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A Gene Encoding the Cytokinin Enzyme Zeatin *O*-Xylosyltransferase of *Phaseolus vulgaris*[1]

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Zeatin is the most active and ubiquitous form of the naturally occurring cytokinins. Glycosyl conjugates of zeatin are found in many plant tissues and are considered important for storage and protection against degradative enzymes. Two enzymes catalyzing the formation of *O*-glycosyl derivatives of zeatin have been characterized, *O*-glucosyltransferase and *O*-xylosyltransferase, occurring in seeds of lima bean (*Phaseolus lunatus)* and bean (*Phaseolus vulgaris),* respectively. Recently, the *ZOG1* gene (zeatin *O*-glucosyltansferase) was isolated from *P. lunatis* (Martin et al., 1999). Based on the *ZOG1* sequence, the *ZOX1* gene (zeatin *O*-xylosyltransferase) was cloned from *P. vulgaris*. *ZOX1* contains an open reading frame of 1362 bp that codes for a 454-amino acid peptide of 51 kD. The recombinant protein has properties identical to the native enzyme: it catalyzes *O*-xylosylzeatin formation with UDP-Xyl as a glycosyl donor but does not recognize UDP-Glucose as a substrate. The *ZOX1* and *ZOG1* genes exhibit 93% identity at the nucleotide level and 90% similarity at the amino acid level. Neither gene contains introns. These zeatin-specific genes and their promoters will be useful for studies of the regulation of active versus storage forms of cytokinins. Comparison of sequences encoding similar enzymes with distinct substrate specificity may lead to identification of epitopes specific to cytokinin and glycosyl donor molecules.

Cytokinins are important in plant cell division and differentiation (Miller et al., 1956). Zeatin, an adenine derivative first found in maize, is ubiquitous and the most active cytokinin (Letham, 1963; Shaw, 1994). Studies concerning structure-activity relationships (Skoog and Armstrong, 1970) have revealed the importance of the N^6 side chain for cytokinin activity. The *trans*-hydroxylated isoprenoid side chain of zeatin confers high cytokinin activity, but it also makes it vulnerable to attack by degradative enzymes, the cytokinin oxidases (Whitty and Hall, 1974; Armstrong, 1994). Modification of the side chain, such as reduction to dihydrozeatin or *O*-glycosylation, confers resistance to cytokinin oxidases. Although dihydrozeatin is active itself, the *O*-glucosyl derivatives are believed to be storage products and transport forms and to be active only after conversion back to zeatin by β-glucosidases (Jameson, 1994; Letham, 1994).

There are pronounced differences between *Phaseolus* sp. in the formation of glycosyl conjugates. In lima bean (*Phaseolus lunatus*) seed exogenous zeatin is rapidly converted to *O*-glucosylzeatin, whereas in bean (*Phaseolus vulgaris*) seed zeatin is metabolized to *O*-xylosylzeatin (Lee et al., 1985; Turner et al., 1987; Dixon et al., 1989). The precise function of *O*-xylosylzeatin is not known, but presumably it is similar to *O*-glucosylzeatin, since *O*-xylosylzeatin can also be reconverted to zeatin and it is active in bioassays (Mok et al., 1987). The corresponding enzymes have been isolated from the two species (Turner et al., 1987; Dixon et al., 1989; Mok and Martin, 1994). The zeatin *O*-xylosyltransferase (EC 2.4.1.204) of *P. vulgaris* uses UDP-Xyl as the sugar donor, whereas the *O*-glucosyltransferase (EC 2.4.1.203) of *P. lunatus* can use both UDP-Glc and UDP-Xyl to form *O*-glucosylzeatin or *O*-xylosylzeatin but has much higher affinity to UDP-Glc (Dixon et al., 1989). The two enzymes can be separated by charge on ion-exchange columns or by PAGE. Thus far, they are the only cytokinin *O*-glycosyltransferases characterized.

Recently, the cDNA and the gene *ZOG1* (zeatin *O*-glucosyltransferase) were isolated from *P. lunatus* by screening an expression library with monoclonal antibodies to the enzyme (Martin et al., 1999). In this paper we describe the cloning of the *ZOX1* (zeatin *O*-xylosyltransferase) gene from *P. vulgaris* using the sequence information from *ZOG1*. The authenticity of the gene is confirmed by the catalytic activity and substrate specificity of the recombinant protein of *ZOX1*, which are identical to the native zeatin *O*-xylosyltransferase.

MATERIALS AND METHODS

Plant Materials

Immature seeds of bean (*Phaseolus vulgaris* L. cv Great Northern [GN]) were the source for isolation of the native zeatin *O*-xylosyltransferase, as described previously (Dixon et al., 1989). Leaves of cv GN were used for isolation of DNA.

PCR, Inverse PCR, and Sequencing

Standard PCR and inverse PCR protocols (Ochman et al., 1990) were used. Inverse PCR was used first to determine

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Abbreviation: ORF, open reading frame.

the 5′ region of the *P. vulgaris* gene. For that purpose, DNA of cv GN was digested with *Hin*dIII. After digestion, the restriction enzyme was inactivated by heating the sample at 75°C for 10 min. After dilution, T_4 DNA ligase (Promega) was added to allow intramolecular ligation (circularization) at 15°C for 24 h. The DNA was precipitated, and inverse PCR was performed with primers CATGGAGATGGGTTCTTTCATTGCAC (primer A) and CAACAACTGAAGCACTCACCAACG (primer B) to amplify the 5′ and 3′ border regions, respectively. The products obtained from inverse PCR reactions were analyzed on a 1% Sea Plaque gel (FMC Bioproducts, Rockland, ME). Bands of interest were excised and DNA was purified with a Qiaex II gel extraction kit (Qiagen, Chatsworth, CA). The products were ligated into a pGem-T vector (Promega) for sequencing. Subsequently, flanking primers CCAAAGTCGACAATGGCTTTGAATGATG (primer C) and GCTATGCGGCCGCCTAAATGGTATGAC (primer D) were used to obtain the complete *ZOX1* gene with standard PCR procedures. PCR products were analyzed as the inverse PCR products above.

Sequencing and primer synthesis were performed by the Central Services Laboratory (Center for Gene Research and Biotechnology, Oregon State University, Corvallis). A DNA sequence analyzer (model 370A, Applied Biosystems) was used for sequencing, and a DNA synthesizer (Applied Biosystems) was used for primer synthesis.

Isolation of Recombinant Proteins

To obtain recombinant proteins, the inserts were excised from the pGem-T plasmid by digestions with the restriction enzymes *Sal*I and *Not*I and cloned into pZL1 plasmid. The plasmids were then used to transform Epicurian Coli BL21(DE3) pLysS competent cells (Stratagene) following the recommended protocol. The induction conditions were the same as described previously by Martin et al. (1999).

RNA Blot

Poly(A^+) RNA was isolated from various cv GN tissues as described previously by Martin et al. (1999). mRNA (4 μg) was separated on a 1.2% formaldehyde gel. RNA blotting (RNA capillary transfer) to Zeta Probe GT membranes (Bio-Rad) was performed according to the manufacturer's instructions. The *ZOX1* ORF was used to synthesize an [α-^{32}P]dCTP-labeled probe with Ready-To-Go DNA-labeling beads (Pharmacia).

DNA Blot

DNA was extracted from young cv GN leaves with the modified cetyltrimethylammonium bromide procedure as described earlier (Martin et al., 1999). DNA (30 μg) was digested with restriction enzymes and separated on a 1.1% gel and transferred to a Zeta Probe GT membrane. Hybridization was performed as for the RNA blotting with [α-^{32}P]dCTP-labeled *ZOX1* as the probe.

Enzyme Assays and Analysis of Reaction Products

Enzyme activity was determined as reported previously (Dixon et al., 1989). The reaction mixture consisted of ^{14}C-labeled cytokinins (specific activity of 24 mCi/mmol), glycosyl donor (3 mM of UDP-Xyl or UDP-Glc), 0.05 M $MgCl_2$, 0.5 mM ATP, and recombinant protein in 100 mM Tris, pH 8.0. The reaction mixture was incubated at 27°C for 4 h. Reaction products were separated by HPLC (Dixon et al., 1989).

RESULTS

Isolation of *ZOX1* by PCR

Initial experiments using *P. vulgaris* DNA as the template and primers covering various segments of the *ZOG1* sequence yielded only products corresponding to the middle and 3′ regions of the *ZOG1* gene (data not shown), suggesting that there was divergence between the 5′ terminus of the *ZOG1* and the *P. vulgaris* gene. To generate a product containing the 5′ end of the *P. vulgaris* gene, inverse PCR was performed with forward primer A at the 3′ end and backward primer B close to the 5′ end. A product of approximately 1.5 kb was obtained, and the sequences containing the ends of the ORF were used to synthesize primers C and D. Amplification of *P. vulgaris* DNA with these primers resulted in a genomic clone of 1390 bp. The clone

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ZOX1    1 MALND.....ETKVVVLLLPFPVQGHLNPFLQLSHLIAAQNIAVHYVGTV 45
          |||||     ||||||||:||| ||||| || || || |||| |||||||
ZOG1    1 MALNDKSIPHETKVVVLLIPFPAQGHLNQFLHLSRLIVAQNIPVHYVGTV 50

         46 THIRQAKLRYHNATSNIHFHAFEVPPYVSPPPNPEDDFPSHLIPSFEASA 95
            |||||| |||.| |||||||||:|||:||||||||||||||||||||||
         51 THIRQATLRYNNPTSNIHFHAFQVPPFVSPPPNPEDDFPSHLIPSFEASA 100

         96 HLREPVGKLLQSLSSQAKRVVLINDSLMASVAQDAANFSNVERYCFQVFS 145
            ||||||||||||||||||||.|||||||||||||||| |||| | |  ||
        101 HLREPVGKLLQSLSSQAKRVVVINDSLMASVAQDAANISNVENYTFHSFS 150

        146 ALNTAGDFWEQMGKPPLADFHFPDIPSLQGCISAQFTDFLTAQNEFRKFN 195
            | ||.|||||:|||||. ||||||: |||:|||.|||  | ||| ||||||
        151 AFNTSGDFWEEMGKPPVGDFHFPEFPSLEGCIAAQFKGFRTAQYEFRKFN 200

        196 NGDIYNTSRVIEGPYVELLERFNGGKEVWALGPFTPLAVEKKDSIGFSHP 245
            |||||||||||||||||||| |||||.||||||| |||||||||||| ||
        201 NGDIYNTSRVIEGPYVELLELFNGGKKVWALGPFNPLAVEKKDSIGFRHP 250

        246 CMEWLDKQEPSSVIYVSFGTTTALRDEQIQELATGLEQSKQKFIWVLRDA 295
            |||||||||||||||:|||||||||||||::|||||||||||||||||:|
        251 CMEWLDKQEPSSVIYISFGTTTALRDEQIQQIATGLEQSKQKFIWVLREA 300

        296 DKGDIFDGSEAKRYELPEGFEERVEGMGLVVRDWAPQMEILSHSSTGGFM 345
            |||||| |||||||||.|||||||||||||||||||:|||||||||||||
        301 DKGDIFAGSEAKRYELPKGFEERVEGMGLVVRDWAPQLEILSHSSTGGFM 350

        346 SHCGWNSCLESLTRGVPMATWAMHSDQPRNAVLVTDVLKVGLIVKDWEQR 395
            ||||||||||||:| |||.||| |||||||||||||:||||||:|||| ||
        351 SHCGWNSCLESITMGVPIATWPMHSDQPRNAVLVTEVLKVGLVVKDWAQR 400

        396 KSLVSASVIENAVRRLMETKEGDEIRKRAVKLKDEIHRSMDEGGVSRMEM 445
             |||||||:|| ||||||||||||.|.|||:||. |||||||||| |||
        401 NSLVSASVVENGVRRLMETKEGDEMRQRAVRLKNAIHRSMDEGGVSHMEM 450

        446 ASFIAHISR 454
            |||||||:
        451 GSFIAHISK 459
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Figure 1. Deduced amino acid sequence of the ORF of *ZOX1* from *P. vulgaris* (top line) compared with that of *ZOG1* of *P. lunatus*. Alignment was performed with the GCG Program, identifying identical (|), highly similar (:), and similar (.) pairings based on the scoring matrix of Henikoff and Henikoff (1992).

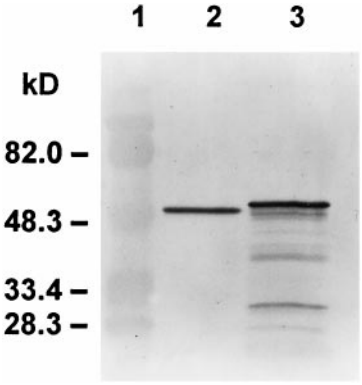

Figure 2. Western analysis of recombinant protein encoded by the ORF of *ZOX1* of *P. vulgaris*. Lane 1, Markers; lane 2, native zeatin *O*-xylosyltransferase; lane 3, recombinant protein as a fusion to α-peptide.

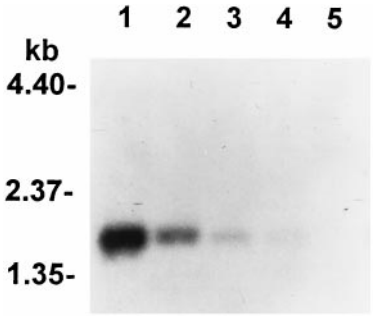

Figure 3. Northern analysis of mRNA from tissues of *P. vulgaris* probed with *ZOX1*. Lane 1, Small seeds (<2 mm); lane 2, medium seeds (2–4 mm); lane 3, large seeds (4–6 mm); lane 4, leaves; lane 5, roots.

contained an ORF of 1362 bp encoding a polypeptide of 454 amino acids (Fig. 1) with a mass of 51 kD.

Biological Activity of the Recombinant Protein

The ORF of the genomic clone was ligated into the *Not*I/*Sal*I site of the pZL1 plasmid. The properties of the recombinant protein were analyzed by western blotting and enzyme assay. The fusion protein (with the α-peptide of the cloning plasmid) was antigenic to an antibody to the zeatin *O*-xylosyltransferase of *P. vulgaris* (Martin et al., 1990) and had the expected size of 54 kD (Fig. 2). The gene product had high enzyme activity, converting ^{14}C-zeatin to *O*-xylosylzeatin and ^{14}C-dihydrozeatin to *O*-xylosyldihydrozeatin with UDP-Xyl as the sugar donor (Table I). For example, in one assay 82% of the labeled zeatin was converted to *O*-xylosylzeatin and 78% of the dihydrozeatin was converted to *O*-xylosyldihydrozeatin in 4 h. UDP-Glc is not a substrate because no *O*-glucosylzeatin was formed with UDP-Glc as the sugar donor under the same reaction conditions. The recombinant protein did not convert *cis*-zeatin or ribosylzeatin to the corresponding xylosyl derivatives in the presence of UDP-Xyl. Thus, the substrate specificity corresponds closely to that of the native enzyme of *P. vulgaris* (Dixon et al., 1989), and therefore, the isolated clone is a gene encoding a zeatin *O*-xylosyltransferase, referred to as *ZOX1* (accession no. AF116858).

ZOX1 Gene Expression and Copy Number

The RNA blot of mRNA isolated from seeds, leaves, and roots revealed strong expression in developing seeds but only very weak signal in vegetative tissues (Fig. 3). This confirms our previous western analyses results with monospecific antibodies to the enzyme, in which high levels of antigenic protein were detected in young seeds, less in older seeds, and very low levels in roots (Martin et al., 1990).

DNA after *Bgl*II or *Bam*HI digestion, with *ZOX1* as the probe, yielded a single, large hybridizing fragment (Fig. 4). *Xba*I-treated samples contained two complementary bands. Because none of these enzymes cut within the *ZOX1* gene, one possible explanation for these results may be that there

Table I. *Substrate specificity of recombinant proteins encoded by the ORF of ZOX1 and a control plasmid without insert*

Protein (125 μL) from culture supernatant was incubated with radiolabeled cytokinins and UDP-Xyl (UDPX) or UDP-Glc (UDPG), as indicated for 4 h. Reaction products were separated by HPLC on a reverse-phase C_{18} column.

Cytokinin Substrate[a]	Glycosyl Donor	Product			Cytokinin Substrate Left	
			cpm	*%*	*cpm*	*%*
ZOX1 ORF						
Z	UDPX	OXZ	35,838	82	7,768	18
Z	UDPG	OGZ	0	0	41,679	100
DHZ	UDPX	OXDHZ	33,237	78	9,244	22
cZ	UDPX	OXcZ	0	0	29,460	100
RZ	UDPX	OXRZ	0	0	39,238	100
Control plasmid						
Z	UDPX	OXZ	0	0	34,715	100
Z	UDPG	OGZ	0	0	37,842	100

[a] cZ, *cis*-zeatin; DHZ, dihydrozeatin; OGZ, *O*-glucosylzeatin; OXZ, *O*-xylosylzeatin; OXDHZ, *O*-xylosyldihydrozeatin; OXRZ, *O*-xylosylribosylzeatin; RZ, ribosylzeatin; Z, *trans*-zeatin.

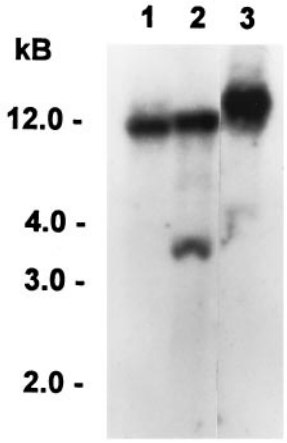

Figure 4. Southern analysis of *P. vulgaris* DNA probed with *ZOX1*. Restriction digestion was with *Bgl*II (lane 1), *Xba*I (lane 2), and *Bam*HI (lane 3).

are two genes close together, with an *Xba*I site between the genes or within the second gene.

Comparison with the *ZOG1* Gene

The nucleotide sequences of the two genes are very similar with an identity of 93% over the length of 1380 bp. The deduced amino acid sequences have a similarity of 90% and an identity of 87% over 449 amino acids (Fig. 1). Based on BLAST analyses, amino acids between positions 329 and 372 of *ZOX1* are common to many glycosyltransferase genes and may therefore contain epitopes conferring glycosyl donor recognition. A 15-base deletion in the *ZOX1* gene at the 5′ terminus accounts for the smaller size of the *O*-xylosyltransferase (Fig. 1) and is likely the cause of the initial difficulty in amplifying the 5′ end of the *ZOX1* gene. The *ZOX1* gene does not contain any introns, as is true for the *ZOG1* gene of *P. lunatus* (Martin et al., 1999).

DISCUSSION

The *ZOX1* gene is the second gene isolated from a family encoding zeatin *O*-glycosyltransferases. Both *ZOX1* and *ZOG1* are highly expressed in seeds. DNA-blot analyses detected the presence of additional homologous fragments in both *P. vulgaris* (Fig. 4) and *P. lunatus* (Martin et al., 1999). These may be genes encoding zeatin glycosyltransferases expressed in vegetative tissues, since *O*-glucosyl derivatives of zeatin and ribosylzeatin also occur in leaves and stems (Letham, 1994). Such genes are expected to have similar coding regions but to differ in their promoters. The isolation of an additional zeatin *O*-glucosyltransferase gene from *P. lunatus* (R.C. Martin, M.C. Mok, and D.W.S. Mok, unpublished data) supports this hypothesis. The activity of *O*-glycosyltransferases may also be affected directly or indirectly by other genes, since a *P. vulgaris* cDNA constitutively expressed in transgenic tobacco stimulated a low level of *O*-xylosyltransferase activity (Martin et al., 1997). A BLAST search of *ZOX* and *ZOG* amino acid sequences revealed homology to other glycosyltransferases such as flavonoid 3-*O*-glucosyltransferases. A C-terminal segment suggested to be the UDP-glycosyltransferase signature is present in these genes. In the *ZOX* gene this region occurs between amino acids 329 to 372 (WAP∞DQ).

The isolation of the *Phaseolus ZOG* and *ZOX* genes will facilitate cloning of similar genes from other species. A collection of zeatin metabolic genes may facilitate determination of domains regulating substrate specificity. For example, the difference in glycosyl donor recognition between *ZOX1* and *XOG1* should make it possible to identify the sequence essential for differences in UDP-Xyl and UDP-Glc affinity through domain swapping and/or site-directed mutagenesis.

Whereas different zeatin *O*-glycosyltransferases occur in *P. vulgaris* and *P. lunatus* seeds, two other *Phaseolus* sp. examined, *Phaseolus coccineus* and *Phaseolus acutifolius*, appear to be closer to *P. vulgaris* since they display mainly *O*-xylosyltransferase activity based on incubation studies with labeled zeatin (Mok and Mok, 1987). The differences between species may reflect the availability of glycosyl donors, leading to the adaptation of specific enzymes using the prevalent glycosyl substrate in each species. Because interspecific hybrid embryos of *Phaseolus* display distinct developmental limits depending on the parental species combination, with *P. vulgaris* × *P. lunatus* crosses being the least successful (Mok et al., 1986), it will be of interest to determine whether abnormal embryo growth is somehow related to interspecific differences in glycosylation preference.

Many cytokinin mutants have been reported (Wang, 1994; Deikman, 1998), and recently several Arabidopsis genes most likely involved in cytokinin-signaling pathways have been identified (Kakimoto, 1996; Brandstatter and Kieber, 1998; Plakidou-Dymock et al., 1998). As to cytokinin metabolic enzymes, the sequence of a maize gene encoding a cytokinin oxidase has recently been obtained (Houba-Hérin et al., 1999; Morris et al., 1999). Other genes related to zeatin metabolism are those encoding β-glucosidases (Brzobohaty et al., 1993; Falk and Rask, 1995). However, these genes are unlikely to be critical to cytokinin metabolism since β-glucosidases degrade a broad range of glycosides (Babcock and Esen, 1994). In comparison, the zeatin *O*-glycosyltransferases of *Phaseolus* are highly specific, discriminating among *trans*- and *cis*-zeatin, dihydrozeatin, and ribosylzeatin. The expression of the *O*-glycosyltransferase genes may be tightly regulated, having a direct bearing on the levels of active cytokinins in plant tissues.

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