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Running title: Molecular wheat allelopathy

Modern genomic approaches to improving allelopathic capability in wheat (*Triticum aestivum* L)

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CONTENTS

1. INTRODUCTION
2. GENETICS OF WHEAT ALLELOPATHY
3. CYTOCHROME P450 ENCODING BIOSYNTHESIS OF WHEAT SECONDARY METABOLITES
4. APPLICATION OF DNA MICROARRAY TECHNOLOGY IN UNVEILING THE MODE OF ACTIONS OF ALLELOCHEMICALS
5. APPLICATION OF FUNCTIONAL GENOMICS APPROACHES IN LOCATING GENES FOR ENCODING THE BIOSYNTHESIS OF ALLELOCHEMICALS
6. DISCUSSION

7. REFERENCES

ABSTRACT

Wheat (*Triticum aestivum* L) has been extensively studied for its allelopathic potential in weed management. Wheat plants can produce and exude allelochemicals into their surroundings to suppress weed growth. Many allelochemicals responsible for weed suppression have been identified. Recently, modern genomics approaches have been exploited to identify genetic markers associated with wheat allelopathy, to study the mode of actions of allelochemicals, and to discover the genes encoding the complex biosynthesis of plant defense compounds. Plant cytochrome P450s catalyse myriad biosynthetic pathways of plant secondary metabolites. A number of cytochrome P450s have been reported and cDNAs of these P450s are now available for further identification of new P450 families and subfamilies. Five P450 genes involved in the biosynthesis of a wheat allelochemical DIBOA have been identified. Parallel analyses of genomics, transcriptomics, proteomics and metabolomics, coupling with the data mining tool bioinformatics, will greatly assist in the identification of genes encoding the biosynthesis of allelochemicals. The genetic manipulation could be performed to regulate the biosynthesis of allelochemicals, thereby resulting in better weed suppression via elevated levels of allelopathic potential in commercial wheat cultivars.

Key words: allelochemicals, allelopathy, cytochrome P450, gene expression, *Triticum aestivum*, weed management.

1. INTRODUCTION

The role of allelopathy in weed management has attracted attention over the last two decades (9, 39). The application of crop allelopathy in weed suppression involves two crop growth stages, i.e. the vegetative stage and post-harvest stage. At the vegetative growth stage, crop seedling allelopathy could be exploited to suppress weeds. At the post-harvest stage, crop residue allelopathy could be used for weed suppression, especially during the establishment period of the following crop (40). Wheat (*Triticum aestivum* L.), as one of the major food crops in the world, has been examined internationally for its allelopathic potential in weed suppression (8, 9, 40).

Early studies have shown that wheat residues or straw aqueous extracts inhibited the germination and growth of a broad range of economically important weeds. Banks and Robinson (5) reported that a straw mulch suppressed the growth of spiny amaranth (*Amaranthus spinosus* L.), tall morningglory [*Ipomoea purpurea* (L.) Roth], and volunteer wheat more than herbicides used on non-mulched areas. The allelochemicals in wheat residues could kill weeds in the next crop sown into the mulched residues under no-till systems (37).

The observed wheat residue allelopathy in the field was further confirmed under controlled laboratory conditions by aqueous extract bioassays (36, 44). Wu et al. (44) found that aqueous extracts of shoot residues derived from certain wheat cultivars significantly inhibited the germination and root growth of a susceptible biotype of *Lolium rigidum*, as well as a biotype of this weed resistant to a number of chemically distinct herbicides. These results suggest that allelochemicals in wheat straws might be leached into the soil to

selectively influence the growth of certain weeds in the vicinity, and that wheat allelopathy might have implications in the management of herbicide resistant weeds.

Similarly to the allelopathic effects of wheat residue on weeds, wheat seedling allelopathy can also be exploited for weed management. Weed suppression by wheat seedling allelopathy during the early establishment period could reduce the need for early season application of commercial herbicides, with late season weed control provided by the heightened advantages of crop competitiveness (40). Wheat seedlings have produced and released toxic root exudates inhibitory to a number of weed species, such as Japanese brome (*Bromus japonicus* Thunb.) and common lambsquarters (*Chenopodium album* L) (35), annual ryegrass (*L. rigidum* Gaud.) (40), white mustard (*Sinapis alba* L.) (7) and perennial ryegrass (*L. perenne* L.) (8, 9). A novel screening bioassay, the 'equal-compartment-agar-method' (ECAM), was developed to evaluate seedling allelopathy against *L. rigidum* in a worldwide collection of wheat accessions originating from fifty countries (40). Using a similar ECAM-based screening bioassay, Bertholdsson (8) found that root exudates of certain wheat genotypes inhibited the root growth of perennial ryegrass (*L. perenne* L.) by as much as 50-60%. An increasing trend in potential allelopathic activity was found in spring wheat during 100 years of breeding, although there is a decreasing trend in barley (9). The high allelopathic activity in some modern spring wheat cultivars could be incorporated into high yielding wheat lines for better weed suppression.

Wheat allelopathy research has progressed rapidly toward the identification of bioactive allelochemicals responsible for both residue and seedling allelopathy on weeds. A number of phytotoxic substances suspected of causing allelopathic effects have been

identified in wheat, including phenolic acids, cyclic hydroxamic acids and short-chain fatty acids (22, 41, 42). Among them, cyclic hydroxamic acids and related benzoxazolinones (Bx) have been a particular focus due to their important roles in wheat's natural defense against pests, diseases and weeds (20, 28, 34). Perez (28) reported that 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and its decomposition product 6-methoxybenzoxazolin-2-one (MBOA) inhibited root growth of wild oats (*Avena fatua* L.). Huang et al. (20) reported that two Bxs DIMBOA and 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) were more inhibitory to *L. rigidum* than the natural lactam benzoxazinones, 2-hydroxy-1,4-benzoxazin-3-one (HBOA) and 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA).

The amounts of DIBOA and DIMBOA exuded by different wheat cultivars showed a positive correlation with their allelopathic activity on the root growth of *S. alba* (7). Similarly, in comparison with weakly allelopathic accessions, strongly allelopathic accessions produced significantly higher amounts of allelochemicals in the shoots or roots of the wheat seedlings, and also exuded larger amounts of allelochemicals into the growth medium (41, 42, 43).

Rapid advances in plant molecular sciences have provided unique opportunities to greatly improve the scientific rigor in wheat allelopathy research. This paper reviews the current application of genomics approaches in the identification of genetic markers associated with wheat allelopathy, the understanding of the mode of actions of allelochemicals, and the discovery of genes encoding the biosynthesis of allelochemicals.

2. GENETICS OF WHEAT ALLELOPATHY

Genetic variations in wheat allelopathic activity and the differential production of allelochemicals between wheat accessions have laid a solid foundation towards developing wheat cultivars with strong allelopathic potential. Although this ultimate breeding goal has not yet been achieved, progress has been made in understanding the genetic control of crop allelopathy. Research has revealed that allelopathic activity is quantitatively inherited in wheat and rice (14, 21, 40, 45). In a study of allelopathic activity of 453 wheat accessions on *L. rigidum*, Wu et al. (40) reported that wheat allelopathic activity was normally distributed, indicating that this weed-suppressing ability is a quantitative trait.

Wu et al. (40) studied the genetic control of wheat allelopathy using near isogenic wheat lines (NILs) derived from Hartog (weakly allelopathic) $\times$ Janz (strongly allelopathic). The allelopathic activity of BC_2-Hartog lines (backcrossed to Hartog) was weak, similar to that of Hartog. Similarly, Janz lines had strong allelopathic activity derived from Janz. These results suggest a strong chemical basis involved in the inhibition provoked by root exudates.

Complex biochemical pathways and distinct categories of allelopathic compounds indicate multiple genes are probably involved in the production of allelochemicals (40). Niemeyer and Jerez (24) investigated the chromosomal location of genes controlling DIMBOA production in wheat using euploid and aneuploid Chinese Spring wheat (high in DIMBOA) and substitution lines derived from a cross between Chinese Spring and the variety Cheyenne wheat (low in DIMBOA). It was found that chromosome 4A and 4B appeared to contain genes for the transformation of DIBOA into DIMBOA and chromosome 5B for the transformation of methoxylated lactam into DIMBOA. In addition, there appeared to be a gene in chromosome 4D inhibiting the accumulation of hydroxamic acids.

Recently, molecular techniques have been used to investigate the genetic markers associated with allelopathic activity in wheat. Genetic mapping of quantitative trait loci (QTLs) has shed light on the inheritance of allelopathy traits. QTLs conferring the allelopathic activity have been identified in wheat (45) and rice (14, 21). A doubled haploid (DH) population derived from cv. Sunco (weakly allelopathic) and cv. Tasman (strongly allelopathic) was developed to investigate the genetic control of wheat allelopathy (45). Analysis of restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP) and microsatellite (SSRs) makers identified two major QTLs on chromosome 2B associated with wheat allelopathy. Regression based on simple interval mapping revealed that one of these QLTs on chromosome 2B accounted for 29% of the total phenotypic variance of wheat seedling allelopathy (45). In rice, Jensen et al. (21) identified four QTLs on three rice chromosomes, explaining 35% of the total phenotypic variation of the allelopathic activity. Ebana et al. (14) also identified seven QTLs associated with rice allelopathic activity. A multiple model estimated that five QTLs explained 36.6% of the total phenotypic variation. These QTLs, once validated, can be used to improve genetic gains for the allelopathic activity through marker-assisted selection in crop breeding programmes.

3. CYTOCHROME P450 ENCODING BIOSYNTHESIS OF WHEAT SECONDARY METABOLITES

Cytochrome P450s are a large group of heme-containing enzymes present in all kingdoms (23). They catalyse diverse biochemical reactions including hydroxylations, dehalogenations, dealkylations, deaminations and epoxidations, and are therefore involved in myriad biosynthetic pathways of plant secondary metabolites, such as the biosynthesis of

pigments, antioxidants and defense compounds, which include phenylpropanoids, flavonoids, phenolic esters, coumarins, glucosinolates, cyanogenic glucosides, benzoxazinones, isoprenoids, alkaloids, terpenoids, lipids, and plant growth regulators such as gibberellins, jasmonic acid, and brassinosteroids (11). Examples of P450s involving the biosynthesis of plant secondary metabolites are given in Table 1. Many of these secondary metabolites are excellent lead molecules for herbicide development (30).

The growing interest in plant cytochrome has led to the identification of many plant P450s encoding the biosynthesis of defense compounds, including the notable Bxs in maize and wheat. The biosynthetic pathway of Bxs branches off from that of tryptophan at indole-3-glycerol phosphate. Five genes involved in the downstream reactions from indole-3-glycerol phosphate were isolated from maize and designated as *Bx*1– *Bx*5 (16). *Bx*1 encodes indole synthase homologous to the tryptophan synthase alpha subunit (TSA), whereas *Bx*2–*Bx*5 genes encode four cytochrome P450 monooxygenases (CYP71C1-CYP71C4) (16). Expression of cDNAs in yeast demonstrated that each of these cloned P450s catalysed sequential hydroxylation of indole to DIBOA.

Further study has demonstrated that *Bx6* and *Bx7* genes are involved in the conversion of DIBOA to DIMBOA (17). *Bx6* encodes a 2-oxoglutarate-dependent dioxygenase that catalyses the hydroxylation of DIBOA at position 7. The resulting product (TRIBOA) is then methylated by the O-methyltransferase *Bx*7 to generate DIMBOA. Two glucosyltransferase genes *Bx8* and *Bx*9 are both able to catalyse the conversion of DIBOA and DIMBOA to DIBOAGlc and DIMBOAGlc, respectively (31). *Bx*s genes (*Bx*1 - *Bx*8), governing the entire DIMBOA biosynthesis, have been shown to cluster on the short arm of

chromosome 4, although *Bx*9 was located on chromosome 1 (17, 31). A schematic biosynthetic pathway of DIMBOA is illustrated in Figure 1.

cDNAs of five P450s (CYP71C6, CYP71C7v2, CYP71C8v2, CYP71C9v1 and CYP71C9v2) involved in DIBOA biosynthesis were also isolated in wheat (25). CYP71C9v1 and CYP71C9v2 shared 97% similarity in amino acid and nucleotide sequences. The cloned P450 species showed 76–79% identity at the amino acid level to the corresponding maize P450 species CYP71C1–C4, suggesting the common origin of DIBOA biosynthesis in wheat and maize. Nomura et al. (26) further found that these cDNAs (TaBx1–TaBx5) genes were separately located on two groups of chromosomes in wheat. TaBx1 and TaBx2 co-existed in specific regions of chromosomes 4AS, 4BL and 4DL. TaBx3 genes were located on 5AS, 5BS, 5DS and 5BL, while TaBx4 and TaBx5 genes were located on the short arms of group-5 chromosomes.

Since the cloning of the first plant P450 gene in 1990, there has been an explosion in the rate at which genes encoding plant P450s have been identified. Overexpression of these P450 genes may modify the flux through biosynthetic pathways that give rise to accumulation of allelochemicals. Alternatively, the introduction of foreign P450 genes into alternative host plants may allow for the engineering of novel biochemical pathways and the synthesis of potent allelochemicals for weed suppression.

4. APPLICATION OF DNA MICROARRAY TECHNOLOGY IN UNVEILING THE MODE OF ACTIONS OF ALLELOCHEMICALS

There has been growing interest in using modern DNA microarray technology for assessing the transcriptome responses induced by allelochemicals (2, 4). DNA microarray or

'chip' technology, in which very large numbers of cDNA species or oligonucleotides are gridded for hybridisation to target DNA molecules, is advancing rapidly. Gene expression profiling using DNA microarrays can be used to characterise novel mechanisms of action of allelochemicals through the identification of gene expression networks (2, 4, 32).

DNA microarray technology has been used extensively in gene expression profiling, and in the identification and genotyping of polymorphisms (1). This technology is widely used to simultaneously monitor the expression levels of thousands to tens of thousands of genes. In microarray data analyses, genes showing 2-fold relative expression levels at least or >2 SDs away from the mean among expression levels are often considered to show precise measurement or significantly different expression from the control. These genes are selected for further analysis (47).

One of the wheat allelochemicals, BOA, derived from a two-step degradation of the glucoside of DIBOA, was studied in detail on its mode of action and its effect on gene expression in the genomic model plant *Arabidopsis thaliana* by using DNA microarray technology (3). It was found that one of the largest functional categories observed for BOA-responsive genes corresponded to protein families known to participate in cell rescue and defense, with the majority of these genes potentially associated with chemical detoxification pathways. Further experiments using a subset of these genes revealed that many are also transcriptionally induced by a variety of structurally diverse xenobiotic compounds, suggesting they comprise components of a co-ordinately regulated, broad specificity xenobiotic defense response. Using several physiological, biochemical and

molecular techniques, Reigosa (32) reported that BOA showed multiple modes of action, which include both primary and secondary effects.

DNA microarray methods have also been used to discover the mode of action of (–)-catechin, an allelochemical isolated from spotted knapweed (*Centaurea maculosa*) (4). Researchers reported that (–)-catechin initiated a wave of reactive oxygen species (ROS) at the root meristem of *Arabidopsis thaliana*, which leads to a genome-wide changes in gene expression and ultimate death of the root system. Analysis of global gene expression showed that after 1 hour of treatment with (–)-catechin (100 µg/ml), 956 genes were induced twofold or greater whereas by 12 hours many of these same genes were repressed, likely reflecting the onset of cell death. A large number of these induced genes were related to oxidative stress and the phenylpropanoid and terpenoid pathways. A global gene expression profile obtained 10 min after (–)-catechin treatment showed a cluster of 10 genes upregulated. These genes were associated with a steroid sulfotransferase-like protein, α-cystathionase, calmodulin, a ribosomal protein L9, peroxidase ATP21a, a chlorophyll binding protein, and four uncharacterized genes. These genes may be implicated in plant-specific early signal transduction events linked to oxidative stress. It seems likely that acclimation to oxidative stress generated by ROS signalling after (–)-catechin treatment involves concerted, long-term potentiation of different sets of antioxidant and defense genes.

Microarray methods are essential for the initial screening of many thousands of genes in a parallel analysis. However, the results obtained often need to be validated. The reliability of microarray experiments may sometimes be questioned (18). Since plants display a high number of multigene families, cross-hybridization between cDNA

representatives of members of gene families on cDNA-based chips may lead to false interpretations. Candidate genes identified from microarray studies are validated with real-time polymerase chain reaction (RT-PCR) and/or in situ hybridization (3, 10). A strategy of combining microarray with RT-PCR analysis is widely used. Microarray experiments can analyse thousands of genes in one single step to point out a handful of potentially interesting genes both up-regulated and down-regulated, whereas confirmation of these candidate genets is performed by RT-PCR analysis.

5. APPLICATION OF FUNCTIONAL GENOMICS APPROACHES IN LOCATING GENES FOR ENCODING THE BIOSYNTHESIS OF ALLELOCHEMICALS

A better understanding of metabolite synthesis and the regulation thereof will be increasingly important for improving plant natural defense mechanisms against pest, diseases and weeds. The availability of the complete genome sequences of *Arabidopsis thaliana* and rice, accompanied by the development of sophisticated molecular tools to assess gene regulation and expression on a large scale (i.e., DNA microarrays), have opened new opportunities for dissecting the biochemical pathways involved in cell metabolism. Gene expression profiling with DNA microarrays and expressed sequence tag (EST) are instrumental in providing a holistic picture of the underlying genetic complexities that orchestrate biochemical pathways and networks.

EST analysis, the generation of single-pass DNA sequence data sets from randomly selected cDNA library clones (6), has in recent years emerged as a highly effective approach for identifying genes involved in plant secondary metabolic pathways. Over three million

sequences from approximately 200 plant species have been deposited in the publicly available plant expressed sequence tag (EST) sequence databases (33). Many of the ESTs have been sequenced as an alternative to complete genome sequencing or as a substrate for cDNA array-based expression analyses. Baerson (3) used EST analysis to identify genes involved in the biosynthesis of the allelochemical sorgoleone. This approach, coupled with high-throughput gene expression analysis using quantitative real-time RT-PCR, has provided a highly efficient means for identifying candidate fatty acid desaturase, polyketide synthase, *O*-methyltransferase, and P450-like sequences preferentially expressed in sorghum (*Sorghum bicolor* L. Moench) root hair cells. This has led to significant progress in the identification of *O*-methyltransferase and polyketide synthase genes potentially involved in sorgoleone biosynthesis.

The availability of the complete rice genome (48) now enables the identification of the genes involved in the production of the more important allelochemicals in rice. Allelopathic rice produces glycosides of lipid resorcinols, flavones, and benzoxazinoids, as well as momilactones and cyclohexenones (12). The genomic information of rice was used to find that the gene for *syn*-copalyl diphosphate synthase plays a regulatory role in the synthesis of the momilactones and structurally related phytoalexins (46). Using a similar approach, the rice genome available from the Institute for Genomic Research (www.tigr.org) was surveyed for the presence of genes encoding for putative polyketide synthase (PKS) (12). The analysis showed that there are 33 PKS-like genes in rice. Of these, 8 sequences were selected as candidates genes because they had significant similarity to the PKS genes involved in the synthesis of lipid resorcinol in sorghum. Genes putatively involved in the ring formation of these unusual resorcinols in rice have been identified. These novel

polyketide synthases accept long chain fatty acid-CoA substrates instead of the usual coumaroyl-CoA substrate used by the more common PKS such as chalcone and stilbene synthases. The substrate specificity of some of these rice enzymes indicates that they may be involved in the biosynthetic pathway of lipid resorcinols (12).

6. DISCUSSION

Crops have not yet been engineered to enhance the biosynthesis of allelochemicals for weed control, although they have been made resistant to insects, pathogens, and herbicides with transgenes. For example a gene from *Bacillus thuringiensis* (Bt) has been successfully engineered into cotton to produce insecticidal toxin (29). The successful metabolic engineering of these plant defense compounds to improve resistance to insects and diseases has provided a novel approach for integrated weed management.

Molecular and biochemical approaches are now being rapidly applied to agricultural research. Multi-parallel analyses of genomics, transcriptomics, metabolomics and bioinformatics provide exciting opportunities for the discovery of novel structural and regulatory genes in the biochemical pathways of specific plant metabolites, such as alkaloids, flavonoids and isoprenoids (27). Metabolomics, the unbiased identification and quantitation of all the metabolites, has emerged as a viable counterpart to transcriptomics and proteomics (15, 38). Advances in instrumentation for metabolite analyses are empowering us with the ability to increase the coverage of metabolites within a single analysis. For example, Hirai et al. (19) used the Fourier transform–ion cyclotron MS, with mass resolution > 100,000 and accuracy < 1 ppm, enabling analysis and separation of complex metabolite mixtures based on isotopic mass alone. More commonly, GC-MS- and

liquid chromatography MS-based approaches have been applied in plant metabolomics applications (15).

All ‘‘-omics’’ approaches have generated a huge amount of data. As a result, novel bioinformatics tools are being developed to integrate the data sets and to generate gene-to-metabolite crosstalk networks. The mining and exploitation of the data obtained from genomics and the related research areas of genome-wide transcriptomics, proteomics, and metabolomics provides possibilities for assigning gene functions based on cellular dynamics (13).

Interdisciplinary approaches, combining expertise from biology, chemistry, instrumentation, computer science, physics and mathematics, will lead us into a new era of understanding of wheat allelopathy. A diverse range of wheat genetic stocks, such as wheat-barley addition lines and wheat-rye addition and translocation lines, could be used to identify genes conferring natural chemical defense on weeds. Transcriptomics and metabolomics analyses of existing wheat genetic stocks would provide comprehensive knowledge on genes conferring the biosynthesis of natural defense compounds and their chromosome locations. These alien elite genes will ultimately be introgressed into wheat via chromosome engineering for improved management on weeds.

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Table 1. Plant cytochrome P450s encoding the biosynthesis of secondary metabolites. (http://members.shaw.ca/P450sinPlants).

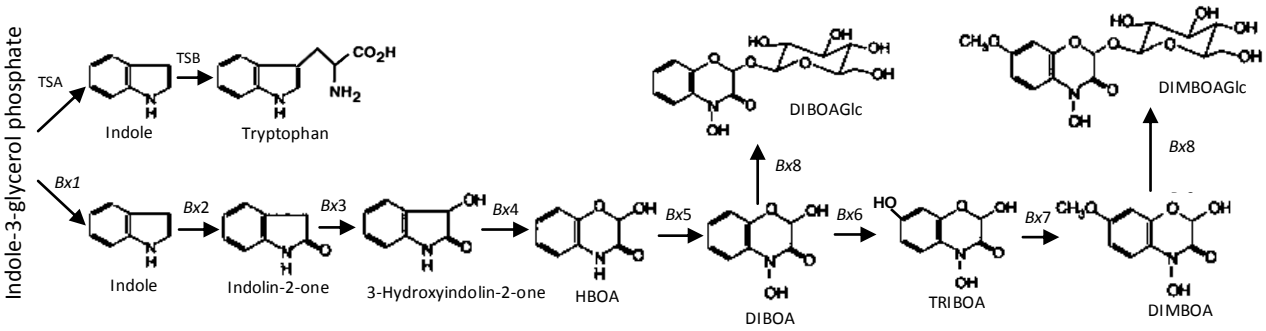
P450	Pathway	Plant Species
CYP71C1-4	cyclic hydroxamic acid	Corn
CYP71C6, C7v2, C8v2, C9v1 C9v2	cyclic hydroxamic acid	Wheat
CYP71D12	indole alkaloid	periwinkle
CYP71D9	flavonoid/isoflavonoid	Soybean
CYP71E1	cyanogenic glucoside	sorghum
CYP72A1	indole alkaloid	periwinkle
CYP73A5	phenylpropanoid	*Arabidopsis thaliana*
CYP75A1	phenylpropanoid	Petunia
CYP75B1	phenylpropanoid	*Arabidopsis thaliana*
CYP76B6	terpenoid indole alkaloid	periwinkle
CYP79A1	cyanogenic glucoside	sorghum
CYP79A2	benzylglucosinolate	*Arabidopsis thaliana*
CYP79B2	indole glucosinolate	*Arabidopsis thaliana*
CYP79D1 / D2	cyanogenic glucosides	Cassava
CYP79E1 / E2	cyanogenic glucosides	seaside arrow grass
CYP79F1	aliphatic glucosinolate	*Arabidopsis thaliana*
CYP80B1	alkaloid	California

		poppy
CYP81E1	flavonoid/isoflavonoid	Licorice
CYP83A1	indole glucosinolate	*Arabidopsis thaliana*
CYP84A1	phenylpropanoid	*Arabidopsis thaliana*
CYP85	brassinosteroid biosynthesis	Tomato
CYP85A1	brassinolide	*Arabidopsis thaliana*
CYP86A1	fatty acids	*Arabidopsis thaliana*
CYP90A1	brassinolide	*Arabidopsis thaliana*
CYP90B1	brassinolide	*Arabidopsis thaliana*
CYP90D2	brassinosteroid biosynthesis	Rice
CYP92A6	brassinosteroid biosynthesis	Pea
CYP93A1	pterocarpanoid phytoalexin biosythesis	Soybean
CYP93B1	flavonoid/isoflavonoid	Licorice
CYP93C1	isoflanoids	Soybean
CYP94A5	fatty acids	Tobacco
CYP96C1	terpenoid indole alkaloid	Periwinkle
CYP97C1	carotenoid	*Arabidopsis thaliana*

CYP98A3	phenypropanoid	*Arabidopsis thaliana*
CYP706B1	sesquiterpene biosynthesis	Cotton

Figure 1. DIMBOA biosynthetic pathway in maize. TSA and TSB stand for the alpha and beta subunits of tryptophan synthase, respectively. *Bx*1 represents a tryptophan synthase activity, *Bx*2 to *Bx*5 represent cytochrome P450 monooxygenases; *Bx*6 and *Bx*7 encode 2-oxoglutarate-dependant dioxygenases, and *Bx*8 and *Bx*9 encode glucosyltransferases.

Figure 1.


Indole-3-glycerol phosphate
TSA
TSB
Indole
Tryptophan
Bx1
Bx2
Bx3
Bx4
Bx5
Bx6
Bx7
Bx8
Indole
Indolin-2-one
3-Hydroxyindolin-2-one
HBOA
DIBOA
TRIBOA
DIMBOA
DIBOAGlc
DIMBOAGlc