CYP79F1* genes were confirmed by PCR using a *SPS/CYP79F1*-specific primer and a *Ds* primer. The PCR products were further verified by DNA sequencing. Both independent double-knockout mutants, designated *sps-7 cyp79F2-1* and *sps-8 cyp79F2-1*, contained *Ds* elements inserted into the second intron of the *SPS/CYP79F1* gene but at different positions (Fig. 5). The presence of *Ds* elements or footprints left by germinal excisions of *Ds* elements in the donor *CYP79F2* gene was investigated by determination of the genomic sequences at the original donor sites. Of the two independent double-knockout mutants isolated, one contained an empty donor site with a footprint that generates a frame shift mutation in the *CYP79F2* gene. The other double-knockout mutant retained the *Ds* element at the original location in the *CYP79F2* gene in addition to the new insertion in the *SPS/CYP79F1* gene. Positions of the mutations in the two independent double mutants are shown in Figure 5.

Phenotypes and Glucosinolate Profiles of the *sps/cyp79F1* and *cyp79F2* Double Mutants

The *sps-7 cyp79F2-1* double-mutant plants were further analyzed for their physiological and metabolic effects. Results from expression patterns of the *SPS/CYP79F1* and *CYP79F2*, as well as phenotypic characterization of single mutants, indicate that the two genes function mainly in different parts of the plant. *sps/cyp79F1* and *cyp79F2* double mutants are defective in both shoot and root systems. As expected from the phenotypic screen used to initially identify the double-knockout mutants, the aerial structure of the double mutants is similar to the previously described supershoot phenotype of *sps/cyp79F1* single mutants in terms of shoot branching (Tantikanjana et al., 2001), but the plants are slightly smaller in size (data not shown). In addition, the lateral root length of the double mutants is also reduced relative to wild-type plants, to a level comparable to that in the single *cyp79F2* mutant,

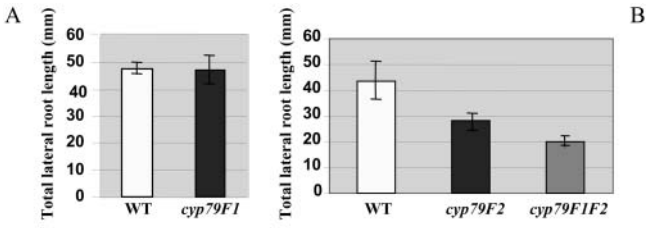

Figure 3. Lateral root length of *sps/cyp79F1*, *cyp79F2* mutants and *sps/cyp79F1 cyp79F2* double mutant. A, Lateral root length of *sps/cyp79F1* and wild-type plants. B, Lateral root length of *cyp79F2*, *sps/cyp79F1 cyp79F2* double-mutant and wild-type plants. The studies of lateral root development between *sps/cyp79F1* and wild-type plants (A), and *cyp79F2*, *sps/cyp79F1 cyp79F2* double-mutant and wild-type plants (B) were done in separate experiments but in the same controlled growth chamber conditions. Error bars indicate SD (n = 21–24).

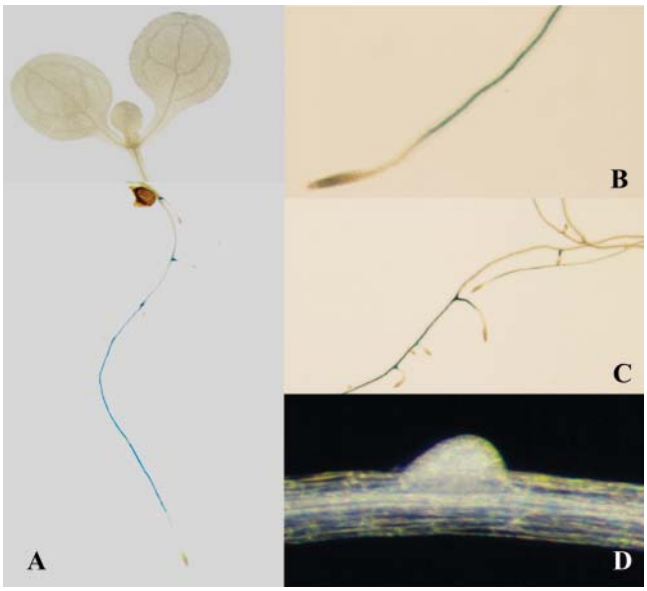

Figure 4. *CYP79F2* expression patterns monitored by GUS expression from the *Ds* gene trap insertion in the *cyp79F2-2* allele. A, GUS-staining pattern detected in the root system of a 10-d-old seedling. The picture is the composite image of the aerial part and root system from the same seedling. B to D, Staining detected in the root of a 2-week-old seedling; B, At the region above root apical meristem; C, Staining is not detectable in more mature regions of the root. D, No GUS staining is detected in the root primodium.

though slightly shorter (Fig. 3B). Therefore the developmental effects of the double knockout are largely additive, as may be expected from their essentially nonoverlapping patterns of expression.

Biochemical studies have previously shown that SPS/CYP79F1 and CYP79F2 have overlapping substrate specificity in the synthesis of aliphatic glucosinolates. SPS/CYP79F1 catalyzes the conversion of homomethionine, di-, tri-, tetra-, penta-, and hexahomomethionine to their aldoximes, whereas CYP79F2 catalyzes the conversion of penta- and hexahomomethionine to their aldoximes (Chen et al., 2003). The biochemical data have been supported by the presence of long-chain aliphatic glucosinolates in both the single null *sps/cyp79F1* mutant and the single null *cyp79F2* mutant, while short-chain glucosinolates are abolished only in *sps/cyp79F1* nulls. Double-knockout mutants obtained in our studies allowed us to test the overlapping metabolic roles of the *SPS/CYP79F1* and *CYP79F2* directly. Glucosinolate profiles of the wild type, *sps/cyp79F1* and *cyp79F2* single-knockout mutants, as well as *sps/cyp79F1* and *cyp79F2* double-knockout mutants, are shown in Table I. The analysis of glucosinolate profiles in the double-knockout mutant plants shows that, in addition to the absence of short-chain aliphatic glucosinolates, long-chain elongated glucosinolates are completely abolished when both genes are disrupted. This result provides genetic confirmation for the partial functional redundancy of these two genes in glucosinolate metabolism and demonstrates that disruption of both genes results in the complete absence of aliphatic glucosinolates in the plant. It has previously been shown that indole glucosinolates are also increased in the *sps/cyp79F1* mutant (Reintanz et al., 2001; Chen et al., 2003). However, the double knockout in both *SPS/CYP79F1* and *CYP79F2* genes did not significantly enhance biosynthesis of indole glucosinolates as compared to the single knockout in *SPS/CYP79F1* gene (Table I).

DISCUSSION

Recent biochemical and genetic studies have shown that disruption of glucosinolate biosynthesis, considered to be secondary metabolism, has important effects on hormone homeostasis in Arabidopsis. The loss-of-function mutations in the *CYP83B1* gene (*SUR2)* and the C-S lyase *(SUR1)* in the glucosinolate pathway result in plants with elevated IAA levels. Consequently, the *sur1* and *sur2* mutants confer high-auxin phenotypes, including severe apical dominance and adventitious root development from hypocotyl tissue. In addition, disruption of *CYP83B1* function also up-regulates Trp biosynthesis and other stress-induced pathways (Smolen and Bender, 2002). Likewise, characterization of the *bushy/sps/cyp79F1* mutants has shown that disrupting the *SPS/CYP79F1* gene required for the synthesis of short-chain and long-chain aliphatic glucosinolates severely affects

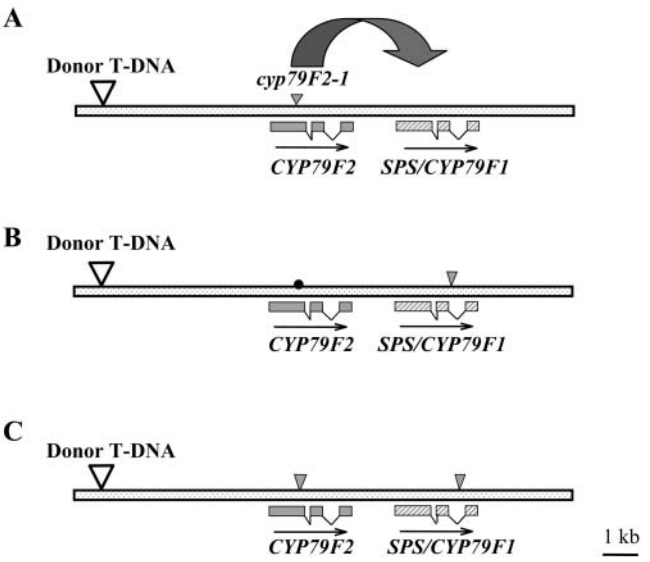

Figure 5. Generation of *sps/cyp79F1 cyp79F2* double mutants. A, Position of the *Ds* element in the *cyp79F2-1* allele used as a starter line for the reactivation of *Ds* transposition. B to C, Positions of the *Ds* elements in the *SPS/CYP79F1* and *CYP79F2* genes of the two independent double-knockout mutants, designated *sps-7 cyp79F2-1* allele (B) and *sps-8 cyp79F2-1* allele (C). Boxes represent exons. Insertion sites of *Ds* elements in the *SPS/CYP79F1* and *CYP79F2* genes are indicated as small arrowheads; positions of the donor T-DNAs are indicated as big arrowheads. Dot represents position of the frame-shift mutation in the *CYP79F2* gene after excision of the *Ds* element.

Table I. *Glucosinolate profile of cyp79F1 and cyp79F2 mutants and cyp79F1 cyp79F2 double mutant*

	Short-Chain Glucosinolates			Long-Chain Glucosinolates			Indole Glucosinolates		
	3-msp	5-msp	3-mtp	7-msh	8-mso	8-mto	i-3ym	4mi-3ym	Nmi-3ym
Rosette Leaves									
Wild type	10.67 ± 0.92	0.24 ± 0.02	0.24 ± 0.02	0.05 ± 0.002	0.61 ± 0.04	0.06 ± 0.008	1.33 ± 0.06	0.25 ± 0.02	0.29 ± 0.06
cyp79F1	ND	ND	ND	0.05 ± 0.006	1.66 ± 0.12	0.14 ± 0.009	2.14 ± 0.07	0.24 ± 0.01	0.013 ± 0.001
cyp79F2	9.88 ± 0.85	0.22 ± 0.03	0.24 ± 0.02	0.05 ± 0.008	0.47 ± 0.05	0.05 ± 0.007	1.42 ± 0.13	0.25 ± 0.01	0.27 ± 0.04
cyp79F1 cyp79F2	ND	ND	ND	ND	ND	ND	2.2 ± 0.08	0.24 ± 0.02	0.03 ± 0.02
Root									
Wild type	7.38 ± 0.69	0.24 ± 0.05	0.22 ± 0.01	0.02 ± 0.002	0.36 ± 0.02	0.12 ± 0.01	0.9 ± 0.06	0.24 ± 0.02	11.3 ± 0.87
cyp79F1	ND	ND	ND	0.03 ± 0.004	0.58 ± 0.04	0.21 ± 0.02	0.73 ± 0.08	0.15 ± 0.01	7.97 ± 0.35
cyp79F2	5.02 ± 0.37	0.32 ± 0.05	0.18 ± 0.02	0.01 ± 0.0002	0.17 ± 0.03	0.37 ± 0.02	0.79 ± 0.04	0.16 ± 0.007	13.3 ± 0.58
cyp79F1 cyp79F2	ND	ND	ND	ND	ND	ND	0.44 ± 0.07	0.11 ± 0.03	6.05 ± 1.43
Stem									
Wild type	8.38 ± 0.22	0.1 ± 0.01	0.07 ± 0.02	0.04 ± 0.01	0.44 ± 0.10	0.005 ± 0.001	0.33 ± 0.01	0.06 ± 0.007	0.007 ± 0.001
cyp79F1	ND	ND	ND	0.19 ± 0.01	5.58 ± 0.39	0.03 ± 0.004	2.15 ± 0.16	0.002 ± 0.0003	0.02 ± 0.005
cyp79F2	14.29 ± 0.86	0.14 ± 0.03	0.13 ± 0.01	0.07 ± 0.01	0.44 ± 0.11	0.02 ± 0.001	0.47 ± 0.02	0.05 ± 0.01	0.01 ± 0.002
cyp79F1 cyp79F2	ND	ND	ND	ND	ND	ND	2.12 ± 0.17	0.002 ± 0.0002	0.018 ± 0.002
Flower									
Wild type	33.02 ± 0.99	0.27 ± 0.03	0.93 ± 0.05	0.57 ± 0.03	7.76 ± 0.46	0.06 ± 0.007	1.32 ± 0.07	0.03 ± 0.005	0.009 ± 0.002
cyp79F1	ND	ND	ND	0.41 ± 0.007	11.8 ± 0.31	0.18 ± 0.008	3.02 ± 0.08	0.04 ± 0.003	0.01 ± 0.002
cyp79F2	28.36 ± 2.11	0.24 ± 0.02	1.17 ± 0.01	0.47 ± 0.008	5.47 ± 0.11	0.08 ± 0.01	1.72 ± 0.07	0.03 ± 0.001	0.02 ± 0.007
cyp79F1 cyp79F2	ND	ND	ND	ND	ND	ND	2.77 ± 0.05	0.03 ± 0.001	0.01 ± 0.0006

3-msp, 3-methylsulfinylpropyl glucosinolate; 5-msp, 5-methylsulfinylpentyl glucosinolate; 3-mtp, 3-methylthiopropyl glucosinolate; 7-msh, 7-methylsulfinylheptyl glucosinolate; 8-mso, 8-methylsulfinyloctyl glucosinolate; 8-mto, 8-methylthiooctyl glucosinolate; i-3ym, indole-3-ylmethyl glucosinolate; 4mi-3ym, 4-methoxyidol-3-ylmethyl glucosinolate; Nmi-3ym, *N*-methoxyindol-3-ylmethyl glucosinolate. ND, Not determined.

hormone homeostasis (Hansen et al., 2001; Reintanz et al., 2001; Tantikanjana et al., 2001). Despite the fact that *sps/cyp79F1* mutants have higher level of both auxin and cytokinin, the mutants resemble plants with cytokinin overproduction. The role of *SPS/CYP79F1* as a modulator for cytokinin homeostasis is unclear, and the lack of detailed understanding of cytokinin metabolism makes it difficult to identify the biochemical link between glucosinolate biosynthetic pathway and cytokinin homeostasis.

In order to relate the changes of auxin and cytokinin levels with developmental defects observed in the *sps/cyp79F1* mutants, we used the cytokinin-responsive reporter *ARR5::uidA* and the synthetic auxin-responsive reporter *DR5::uidA* to detect changes of hormone levels at particular sites. The study reveals that higher levels of cytokinin, particularly at the site of bud initiation correlates well with the increase in branching in the *sps/cyp79F1* mutants, whereas the dramatic increase in auxin content is in the leaf blade. The pattern of increased cytokinin levels in the mutants revealed by the reporter gene correlated well with the expression pattern of the *SPS/CYP79F1* gene. These data support the hypothesis that the primary effect of disrupting *SPS/CYP79F1* function is on cytokinin homeostasis rather than auxin homeostasis. It is well documented that both auxin and cytokinin can influence the hormone levels of each other. Even though the exact mechanisms that regulate these hormone interactions are not fully understood, it has been shown that auxin can stimulate oxidative breakdown of active cytokinin (Palni et al., 1988; Zhang et al., 1995). In addition, it has been proposed that auxin levels may influence cytokinin biosynthesis, because removal of the endogenous source of auxin by decapitation leads to an increase in the cytokinin content of xylem exudates (Bangerth, 1994). Unlike the effect of auxin that results in decreased cytokinin levels, the effect of cytokinin on auxin levels is less well understood. Manipulation of cytokinin levels in plants by transformation with a bacterial cytokinin biosynthesis gene results in accumulation of increased levels of auxin (Binns et al., 1987; Makarova et al., 1996). Complex interactions are also observed between different glucosinolate biosynthetic pathways. Disruption of the *SPS/CYP79F1* gene in the aliphatic glucosinolate biosynthesis leads to increased levels of indole glucosinolates (Reintanz et al., 2001; Chen et al., 2003). The connection between disruptions of different glucosinolate biosynthetic pathways that can influence the levels of different hormones opens a possibility that hormone interaction can occur, in part, through a complex network of secondary metabolites and/or common intermediates. A direct connection between aliphatic and indole glucosinolate biosynthetic pathways is not well understood. It is unlikely that the higher cytokinin levels and the consequent developmental effects in these mutants is due to the higher indole glucosinolate levels, as the double mutants, despite their more extreme phenotypes, show comparable levels of indole glucosinolates to the single *CYP79F1/sps* mutant (Table I). A more likely possibility arises from the observation that the *CYP79B2/B3* genes are up-regulated in stressed plants, resulting in

increased indole glucosinolates and IAA (Mikkelsen et al., 2003). Therefore, it is also possible that *sps/cyp79F1* mutants are stressed because of the perturbation of cytokinin homeostasis, which in turn up-regulates *CYP79B2/B3* genes. Glucosinolates are the largest group of secondary metabolites known in Arabidopsis and other genera of Brassicaceae (Halkier, 1999). Several glucosinolate biosynthetic enzymes have been cloned and shown to be members of the cytochrome P450 superfamily. Gene duplication, tandem clustering of the duplicated genes, and gene conversion of the cytochrome P450 genes provide evidence that rapid evolution led to generation of diverse enzymatic reactions. Analysis of the highly related *SPS/CYP79F1* and *CYP79F2* genes provide a clear picture of a recent gene duplication undergoing diversification through the utilization of distinct but overlapping substrates, as well as through different spatial expression profiles.

Reintanz et al. (2001) and Chen et al., (2003) showed that, by using *SPS/CYP79F1* and *CYP79F2* promoter-GUS fusion, both promoters conferred GUS expression in aerial parts as well as in root. However, we showed by using gene trap insertion that *SPS/CYP79F1* and *CYP79F2* genes were expressed mainly in aerial parts and in root, respectively. These discrepancies in the expression patterns of *SPS/CYP79F1* and *CYP79F2* genes may derive from different types of reporter gene fusions used in the studies, i.e. gene trap fusions in this study versus ectopic transgene fusions. We also cannot rule out the possibility that the distinct expression patterns of the *SPS/CYP79F1* and *CYP79F2* genes observed by different groups may reflect ecotype-dependant allelic variations, as different Arabidopsis ecotypes were used in the studies, i.e. the Wassilewskija ecotype in this study, versus the Columbia ecotype. Recently, it has been reported that glucosinolates show extensive variations in both the composition and concentration among different ecotypes (Kliebenstein et al., 2001; Petersen et al., 2002; Reichelt et al., 2002; Brown et al., 2003).

Arabidopsis has an estimated 273 different cytochrome P450 genes in the genome (for review, see Nelson, 1999; Schuler and Werck-Reichhart, 2003; http:/biobase.dk/P450). With the large number of related cytochrome P450 sequences in the genome, it is not surprising that functions of some of the closely related cytochrome P450 genes are redundant or overlapping. The construction of double mutants by genetic recombination is the most direct approach to uncover redundant functions of duplicated genes. However, this approach can be very cumbersome when the duplicated genes are tightly linked as a tandem duplication, as is the case with nearly 17% of the Arabidopsis genes (The Arabidopsis Genome Initiative, 2000). We have successfully used the *Ac-Ds* transposition system to generate double mutations of two closely related genes in tandem, i.e. the *SPS/CYP79F1* and *CYP79F2* genes, to study their overlapping metabolic and physiological functions. The isolation of *sps/cyp79F1* and *cyp79F2* double mutants from a small set of transposed lines (approximately one in 100 lines) demonstrates that the approach is efficient. This approach of using multiple transpositions of the *Ds* transposon may be of general utility for elucidating functions of other clustered duplicated genes in the genome.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis mutants and wild-type plants were derived from Wassilewskija ecotype. Plants were grown on soil under controlled growth chamber conditions at 20°C, 60% to 70% relative humidity, 16/8 photoperiod, and a photosynthetic flux of 100 μmol photons m^{-2} s^{-1}. Plants used for glucosinolate analysis were grown under a photosynthetic flux of 250 μmol photons m^{-2} s^{-1} for 3 weeks before the samples were collected as pooled materials.

Isolation of *cyp79F2* Mutants

A collection of *Ds* insertion lines, generated by transpositions of a *Ds* gene trap element from a single T-DNA (Tantikanjana et al., 2001), was used to screen for insertion lines containing *Ds* element in the *CYP79F2* genes. Approximately 900 insertion lines were used for the screening using *CYP79F2* gene-specific primers (5′-TCTTCATCGCATCAATCACTTTAC-3′; 5′GCGTGGCTCAACAGACAA-3′) and transposon-specific primer (5′-CCGTTTACCGTTTTGTATATCCCCG-3′; 5′-CGATTACCGTATTTATCCCGT-3′). Young leaves from four independent insertion lines were pooled for DNA extraction, and pooled DNA from a total of 24 insertion lines were used for each PCR reaction. The putative lines were then identified and verified by sequencing.

Generation of *sps/cyp79F1* and *cyp79F2* Double-Knockout Mutants

The mutant *cyp79F2-1* allele containing a *Ds* element inserted into the first exon of the gene was used as a starter line for the reactivation of *Ds* transposition. Because expression of the transposase used in this tagging system is driven by the induction of a heat shock promoter (Balcells et al., 1994; Tantikanjana et al., 2001), transactivation of the *Ds* element was performed by subjecting the starter line to high temperature (40°C to 42°C) as described (Balcells et al., 1994). The heat-shocked seeds were then sowed and allow to set seeds. Screening for families, segregating for plants with abnormal aerial structure resembling to the *sps/cyp79F1* mutant, was done in the next generation. Approximately eight to 12 plants derived from each family were used for the screening. Positions of the *Ds* elements in *SPS/CYP79F1* gene were verified by PCR and sequencing using *SPS1/CYP79F1*-specific primer (5′-GGAGGATGCAAGAACCA-3′) and 3′ *Ds*-specific primers (5′-CGATTACCGTATTTATCCCGT-3′ or 5′-CCGGTATATCCCGTTTTCG-3′). A footprint of the *Ds* element after excision from *CYP79F2* gene in the *sps-7 cyp79F2-1* allele was verified by PCR and sequencing using *CYP79F2*-specific primers (5′-TCTTCATCGCATCAATCACTTTAC-3′ and 5′-GCGTGGCTCAACAGACAA-3′).

Analysis of Lateral Root Length and Lateral Root Number

Seedlings were grown on soil for 8 d before root samples were washed gently to maintain integrity and collected for the analysis. The lateral root length of mutant and wild-type plant was derived from the analysis of 21 to 24 seedlings. For the analysis of lateral root length and lateral root number, total lateral root length and lateral root number derived from the first 1 cm of the primary roots, starting from the hypocotyls-root junction, were used in the investigation.

Staining for GUS Expression

Plant materials were stained in GUS-staining solution (100 mM Na Phosphate at pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 1 mg/mL of X-Gluc [Biosynth AG]), containing 5 or 10 mM potassium ferricyanide and 5 or 10 mM

potassium ferrocyanide. After being placed under vacuum for 10 min in a dessicator, the samples were incubated at 37°C for 2 h to overnight. The stain solution was removed and the tissues were cleared by incubating with several changes of 70% ethanol.

Glucosinolate Analysis by HPLC

Glucosinolates were extracted from approximately 20 mg slightly homogenized freeze-dried rosette leaves, stems, roots, or flowers, either from five to eight single plants or from three to six pools of three to eight plants, by boiling 4 min in 4 mL 70% methanol. The supernatant was collected, and the plant material was washed with 2 mL 70% methanol. The extracts were combined and applied to 200 μL DEAE Sephadex CL-6B (Amersham-Pharmacia Biotech, Uppsala) columns (Bio-Rad Polyprep; Copenhagen) equilibrated with 1 mL 0.02 M KOAc, pH 5.0 and washed with 1 mL water. The columns were washed with 2 mL 70% methanol, 2 mL water, and 0.02 M KOAc, pH 5. Following addition of 100 μL of 2.5 mg/mL *Helix pomatia* sulfatase (Sigma-Aldrich, St. Louis), the columns were sealed and left overnight. The resulting desulphoglucosinolates were eluted with 2 $\times$ 1 mL water. The eluate was lyophilized until dryness and resuspended in 200 μL water. Aliquots of 100 μL were applied to a Shimadzu Spectachrom HPLC system equipped with a Supelco supelcosil LC-ABZ 59142 RP-amid C-16 (25 cm $\times$ 4.6 mm, 5 mm; Supelco, Bellfonte, PA; Holm & Halby, Brendby, Denmark) and an SPD-M10AVP photodiode array detector (Shimadzu, Columbia, MD). The flow rate was 1 mL min^{-1}. Desulphoglucosinolates were eluted with water for 2 min followed by a linear gradient from 0% to 60% methanol in water (48 min), a linear gradient from 60% to 100% methanol in water (3 min), and with 100% methanol (14 min). Detection was performed at 229 nm and 260 nm using a photodiode array. Desulphoglucosinolates were quantified based on response factors (Buchner, 1987; Haughn et al., 1991) and an internal *p*-hydroxybenzylglucosinolate (Bioraf, Akirkeby, Denmark) standard as previously described (Petersen et al., 2001, 2002). The standard was added at the beginning of the extraction procedure.

Sequence data used in this article are taken from GenBank accession number AC0006341.

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