Phytochemistry 70 (2009) 1645–1651

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Review

Benzoxazinoid biosynthesis, a model for evolution of secondary metabolic pathways in plants

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ARTICLE INFO

Article history:
Received 6 March 2009
Received in revised form 23 April 2009
Available online 3 July 2009

Keywords:
Hydroxamic acids
Cytochrome P450 monooxygenase
DIBOA
DIMBOA
Evolution of secondary metabolism
Functional gene cluster
Glucosyltransferase
O-methyltransferase
2-Oxoglutarate dependent dioxygenase
Tryptophan synthase

ABSTRACT

Benzoxazinoids are secondary metabolites that are effective in defence and allelopathy. They are synthesised in two subfamilies of the Poaceae and sporadically found in single species of the dicots. The biosynthesis is fully elucidated in maize; here the genes encoding the enzymes of the pathway are in physical proximity. This "biosynthetic cluster" might facilitate coordinated gene regulation. Data from *Zea mays*, *Triticum aestivum* and *Hordeum lechleri* suggest that the pathway is of monophyletic origin in the Poaceae. The branchpoint from the primary metabolism (*Bx1* gene) can be traced back to duplication and functionalisation of the alpha-subunit of tryptophan synthase (TSA). Modification of the intermediates by consecutive hydroxylation is catalysed by members of a cytochrome P450 enzyme subfamily (*Bx2–Bx5*). Glucosylation by an UDP-glucosyltransferase (UGT, *Bx8*, *Bx9*) is essential for the reduction of autotoxicity of the benzoxazinoids. In some species 2,4-dihydroxy-1,4-benzoxazin-3-one-glucoside (DIBOA-glc) is further modified by the 2-oxoglutarate-dependent dioxygenase BX6 and the *O*-methyltransferase BX7. In the dicots *Aphelandra squarrosa*, *Consolida orientalis*, and *Lamium galeobdolon*, benzoxazinoid biosynthesis is analogously organised: The branchpoint is established by a homolog of TSA, P450 enzymes catalyse hydroxylations and at least the first hydroxylation reaction is identical in dicots and Poaceae, the toxic aglucon is glucosylated by an UGT. Functionally, TSA and BX1 are indole-glycerolphosphate lyases (IGLs). *Igl* genes seem to be generally duplicated in angiosperms. Modelling and biochemical characterisation of IGLs reveal that the catalytic properties of the enzyme can easily be modified by mutation. Independent evolution can be assumed for the BX1 function in dicots and Poaceae.

Contents

1. Introduction

Plants produce a vast array of secondary metabolites. Many of these natural products are specialised metabolites that are produced only by certain taxa (Dixon, 2001). Benzoxazinoids represent protective and allelophatic metabolites that are found in a multitude of species of the family Poaceae (Gramineae) of the

Abbreviations: 2-ODD, 2-oxoglutarate dependent dioxygenase; bp, basepair; P450, cytochrome P450 enzyme; DIBOA, 2,4-dihydroxy-1,4-benzoxazin-3-one; DIMBOA, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one; HBOA, 2-hydroxy-1,4-benzoxazin-3-one; TRIBOA, 2,4,7-trihydroxy-1,4-benzoxazin-3-one; UGT, UDP-glucosyltransferase.

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0031-9422/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2009.05.012

monocot plants (Table 1), including the major agricultural crops maize, wheat and rye. Outside the Poales, benzoxazinoids are detected in two distant orders of the eudicots, the Ranunculales and the Lamiales. In contrast to the situation in the Poales, benzoxazinoid biosynthesis in these orders is restricted to single isolated species within the families Ranunculaceae (i.e. *Consolida orientalis*), Lamiaceae (*Lamium galeobdolon*) and Plantaginaceae (*Scoparia dulcis*) (Sicker et al., 2000; Alipieva et al., 2003) and to several species in the Acanthaceae (*Acanthus mollis*, *Aphelandra tetragona*, *Aphelandra squarrosa*, *Blepharis edulis;* Baumeler et al., 2000). DIBOA [2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one] and its C-7-methoxy derivative DIMBOA (Fig. 1) are the predominant representatives of benzoxazinoids in plants (Niemeyer, 1988). The end product of the benzoxazinoid biosynthesis is the glucoside that has reduced toxicity compared to the aglucon. The glucoside is stored in the vacuole; the specific glucosidase is located in the plastid. The toxic aglucon is produced upon disintegration of the cell due to pathogen or pest attack. The reactivity of DIBOA and DIMBOA with e.g. NH_2 and SH nucleophilic groups in biomolecules (Sicker et al., 2000) confers protection against a wide range of herbivores, pathogenic fungi and bacteria. Benzoxazinoid biosynthesis is fully elucidated in maize (Frey et al., 1997, 2003; Rad et al., 2001; Jonczyk et al., 2008), is characterised in part for wheat (Nomura et al., 2002, 2005, 2008), and diploid Triticales (Nomura et al., 2007) and wild barley (Grün et al., 2005). Benzoxazinoid biosynthesis commences in dicots with the same precursor; however, the complete pathway remains to be elucidated (Schullehner et al., 2008).

2. Benzoxazinoid biosynthesis in maize

The benzoxazinoid biosynthetic pathway was established in maize (Fig. 1). The branchpoint reaction was defined by the isolation of the *Bx1* gene (Frey et al., 1997; Melanson et al., 1997) that is characterised by the benzoxazinless phenotype of the mutant maize plant (Hamilton, 1964). Indole-3-glycerolphosphate is converted by BX1 to indole, the first committed intermediate of the pathway. The same reaction is performed by the alpha-subunit of the tryptophan synthase (TSA) in tryptophan biosynthesis. BX1 and TSA both are indole-3-glycerolphosphate lyases (IGLs), encoded by paralogous genes. Modifications have changed the enzymatic activities of the homologs. While TSAs form a complex with the beta-subunit of the tryptophan synthase (TSB), BX1 functions as monomer, optimised for the production of free indole (Frey et al., 2000). In the following, we name indole-3-glycerol phosphate lyases 'IGL' as a neutral designation for enzyme function, 'BX1' when the catalytic parameters are in the range of ZmBX1 and we restrict the name 'TSA' to subunits of the tryptophan synthase. Like TSA, BX1 is localised in the stroma of the chloroplast.

The following reactions in biosynthesis comprise the conversion of indole to DIBOA. The introduction of four oxygen atoms is catalysed by four cytochrome P450 dependent monooxygenases (P450s) BX2 to BX5, which are members of the CYP71 family (Frey et al., 1995). Indole is converted by BX2 to indolin-2-one, which is transformed to 3-hydroxy-indolin-2-one by BX3. Subsequently an unusual ring expansion is catalysed by BX4: the conversion of 3-hydroxy-indole-2-one to 2-hydroxy-2-1,4-benzoxazin-3-one (HBOA, Spiteller et al., 2001). The *N*-hydroxylation of HBOA to DIBOA is catalysed by BX5. All four P450s are specific with respect to the substrate and the regioselective introduction of oxygen atoms. In maize, the two UDP-glucosyltransferases (UGTs) BX8 and BX9 specifically glucosylate benzoxazinoids (Rad et al., 2001). This glucosylation of benzoxazinoids takes place prior to hydroxylation by the 2-oxoglutarate dependent dioxygenase (2-ODD) BX6 (Frey et al., 2003) and the *O*-methylation by *O*-methyltransferase (OMT) BX7 (Jonczyk et al., 2008). DIBOA is the first toxic intermediate of the pathway. Reduction of DIBOA reactivity by glucosylation might be required in order to reduce autotoxicity and to provide a stable metabolite for further modifications by BX6 and BX7 that are localised in the cytoplasm (Jonczyk et al., 2008). Mutants of *Bx8* and *Bx9* are not yet available, hence the mutant phenotype is unknown. However, the example of the maize UGT BZ1 demonstrates that mutation in a glucosyltransferse of secondary metabolism results in a phenotypic alteration (Schiefelbein et al., 1988). Other examples for glucosylation of biosynthetic intermediates in plant secondary pathways are rare but occur in glucosinolate biosynthesis of *A. thaliana* (Grubb et al., 2004), in loganin biosynthesis of *Lonicera japonica* (Katano et al., 2001) and in aurone biosynthesis of *Antirrhinum majus* (Nakayama et al., 2000).

Table 1
Distribution of benzoxazinoids in angiosperms.

	Tribe	Genus	Benzoxazinoid
Monocots			
Poaceae[a]			
Subfamily Pooidae	Triticeae	*Aegilops*	DIBOA/DIMBOA
		Agropyron	DIBOA/DIMBOA
		Elymus	DIBOA/DIMBOA
		Elytrigia	DIBOA/DIMBOA
		Hordeum	DIBOA
		Leymus	DIBOA/DIMBOA
		Pascopyrum	DIBOA/DIMBOA
		Psathyrostachys	DIBOA
		Pseudoroegneria	DIBOA/DIMBOA
		Secale	DIBOA/DIMBOA
		Thinopyrum	DIBOA/DIMBOA
	Aveneae	*Deschampsia*	DIBOA/DIMBOA
		Phalaris	DIBOA/DIMBOA
Subfamily Pancoideae			
	Maydeae	*Coix*	DIMBOA
		Tripsacum	DIBOA/DIMBOA
		Zea	DIBOA/DIMBOA
Dicots			
		Species	
Ranunculaceae		*Consolida orientalis*	DIBOA
Acanthaceae		*Aphelandra auriantica*	DIBOA/DIMBOA
		Aphelandra squarrosa	DIBOA/DIMBOA
		Acanthus mollis	DIBOA
		Aphelandra tetragona	DIBOA
		Blepharis edulis	HBOA
Plantaginaceae		*Scoparia dulcis*	DIBOA
Lamiaceae		*Lamium galeobdolon*	DIBOA

Data from Baumeler et al. (2000), Gianoli and Niemeyer (1998), Niemeyer et al. (1992), Schullehner et al. (2008) and Sicker et al. (2000).
[a] Several species within the genera indicated synthesise benzoxazinoids.

3. Monophyletic origin of DIBOA biosynthesis in the grasses

In the Poaceae, the expression of the pathway is developmentally regulated and highest benzoxazinoid levels are displayed at about four days after germination, both in root and shoot. Root exudation has been shown to be an important allelopathic function and has been recognised in the 1980s for rye (Barnes and Putnam, 1987). While the main aglucon in maize and wheat is DIMBOA, DIBOA is predominant in rye leaves, DIMBOA and DIBOA are found in the rye root (Copoja et al., 2006). DIBOA-glucoside is the final product in wild *Hordeum* species and several other species of the Poaceae (Niemeyer et al., 1992).

The sequence of reaction steps to DIBOA is identical in maize, diploid and hexaploid wheat and the wild barley *Hordeum lechleri*. The genes *Bx1* to *Bx5* have been isolated from hexaploid (*Triticum aestivum*, Nomura et al., 2002, 2003) and diploid wheat (*Triticum monococcum, Triticum urartu,* and *Triticum boeoticum*, Nomura et al., 2007) and the wild barley (*H. lechleri*, Grün et al., 2005). All