AN ABSTRACT OF THE THESIS OF

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Abstract approved: David W. S. Mok

Cytokinins are a group of plant hormones mediating cell division and differentiation. Zeatin is a naturally occurring and highly active cytokinin that is rapidly metabolized in *Phaseolus* seeds. The metabolites and enzymes mediating the conversion have been studied extensively. Zeatin metabolic enzymes may be utilized in studying the regulation of cytokinin metabolism. This thesis describes such an approach using zeatin O-xylosyltransferase as the candidate.

In immature seeds of *P. vulgaris*, zeatin is rapidly converted to O-xylosylzeatin; while in *P. lunatus*, O-glucosylzeatin is formed. The enzymes that mediate both of these conversions have been isolated and characterized. Samples containing the O-xylosyl-transferase, purified over 2500-fold, were used as antigen to produce monoclonal antibodies (MAbs) which recognized both O-glycosyltransferases. The antibody was used for localization and gene cloning experiments.

Tissue printing and immunolocalization studies revealed that the enzyme occurred predominantly in the endosperm, and is associated with both the cytoplasm and the nucleus. An expression cDNA library derived from mRNAs of immature *P. vulgaris*

seeds was screened with the antibodies and two full length cDNAs were chosen for further analyses. The clones were highly homologous. The ORFs encode proteins of approximately 70 kD and the amino acid sequences were 90% identical. Recombinant proteins generated in prokaryotic (*E. coli*) and eukaryotic (insect cells via baculovirus) expression systems were insoluble in aqueous solutions and no enzyme activity was detected. Proteins obtained from *in vitro* transcription/translation were soluble and could be processed to smaller proteins with the addition of bean endosperm extracts. Processing of preproteins and the effects of the cDNA on plant development were examined by generating transgenic tobacco containing the ORF under the control of a constitutive promoter. Plants harboring the transgene were sensitive to auxin in comparison to no-insert control plants. Auxin also induced processing of the transgenic protein. These results suggest that the cDNAs encode preproteins that require precise processing to yield active enzymes and that hormonal interactions such as cytokinin/auxin balance may be mediated via auxin stimulation of cytokinin metabolic enzymes.

Zeatin O-xylosyltransferase in *Phaseolus vulgaris*: Immunological and Molecular Analyses

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I understand that my thesis will become part of the permanent collecton of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Ruth C. Martin, Author

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CONTRIBUTION OF AUTHORS

Dr. David and Machteld Mok provided support, guidance, and constructive criticism throughout the course of these studies. Dr. Robert Martin aided in the production of the monoclonal antibodies (see chapter 2).

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CHAPTER 1

INTRODUCTION[1]

Cytokinins are a group of adenine derivatives modulating cell division and redifferentiation of callus cells. The genetic controls of biosynthesis and metabolism of this group of hormones are not well understood. Extensive metabolism studies led to the identification of a comprehensive array of derivatives including nucleosides, nucleotides, N-glucosides, O-glycosides, amino acid conjugates and reduced forms of parent compounds. Analytical methods were designed to detect and measure various forms of endogenous cytokinins. Traditionally, quantitative variations in cytokinin content have been regarded as having a dominant effect on growth and development. However, as the mode of cytokinin action is still unknown, it is prudent to take into consideration qualitative differences of various cytokinin derivatives. It is possible that these specific metabolic structural modifications are required for cytokinin activity and that switching between distinct metabolic pathways is correlated with particular developmental events. In *Mercurialis*, for example, fertile and male sterile flowers are associated with the occurrence of *trans* or *cis*- zeatin pathway respectively (1). Genes governing the switching of metabolic pathways may in turn regulate

[1]**Modified with permission from: Mok, D.W.S. and Martin, R.** (1994) Cytokinin metabolic enzymes, in CYTOKININS Chemistry, Activity, and Function eds David W.S. Mok and Machteld C. Mok, published by CRC Press, Inc. pp 129-137.

development. Identification of enzymes mediating interconversion of cytokinins may lead to the isolation of such genes. This section reviews key properties of known enzymes involved in cytokinin metabolism and discusses the possible significance of these enzymes in cytokinin action and plant development.

Enzymes Involved in the Conversion of Cytokinin Bases, Nucleosides and Nucleotides

The conversion of cytokinin bases, nucleosides, and nucleotides are modifications similar to the metabolism of adenine,i.e. the interconversion between free base, nucleoside and nucleotides (Table 1-1). An enzyme, adenosine phosphorylase, which catalyzes the ribosylation of N^6-(Δ^2-isopentenyl)adenine (i^6Ade) was identified from wheat germ (2). The enzyme had higher affinity for adenine (Ade) (Km 32 μM) than i^6Ade (Km 46 μM) and also mediated ribosylation of kinetin. The enzyme was shown to be distinct from the inosine-guanosine phosphorylase based on chromatographic properties and on the competitive inhibition of the two enzymes by adenosine and guanosine-inosine respectively.

An enzyme mediating the opposite reaction (deribosylation) was also isolated from the same tissue. The adenosine nucleosidase was capable of deribosylating both N^6-(Δ^2-isopentenyl)adenosine (i^6Ado) and adenosine (Ado) to their respective free bases. The affinity for adenosine was about twice that of i^6Ado with the Kms of 1.4 and 2.4 μM respectively (3). The molecular weight of the enzyme was 59 kD and the reaction was inhibited by adenosine and other cytokinin nucleosides but was insensitive to guanosine, uridine and 3'-deoxyadenosine.

Table 1-1. Key properties of general cytokinin metabolic enzymes.

Name	Source	Substrate[a]	Km (μM)	Optimal pH	Mol. Mass
Adenosine phosphorylase	wheat germ	i^6Ade kinetin adenine	57 46 32	6.5-7.5	NA[b]
Adenosine nucleosidase	wheat germ	i^6Ado Ado	2.4 1.4	4.7	59 kD
Adenosine kinase	wheat germ	i^6Ado Ado	31 8.7	6.8-7.4	NA
Adenosine kinase	lupine	Ado	1.5	7.0-7.5	38 kD
Adenine phosphoribosyl-transferase	wheat germ	i^6Ade Ade	130 74	7.5	23 kD
5'-Nucleotidase I	wheat germ	i^6AMP AMP	3.5 3.2	7.0	57 kD
5'-Nucleotidase II	wheat germ	i^6AMP AMP	12.8 11.5	7.0	110 kD
Cytokinin-7-glucosyl-transferase	Radish	*t*-io^{6}Ade *c*-io^{6}Ade ipn^{6}Ade bzl^{6}Ade Ade	150	7.0	46 kD
ß-(9-cytokinin)-alanine synthase	Lupine	*t*-io^{6}Ade i^6Ade dihydrozeatin bzl^{6}Ade Ade	800	NA	NA
Cytokinin oxidase	Tobacco callus tissue	i^6Ade	4.0	9.0	NA
Cytokinin oxidase	*P. vulgaris* callus	i^6Ade	$<.1$	6.5	67 kD
Cytokinin oxidase	*P. lunatus* callus	i^6Ade	NA	8.4	60 kD
Cytokinin oxidase	*Populus euroamericana*	i^6Ade	4.0	8.5	NA

Table 1-1, Continued

Name	Source	Substrate[a]	Km (μM)	Optimal pH	Mol. Mass
Cytokinin oxidase	Wheat germ	i^6Ade	.3	7.5	40 kD
Cytokinin oxidase	*Vinca rosea* crown gall tumor tissue	i^6Ade *t*-io^6Ade *t*-io^6Ado	27.7 25.6 28.6	7.0	25 kD
Cytokinin oxidase	*Zea mays* kernals	i^6Ade i^6Ado *t*-io^6Ade *t*-io^6Ado	19.2 33.3 28.6 33.3	6.0	78 kD 94 kD 88 kD

[a]i^6Ade: N^6-(Δ^2-isopentenyl)adenine; i^6Ado: N^6-(Δ^2-isopentenyl)adenosine; i^6AMP: N^6-(Δ^2-isopentenyl)adenosine monophosphate; *t*-io^6Ade: *trans*-zeatin; *t*-io^6Ado: *trans*-zeatin riboside; *c*-io^6Ade: *cis*-zeatin; ipn^6Ade: N^6-(Δ^2-isopentyl)adenine; bzl^6Ade: N^6-benzyladenine.
[b]NA: not available.

For the formation of nucleotides, phosphorylation of adenosine and N^6-(Δ^2-isopentenyl)adenosine (i^6Ado) by an adenosine kinase from wheat germ was reported (4). The reaction products of i^6Ado (Km of 31 μM) appeared to be primarily the corresponding monophosphate; while adenosine (Km of 8.7 μM) as the substrate resulted in the formation of 55% AMP, 45% ADP and traces of ATP. The property of the enzyme is very similar to an adenosine kinase isolated from lupine (5). The lupine enzyme utilized ATP as the phosphate donor. The two enzymes are probably the same as the ATP:adenosine-5'-phosphotransferase (EC 2.7.1.20) isolated from animal systems (6,7) which utilized N^6-substituted adenosines, including N^6-(Δ^2-isopentenyl)adenosine and N^6-benzyladenosine, as substrates.

In the course of studying adenosine kinase, crude extracts from wheat germ and tobacco callus tissues were found to contain enzyme activity catalyzing the phosphoribosylation of adenine and N^6-(Δ^2-isopentenyl)adenine (i^6Ade). Presumably an adenine phosphoribosyltransferase which uses phosphoribosyl pyrophosphate (PRPP) as a phosphoribosyl donor was present in such preparations. Subsequently, the enzyme was purified about 200 fold from crude extracts (8). The affinity of the enzyme for adenine (Km 74 μM) was higher than N^6-(Δ^2-isopentenyl)adenine (Km 130 μM). These results indicated that cytokinin nucleotides may be formed directly from cytokinin bases or via their ribosyl derivatives. It is expected that most adenine metabolic enzymes will have an effect on cytokinin metablism, as indicated by the observation that the formation of N^6-benzyladenine nucleotides were impaired in an *Arabidopsis* mutant defective in adenine phosphoribosyltransferase (9).

Dephosphorylation of these nucleotides by nucleotidases from wheat germ also favored adenosine monophosphate as the substrate (10). Two forms of 5'-nucleotidases (57 and 110 kDs respectively) were compared and both were able to dephosphorylate AMP and N^6-(Δ^2-isopentenyl)adenosine monophosphate (i^6AMP). The rate of hydrolysis of the cytokinin nucleotide by the 57 and 110 kDa enzymes was 72 and 86% of that of AMP, respectively.

These studies indicate that the inter-conversion of cytokinin bases, nucleosides and nucleotides is most likely related to the adenine or purine metabolic pathway (11). As the configuration of the N^6-side chain does not confer specificity in the conversion,

the enzymatic controls of such steps are probably shared with those regulating adenine and adenosine metabolism.

Enzymes Modifying the Purine Ring of Cytokinins

Next to ribosylation and phosphoribosylation, the most frequently observed modifications of the purine ring of cytokinins involve glucosylation and the formation of alanine conjugates. N-Glucosylation at the 3-, 7- or 9-position of zeatin and dihydrozeatin have been studied extensively in tobacco, radish and lupine (12-14). Two peaks of enzyme activity mediating the formation of both N^6-benzyladenine 7- and 9- N-glucosides were separated on a DEAE cellulose column (15). The enzyme from radish present in larger quantity was examined further (16,17). The molecular weight of the enzyme, designated as "cytokinin-7-glucosyltransferase", was 46 kD and catalyzed the formation of both 7- and 9-glucosides but favored the 7-position. Both UDPG (Km of 1.9×10^{-4} M) and TDPG served as the glucosyl donor. Cytokinin substrates included *trans*-zeatin (Km of 1.5×10^{-4} M), *cis*-zeatin, dihydrozeatin, N^6-benzyladenine and a number of chemical variants. The structural requirements for substrates *in vitro* appeared to be similar to those needed for high cytokinin activity, an intact purine ring with aliphatic or cyclic N^6-substituents with at least a 3-carbon chain. Although the requirement for a purine ring seemed stricter than the configuration of the side chain, the relative amounts of the product varied with the substrate, most notably the formation of appreciable quantities of 9-glucosides of zeatin and N^6-benzyladenine versus only trace amounts when dihydrozeatin and zeatin-O-glucoside were used as the substrates. The possibility of other adenine

derivatives serving as substrates for this enzyme was not tested due to the difficulty in extracting sufficient amounts of the enzyme.

There were also differences between *in vitro* assays and *in vivo* feeding experiments in radish tissues. For example, feeding labeled zeatin yielded only 7-glucoside while dihydrozeatin gave 3- and 9-glucosides (18). These differences may suggest the presence of additional N-glucosylation enzymes which have not been identified or interconversion of the products *in vivo*. N-glucosides of cytokinins are biologically inactive and very stable. It is assumed that the N-glucosylation may be important in regulating levels of active cytokinin, possibly through inactivation. Nevertheless, the precise function of these compounds is not known. Although the affinity of the N-glucosylation enzyme *in vitro* showed some correlation with the cytokinin activity of the substrates, the structural requirement is broad. Inhibition by an inhibitor of zeatin, 3-methyl-7- (n-pentylamino)pyrazolo[4,3-d]-pyrimidine, was probably related to the structural similarity of the two compounds and may not indicate specificity of the enzymes to cytokinins.

The formation of 9-alanine conjugates of zeatin and benzyladenine was first observed in *Lupinus* and *Phaseolus* (17, 19-21). The zeatin conjugate was named lupinic acid. An enzyme, β-(6-allylaminopurine-9-yl)adenine synthase, was analysed in lupine. The Km of the enzyme for *trans*-zeatin was 0.8 mM and O-acetylserine (Km of 26 mM) served as the alanine donor. A large number of purines were also substrates for the enzyme. The relative affinity of the enzyme to some cytokinins was: i^6Ade $>>$ *trans*-zeatin $=$ N^6-(Δ^2isopentyl)adenine $>>$ (io^6Ade) $>>$

dihydrozeatin >> *cis*-zeatin (17). Benzyladenine, O-glucosylzeatin, adenine, methyladenine, hydroxyethyladenine, propyladenine and hydroxylhexyladenine were also substrates. Similar to N-glycosylation of cytokinins, the alanyl conjugation probably represents a process of cytokinin inactivation since the alanine conjugates are also biologically inactive and stable.

Enzymes Modifying the N^6-Substituted Side Chain

Cytokinins with a hydroxylated N^6-side chain are often glycosylated to form the O-glucoside and O-xyloside (22-27). The O-glucosyl derivatives are biologically active in bioassays (28). The activity was suggested to be related to the ubiquitous β-glucosidases resulting in conversion to cytokinin bases. The enzymes mediating the O-glycosylation were isolated much later than the discovery of O-glucosylzeatin and the work was centered on zeatin and dihydrozeatin in *Phaseolus* species.

Two enzymes, zeatin O-xylosyltransferase and zeatin O-glucosyltransferase have been isolated from immature seeds of *Phaseolus vulgaris* and *P. lunatus*, respectively (29,30). The enzymes are similar in molecular mass (50 kD) but can be separated on anion exchange columns. In addition, they can be distinguished by their substrate specificity (Table 1-2). The O-xylosyltransferase isolated from *P. vulgaris* utilizes *trans*-zeatin as the main substrate and UDPX as the donor of the xylosyl moiety. The enzyme also accepts dihydrozeatin as substrate but not *cis*-zeatin or ribosylzeatin. The O-glucosyltransferase occurring in *P. lunatus* seeds recognizes *trans*-zeatin but not dihydrozeatin, *cis*-zeatin or ribosylzeatin. In addition, both UDPG and UDPX serve as glycosyl donors for the enzyme, although UDPG is the favored substrate. The

products of the enzymatic reaction have been found to be endogenous in *Phaseolus* (25). In terms of cytokinin action and plant development, it is interesting to note that the occurrence of the enzymes is species specific and the activity is pronounced in immature seeds. These observations may indicate genetic as well as developmental regulation of cytokinin metabolism (31).

Conversion of *trans*-zeatin to dihydrozeatin mediated by a reductase was also discovered in *Phaseolus* (33). The enzyme requires NADPH. Two forms of the enzyme, 50 and 25 kD, have been identified with Kms for *trans*-zeatin of 70 and 100 μM respectively (Table 1-2). However, *cis*-zeatin, ribosylzeatin and N^6-(Δ^2-isopentenyl)denine are not substrates for the enzyme, indicating a high substrate specificity. The level of zeatin reductase was high in *Phaseolus vulgaris*, a species known to have high cytokinin oxidase activity (34,35). As cytokinin oxidases selectively cleave unsaturated N^6-side chains of cytokinins, reduction of the side chain may be a mechanism to maintain the level of active cytokinins.

The enzymes involved in the degradation of cytokinins via the removal of the side chain have recently been reviewed by Armstrong (36). Cleavage of the N^6-side chain by cytokinin oxidases was initially observed in cell free extracts of tobacco callus tissues (37). The enzyme was capable of degrading N^6-(Δ^2-isopentenyl) adenosine (i^6Ado) to adenosine (Ado)(Table 1-1). Subsequently cytokinin oxidases have been isolated from other callus tissues including *Phaseolus* (38,39) and *Populus euroamericana* (40). The enzyme has also been isolated from various plant organs

including *Triticum aestivum* wheat germ (41), *Vinca rosea* crown gall tumor tissue (42) and *Zea mays* kernals and seedlings (43,45-47).

Table 1-2. Zeatin metabolic enzymes isolated from *Phaseolus*.

	Zeatin O-xylosyltransferase of *P. vulgaris*.
Reaction	*t*-zeatin + UDPX---> O-xylosylzeatin
Km	2 μM 3 μM
Reaction	dihydrozeatin + UDPX---> O-xylosyldihydrozeatin
Km	10 μM 3 μM
Optimal pH	8.5
Mol. Mass	50 kD
	Zeatin O-glucosyltransferase of *P. lunatus*.
Reaction	*t*-zeatin + UDPG---> O-glucosylzeatin
Km	28 μM 200 μM
Reaction	*t*-zeatin + UDPX---> O-xylosylzeatin
Km	28 μM 2700 μM
Optimal pH	8.0
Mol. Mass	50 kD
	Zeatin reductase
Reaction	*t*-zeatin ---> dihydrozeatin
Km	70 μM, high molecular weight (HMW) form 100 μM, low molecular weight (LMW) form
Optimal pH	7.5
Mol. Mass	55 kD (HMW), 25 kD (LMW)
	Zeatin *cis-trans* isomerase
Reaction	*c*-zeatin <----------> *t*-zeatin

When i^6Ade, i^6Ado, zeatin and zeatin riboside are substrates for the cytokinin oxidase the expected products Ade, Ado, Ade and Ado are obtained in addition to the side chain fragments. The oxidase requires oxygen (38,41,43,46) and the addition of copper imidazole to the reaction mixture enhanced the activity of cytokinin oxidase (44), while cyanide (43,46) and aminoacetonitrile (45) were found to be inhibitory to oxidase activity. Glycosylation and reduction of the side chain confer resistance to attack by cytokinin oxidase (36). Cytokinin active phenylurea derivatives were able to inhibit the activity of cytokinin oxidases isolated from several sources (38-41). The cleavage of the N^6-side chain inactivates cytokinins and may therefore play a role in regulating active levels of cytokinins (36).

An isomerase mediating the interconversion of *cis*- and *trans*-zeatin was also detected in immature seeds of *Phaseolus* (48,49). The balance of the interconversion favors *trans*-zeatin formation. The reaction requires flavin nucleotides (FMN or FAD) and light. The enzyme was purifed over 2,000 fold using a series of chromatographic steps including molecular sieving, ion exchange and hydrophobic interaction FPLC and Concanavalin A columns (49). Retention on a ConA column suggests that it is a glycoprotein. The molecular weight of the enzyme was estimated to be 68 kD by gel filtration. As the *cis*-isomer of zeatin is not or only weakly active, isomerization is a direct and efficient mechanism of increasing the level of active cytokinins. More importantly, it may provide a link between nucleic acid metabolism and cytokinin biosynthesis.

The Significance of Cytokinin Metabolic Enzymes

Both naturally occurring (e.g. zeatin) and synthetic (e.g. N^6-benzyladenine) cytokinins have been used as the parent compounds to trace metabolic products. Greater progress has been made in the chemical identification than in the enzymology or functional analysis of the metabolites. The importance of those enzymes catalyzing steps in common with adenine metabolism is more difficult to fathom, since it is expected that the structural similarities between adenine-type cytokinins and purines could render them substrates for enzymes with broad specificity. For example, of the five zeatin metabolic enzymes described above, the N-glucosyltransferase recognizes a wide range of adenyl compounds as substrates including zeatin, N^6-benzyladenine and others. The product of the enzymatic reaction is not biologically active and no functional role has been demonstrated. Therefore, it is likely that the enzyme is involved in the general metabolism of purines. The other four enzymes have high substrate specificity to cytokinins, thus the properties of these enzymes and their products may be more relevant to the understanding of cytokinin metabolism.

O-Glucosylzeatin is active in bioassays. Its ready conversion to the corresponding free bases may account for the biological activity. In addition to possibly having a storage role, it may also be involved in transport between and within cells. In *Chenopodium*, O-glucosides of dihydrozeatin and ribosyldihydrozeatin are localized in the vacuoles, while dihydrozeatin and its riboside are present in the cytosol and in the medium (50). These findings suggest that O-glucosylation may result in intracellular compartmentalization. The O-glucosyltransferase obtained from

Phaseolus lunatus appears to be specific to *trans*-zeatin. In *in vitro* assays, dihydrozeatin is not a substrate for the enzyme. Thus, it may be argued that the specificity of this enzyme does not reside in the presence of the hydroxyl moiety *per se*. This observation is puzzling since the O-glucoside of dihydrozeatin has been reported in leaves of *Phaseolus vulgaris* plants (51). It is possible that other O-glucosyltransferases exist but have not been identified. The O-glucosyltransferase of *P. lunatus* can utilize both UDPG and UDPX for the formation of the corresponding O-glycosides of *trans*-zeatin. It is likely that this enzyme and the O-xylosyltransferase are variants of a family of enzymes. Since the O-xylosytransferase recognizes *trans*-zeatin and dihydrozeatin, but only uses UDPX as the glycosyl donor, it is conceivable that other enzymes adapted to catalysing the formation of O-glucosyldihydrozeatin using UDPG may also occur. The expression of individual enzymes may be tissue specific which would account for the presence of O-glucosyldihydrozeatin in *Phaseolus vulgaris* leaves. In any event, the information on the O-glycosyltransferases in *Phaseolus* indicates that the control of zeatin metabolism is precise and is likely to be species and organ specific. The evolutionary relationship between these enzymes may be further clarified by analysing the genes encoding these enzymes.

It is generally accepted that cytokinin biosynthesis can occur via either a *de novo* pathway or indirect synthesis via tRNAs (52,53). The *de novo* synthesis involves the formation of i^6AMP from AMP and iPP mediated by an isopentenyl transferase (perhaps similar to the *ipt* enzyme of *Agrobacterium*), followed by

hydroxylation of the side chain. The isopentenyl transferase activity has been detected from cytokinin autonomous tobacco callus (54), but isolation of the enzyme mediating side chain hydroxylation has not been reported although such conversion may occur in *Actinidia* tissue culture (55,56). Moreover, no sequence homology to the *ipt* genes of microbial systems could be detected in plant DNA preparations (57,58) and the occurrence of zeatin in *E. coli* harboring the *ipt* gene is attributed to hydroxylation enzymes of the host (59). Incubation of maize embryos with labeled adenine led to the formation of radio-labeled zeatin without the recovery of isopentenyl derivatives or nucleotides (60). Therefore, the *de novo* pathway (or pathways) of cytokinin biosynthesis in plants remains to be defined. Another pathway suggested for zeatin (and N^6-(Δ^2-isopentenyl)adenine) biosynthesis involves breakdown of cytokinin bearing tRNAs (61). A major challenge to this pathway is the finding that plant tRNAs contain *cis*-zeatin derivatives (62) which are not biologically active. The occurrence of the isomerase mediating the conversion from *cis*-zeatin to its *trans*-isomer removes such a theoretical constraint for the indirect pathway of zeatin biosynthesis.

Molecular Analyses

The molecular mechanism regulating cytokinin function is unknown. However, Mendelian factors related to cytokinins have been reported. These include genes controlling cytokinin autonomy in *Phaseolus* and *Nicotiana* (63-65), cytokinin resistance in *Nicotiana* and *Arabidopsis* (66-68), and cytokinin overproduction in moss (69). It is difficult to isolate genes involved in these genotypic variations since the

gene products are not known. Zeatin- or other cytokinin-specific enzymes identified from *Phaseolus* and other species can be used more readily in cloning cytokinin genes.

This thesis explores the possibility of utilizing zeatin metabolic enzymes to conduct molecular analyses of the mechanisms regulating zeatin metabolism. The O-xylosyltransferase was chosen as an initial candidate for these studies due to its stability and high substrate affinity. The second chapter describes the use of purified enzyme preparations to generate monoclonal antibodies. The third chapter describes the use of these antibodies for localization and developmental studies. The utilization of the antibody for gene cloning experiments is described in chapter four.

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CHAPTER 2

A MONOCLONAL ANTIBODY SPECIFIC TO ZEATIN O-GLYCOSYLTRANSFERASES OF *PHASEOLUS*

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Abstract

Zeatin O-xylosyltransferase and zeatin O-glucosyltransferase occur in immature embryos of *Phaseolus vulgaris* and *P. lunatus* respectively. Purified preparations of the O-xylosyltransferase were used as antigen to elicit the formation of antibodies in mice. Hybridoma clones were produced by fusion of mouse spleen cells with myeloma cell line Fox-NY. A clone secreting monoclonal antibody (MAb), XZT-1, capable of immunoprecipitating both enzymes was obtained. The MAb detected a unique protein band from crude embryo extracts of each species with the correct Mr (50 kD) and relative charge (Rfs 0.5 and 0.3) of the respective enzymes. Competition experiments with substrates indicated that the glycosyl dinucleotide binding sites of the enzymes are probably not involved in MAb-enzyme recognition. The MAb will be used to screen expression libraries and for the production of additional MAbs.

Introduction

The mechanisms controlling the appropriate levels of cytokinins in plants are not well understood. We are interested in identifying genetic elements regulating the metabolism of zeatin, a naturally occurring and highly active cytokinin. In order to detect useful genetic variation, zeatin metabolism has been examined in immature *Phaseolus* embryos (4), leading to the discovery of major differences between *P. vulgaris* and *P. lunatus*. Zeatin is rapidly converted to O-xylosylzeatin in *P. vulgaris*, whereas O-glucosylzeatin is formed in embryos of *P. lunatus*. Subsequently, two enzymes, O-xylosyltransferase and O-glucosyltransferase, have been isolated from *P. vulgaris* and *P. lunatus* embryos respectively (1, 11). UDPX serves as the only

glycosyl donor for the xylosyltransferase while both UDPG and UDPX can be utilized by the glucosyltransferase to form O-glucosyl or O-xylosylzeatin, although UDPG is the preferred substrate. Competition experiments and enzyme separation by anion exchange HPLC indicate that a single, distinct enzyme occurs in embryos of each of these two species. As both enzymes are highly specific to zeatin (they do not mediate glycosyl transfer to ribosylzeatin or cis-zeatin) and the products of the enzymatic reaction are more stable than zeatin in some *Phaseolus* bioassays (9), probably due to their resistance to cytokinin oxidases, the enzymes may play an important role in maintaining cytokinin activity. Future efforts will be directed at the cloning of the genes encoding these enzymes. Towards this objective, we have used partially purified preparations of O-xylosyltransferase as antigen to generate a monoclonal antibody. This chapter describes the procedures of antibody generation and selection, the properties of the antibody, and its use in detecting enzyme expression in vegetative tissues.

Materials and Methods

Plant Materials

Zeatin O-xylosyltransferase and O-glucosyltransferase were isolated from immature embryos (5-10 mm in length) of field grown plants of *P. vulgaris* cv. Great Northern (GN) and *P. lunatus* cv. Kingston (K) respectively. Roots and hypocotyls were obtained from seeds germinated aseptically; leaves and stems were obtained from

plants maintained in greenhouse. Callus cultures of GN were established and maintained as previously described (8).

Enzyme Purification

The enzymes were isolated and partially purified using ammonium sulfate fractionation (30-60% saturation), affinity column chromatography (AgAMP-agarose and Blue Sepharose 6B, Sigma) and anion exchange (AX-300, BrownLee) HPLC as described previously (1). The amount of protein was determined using a Bio-Rad protein assay kit following procedures recommended by the manufacturer.

Polyacrylamide Gel Electrophoresis

A Bio-Rad Protein II electrophoresis apparatus for vertical slab gels was used routinely. Proteins were separated on a 10% acrylamide gel (1.5 mm thick) layered with a stacking gel of 2.5% acrylamide. The running buffer contained Tris-HCl (0.005 M) and glycine (0.038 M), pH 8.5. Electrophoresis was performed at 100 V overnight.

At the completion of PAGE, the separation gel was sliced horizontally into 3 mm sections. The gel slices were placed in standard scintillation vials containing 5 ml of extraction buffer (0.055 M Tris-HCl, 0.0005 M EDTA, 0.005 M DTT at pH 7.2) and placed on a rotary shaker for 2 hrs. An aliquot (100 ul) was taken from each vial and assayed for enzyme activity under established conditions (below). Gels containing the highest enzyme activity were identified and transferred to dialysis tubings containing 2 ml of extraction buffer. The tubings were placed in a horizonal

electrophoresis container and the enzyme eluted at 200 V for 2 hrs. The eluates from comparable sections of different gels were combined and concentrated with a Centriprep 30 (Amicon) concentrator. All operations described above were carried out at 4° C.

SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed with an apparatus for mini-slab gels (Bio-Rad) using a separation gel (0.75 mm thick) containing 12% acrylamide-Bis (pH 8.8) and a stacking gel of 4% (pH 6.8). The running buffer consisted of Tris-HCl (0.025 M), glycine (0.2 M) and SDS (0.1%), pH 8.3. A reduction buffer (0.125 M Tris-HCl, 20% glycerol, 4% SDS, 10% mercaptoethanol, 0.0025% bromophenol Blue) of pH 6.8 was mixed with the protein sample and boiled before loading. The samples were electrophoresed at 10 mA (for the stacking gel) and 20 mA (separation gel) successively.

Western Transfer

Proteins separated by electrophoresis were transferred to Immobilon (Millipore) using a Bio-Rad Mini Trans-Blot Cell. The transfer was accomplished in a Tris buffer (0.025 M, pH 8.3) with glycine (0.192 M) and methanol (20%) at 90 V for 1 hr at 4° C.

Immunoblotting

Immobilon membranes with protein samples were incubated with a TBS blocking solution (0.05 M Tris-HCl, pH 8.0, 0.15 M NaCl, 0.05% Tween 20, 2%

nonfat dry milk) for 1 hr at room temperature under constant agitation. Filters were transferred to blocking solution containing antibody (usually 1 μg of MAb in 1 ml) and incubated for 1 hr at room temperature. Unbound antibody was removed by washing with blocking buffer. Rabbit anti-mouse antibody (Jackson Laboratories) conjugated with alkaline phosphatase was applied for 1 hr. After washing, positive bands were visualized by adding 5 ml substrate buffer (Alkaline Phosphatase Substrate Kit, Vector Laboratories). For testing of more than one type of antibody on a single sheet of Immobilon, a multi-channel mini-blotter (Immunetics) was used. Immobilon membranes were held in place by the blotting plates and appropriate amounts of antibody (Ab) in blocking solution were applied to individual lanes.

Hybridoma Formation and Identification of Positive Clones

Previously established immunological procedures (3, 5) were followed for the generation of hybridomas. Briefly, 10 week old BALB/c mice were immunized with O-xylosyltransferase obtained from PAGE purified preparations. Two additional injections were administered at three-week intervals. A booster was given three days before the fusion. Spleen cells were fused with myeloma cells (Fox-NY) and plated in standard 96-well plates. ELISA was used for the initial screening using anion exchange purified enzyme preparations and rabbit-antimouse IgG (Jackson Laboratories) conjugated to alkaline phosphatase. The procedures were those recommended by the manufacturer. ELISA positive cultures were again screened by immunoblotting and selected cultures were recloned 12-14 days post-fusion. Wells on the reclone plates containing single colonies were again screened using ELISA and

immunoblotting. Clones of interest were transferred to 1 ml cultures and subsequently 10 ml petri plates. Media and conditions for hybridoma selection, maintenance and storage have been described previously (5).

Purification of Ab Obtained From Hybridomas

Culture fluids were collected after centrifuging cultures at 3800xg for 15 min. Ammonium sulfate (50% saturation) was used to precipitate the proteins. After centrifugation (15,300x g for 25 min), the pellet was redissolved in equilibration buffer (0.01 M sodium phosphate, 0.5 M NaCl, pH 7.2). The solution was concentrated and washed with equilibration buffer to remove residual ammonium sulfate using Centriprep 30 Concentrators (Amicon). The protein samples were applied to affinity columns of goat anti-mouse IgG agarose (Sigma). A ratio of one ml of column material to 10 ml equivalent of culture fluid was used. Mouse Abs were retained while other proteins were removed by five bed volumes of equilibration buffer. The Abs were eluted from the column using an elution buffer (0.1 M glycine, 0.15 M NaCl, pH 2.4) and immediately neutralized with a 2 M solution of Tris-HCl, pH 7.5.

Immuno-precipitation and Assays for Inhibition of Enzyme Activity

The standard assays involved incubating enzyme with varying amounts of purified Ab (3.5 hrs at room temperature, total volume, 250 μl). Appropriate amounts of BSA were included to adjust the total amount of protein to 1 mg. A solution of 100 μl of protein A-Sepharose beads (Sigma) dissolved in PBS was then added. After 1.5 hr, the enzyme-antibody-protein A complex was pelleted by centrifugation (325x g for

5 min). A portion of the supernatant (280 μl) was removed to assay for enzyme activity. Purified MAbs from negative hybridoma lines (PLRV-371A and 36A-1) were used as controls.

Enzyme Assays

Enzyme activity was determined under the following conditions: enzyme, UDPX (3 mM), ATP (0.5 mM), $MgCl_2$ (0.05 M) and 0.025 μCi of ^{14}C zeatin (0.001 μmol) in 320 μl at pH 8.0 (buffered with 1 mM Tris) at 27° C. One ml of cold ethanol was added after 30 min and the mixture was placed at 4° C for 15 min and then centrifuged at 27,000x g for 20 min. The supernatant was concentrated to 100 μl in vacuo (Speed Vac Concentrator, Savant) and analysed by HPLC using a reversed phase C_{18} column.

Cytokinin Analysis

To detect and quantify cytokinins resulting from enzymatic reactions, a Beckman model 110A dual-pump HPLC system with a reversed-phase column (Ultrasphere ODS C_{18}, 5 μm particle size, 4.6 x 250 mm; Altex) was used. The aqueous buffer consisted of 0.2 M acetic acid adjusted to pH 4.8 with triethylamine (TEA). Samples were eluted with a linear gradient of methanol (5-50% over 90 min) in TEA buffer. The flow rate was 1 ml/min and 0.5 ml fractions were collected. A combination of a Beckman model 117 flow-through isotope detector and ISCO UV monitor allowed the initial identification of fractions of interest. Radioactivity in these

fractions was determined in Ready-Gel scintillation fluid (Beckman) with a Beckman LS 7000 scintillation counter.

Results

Preparation of Antigen

The electrophoretic profiles of proteins retained after each step of O-xylosyltransferase purification (ammonium sulfate precipitation, AgAMP-agarose and Blue Sepharose 6B chromatography, and anion exchange HPLC) are illustrated in Figure 2-1. After further purification by PAGE, enzyme activity was detected at Rf 0.55 (Figure 2-2). The gel sections contained several other polypeptides as demonstrated by SDS-PAGE (Figure 2-3). The section with the highest enzyme activity contained 12 visible polypeptides (Figure 2-3, lane 3, corresponds to Rf 0.55-0.58 in Figure 2-2). Eluate from the two PAGE gel sections with the highest O-xylosyltransferase activity was used to inoculate mice.

Hybridoma Screening and the Identification of Monoclonal Antibody Against the O-xylosyltransferase

Each mouse was inoculated with PAGE-purified enzyme preparations obtained from 60 g of embryos. The amount of enzyme was about 60 pmol as determined by measuring the protein content of a single SDS-PAGE band on Immobilon. Two thousand hybridoma lines were initially obtained and 55 lines were selected based on ELISA. Immunoblotting after SDS-PAGE indicated that 43 lines were positive and

these were recloned. A line, designated XZT-1, which exhibited immuno-affinity to a single band of Mr 50 kD was chosen for further analyses.

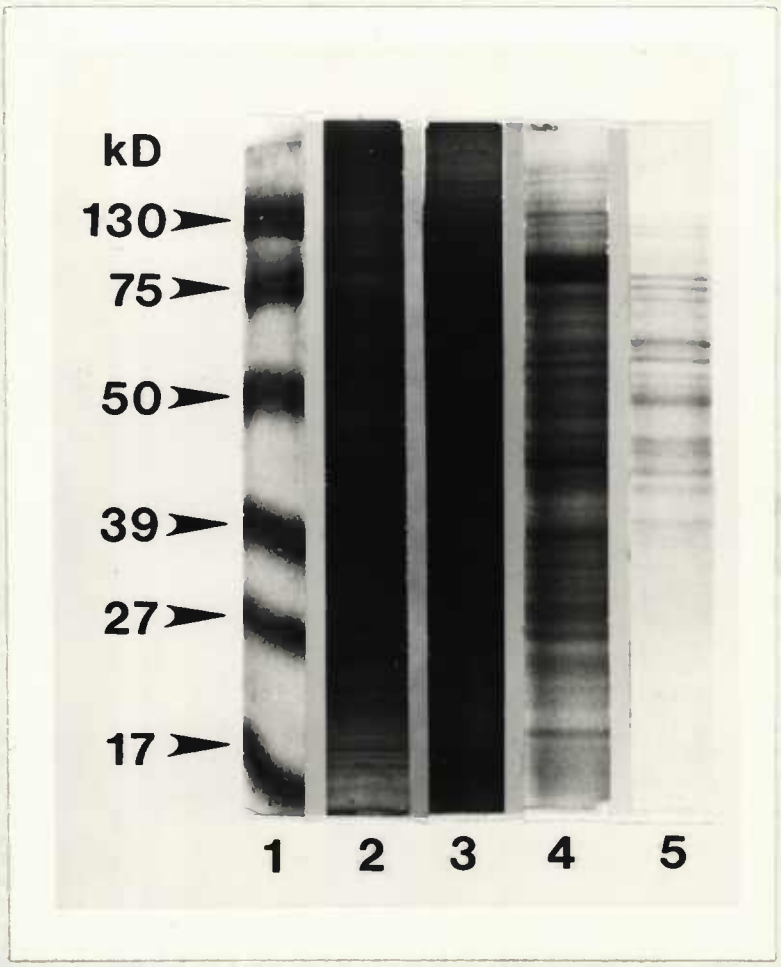

Figure 2-1. Profiles of polypeptides separated by SDS-PAGE after successive purification steps. Lane 1, molecular mass markers; lane 2, crude extract (0.005 g of embryos); lane 3, 30 to 60% ammonium sulfate precipitation (0.01 g of embryos); lane 4, AgAMP and Blue Sepharose 6B affinity column chromatography (0.2 g of embryos); lane 5, anion exchange HPLC (5 g of embryos).

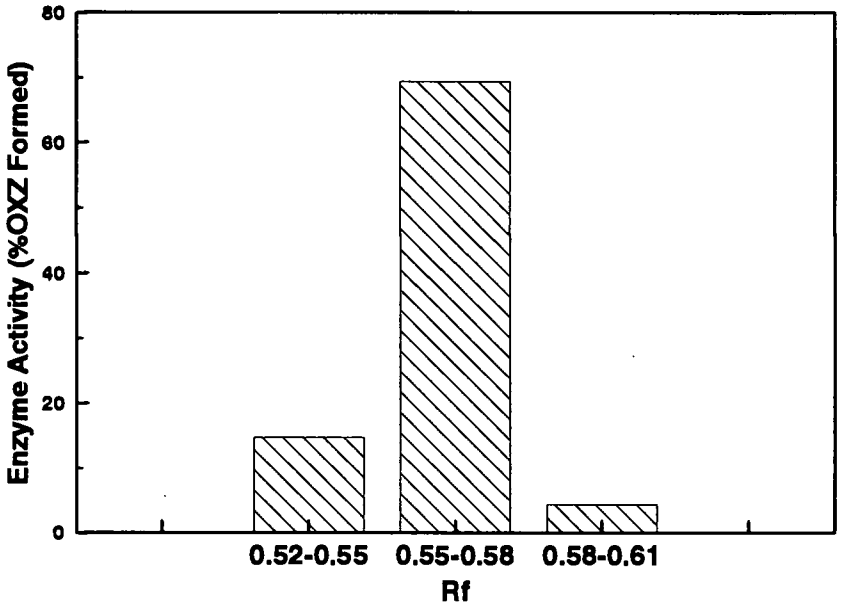

Figure 2-2. Enzyme activity and R_f value of O-xylosyltransferase after PAGE. Appropriate fractions eluted from AX-300 HPLC were combined and separated by PAGE. A gel was sliced into horizontal sections, and enzyme was recovered by electro-elution and assayed for activity.

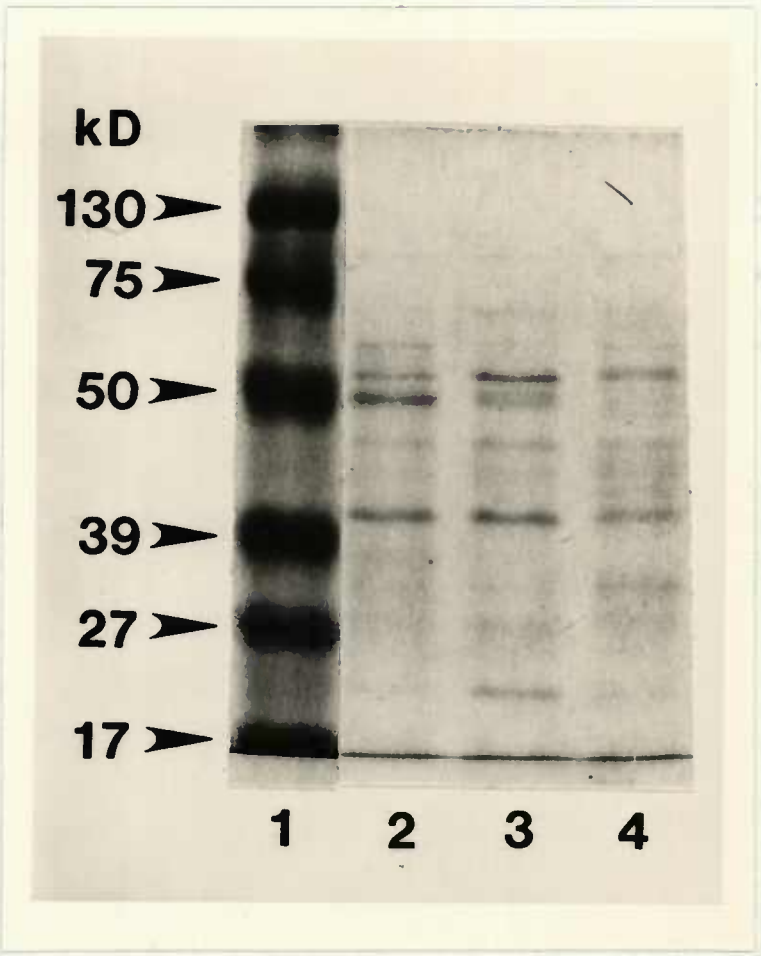

Figure 2-3. Purity of O-xylosyltransferase preparations used as the antigen. SDS-PAGE of polypeptides contained in eluates of PAGE gel sections obtained from Figure 2. Lane 1, molecular mass markers; lane 2, R_f 0.52-0.55; lane 3, R_f 0.55 to 0.58; lane 4, R_f 0.58 to 0.61. Sample represents proteins obtained from 10 g of immature embryos of *P. vulgaris*.

Antibody-Enzyme Interaction

Antibody from the hybridoma line XZT-1 proportionally immunoprecipitated the zeatin O-xylosyltransferase of *P. vulgaris* (Figure 2-4). Purified Ab, ranging from 100 μg to 1 mg, was incubated with enzyme extracted from 0.3 g of embryos purified by affinity and anion exchange chromatography (containing approximately 25 μg of protein). Under the assay conditions described, the enzyme activity was reduced by 50% after incubating with 700 to 800 μg of Ab.

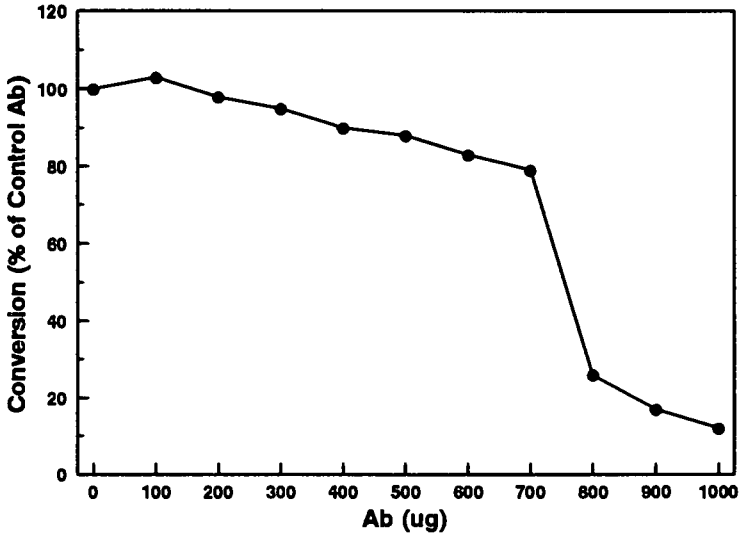

Figure 2-4. Reduction of O-xylosyltransferase activity by immunoprecipitation. Enzyme extracted from 0.3 g of embryos purified by affinity chromatography and anion exchange HPLC (containing 25 μg protein) was incubated with MAb (total volume 250 μl). Appropriate amounts of BSA were added to bring the protein level to 1 mg. Enzyme-MAb complex was precipitated with 100 μl of Protein A-agarose beads and 280 μl of supernatant was used to assay for enzyme activity. MAb secreted by hybridoma line PLRV was used as the control.

Control assays in which Abs secreted by negative hybridoma lines (PLRV-3-71A and 36A-1) were incubated with the enzyme yielded the same level of O-xylosylzeatin (50% conversion) regardless of the amount of control Ab used. The antibody cross-reacted with the O-glucosyltransferase of *P. lunatus*, since it was capable of immunoprecipitating the enzyme from crude samples (obtained from affinity chromatography). For example, a reduction of 13% to 24% in enzyme activity was observed when O-glucosyltransferase obtained from 1 g of embryos (containing 80 μg of protein) was incubated with 200 to 600 μg of the MAb. For immunoblotting, proteins purified by affinity chromatography (Figure 2-1, lane 4) were separated by either SDS-PAGE or PAGE and transferred to Immobilon. Only a single antigenic band occurred in preparations of each species (Figure 2-5). A band of Mr 50 kD was detectable in *P. vulgaris* SDS-blots (Figure 2-5, lane 1) and a band of the same molecular mass was visible in *P. lunatus* preparation (lane 2). Analyses of protein samples separated by PAGE also revealed only one band, at R*f* 0.5, in *P. vulgaris* extracts (lane 3), and another band, at R*f* 0.3, in *P. lunatus* extracts (lane 4). The location of the respective bands was in agreement with the previous finding that O-xylosyltransferase from *P. vulgaris* is more negatively charged than the O-glucosyltransferase of *P. lunatus* as determined by anion exchange HPLC (1).

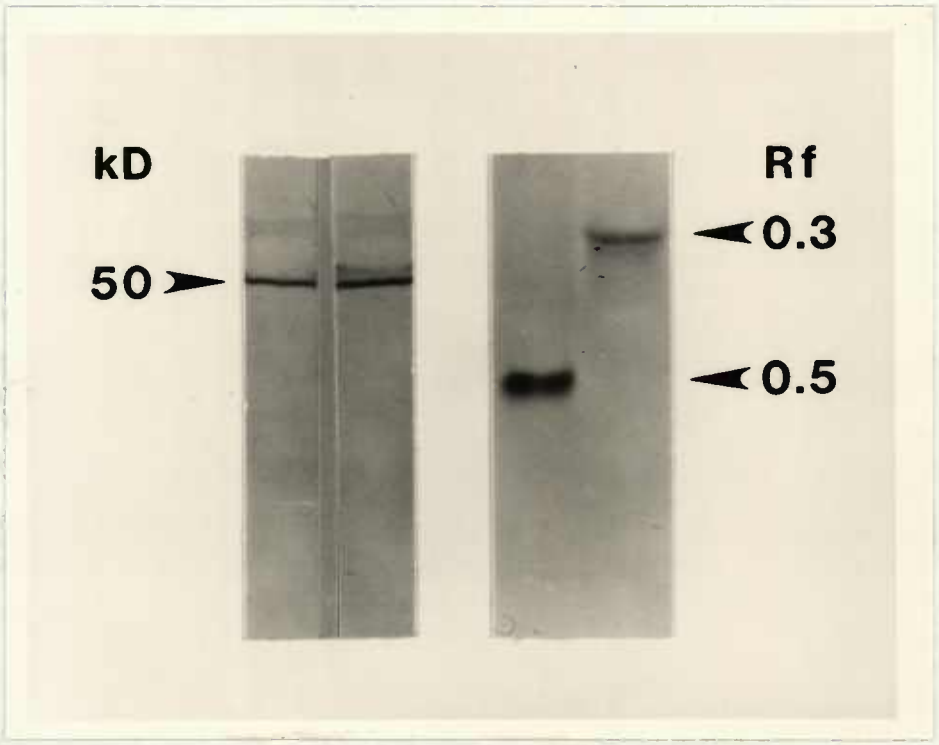

Figure 2-5. Immunoblots using XZT-1 and enzyme preparations from embryos of two *Phaseolus* species after SDS-PAGE and PAGE. Lane 1, SDS-PAGE of *P. vulgaris* preparation; lane 2, SDS-PAGE of *P. lunatus* preparation; lane 3, PAGE of *P. vulgaris* preparation; lane 4, PAGE of *P. Lunatus* preparation. Samples purified by affinity column chromatography (Fig. 1, lane 4) were used for immunoblotting. Each lane contained proteins extracted from 0.2 g of immature embryos.

Discussion

We have been successful in generating a monoclonal antibody to zeatin O-xylosyltransferase, an enzyme with low abundance but high specific activity in embryos of P. vulgaris. The antibody recognized not only the zeatin-O-xylosyltransferase, but also the zeatin-O-glucosyltransferase found in embryos of *P. lunatus*, as demonstrated by Western analyses and immunoprecipitation. However, no other protein bands were visible in immunoblots of crude embryo extracts, indicating that the antibody is highly specific to the zeatin O-glycosyltransferases. Thus, these zeatin-specific enzymes may have a common antigenic site not present in other glycosyltransferases. As blocking with UDPX or UDPG before incubating with the MAb did not affect the ability of the MAb to detect either enzyme in Western analyses (unpublished), the MAb-enzyme recognition does not appear to involve glycosyl dinucleotide binding sites of the enzyme. Whether the antibody recognition site has any role in the binding of zeatin to the enzyme remains to be determined.

In addition to identifying the enzymes in various tissues of *Phaseolus* species, we are using the antibodies to prepare immunoaffinity columns in order to obtain pure enzyme in relatively large quantity. The pure enzyme will be used as antigen to elicit the formation of additional MAbs recognizing different epitopes of the enzyme, possibly also the zeatin-binding site. Such MAbs could then be used to isolate other zeatin-specific proteins, including enzymes involved in zeatin metabolism and proteins related to cytokinin action.

Three zeatin-specific metabolic enzymes, the two glycosyl transferases and a reductase, have been isolated from *Phaseolus* embryos (6,7). These enzymes distinguish both the trans- and cis-isomers of zeatin and the free base and nucleoside forms. The high specificity of the enzymes support their importance in regulating cytokinin activity in plant tissues. Therefore, it will be of primary importance to isolate the genes encoding the enzymes and to study their regulation. The availability of a MAb will greatly facilitate gene cloning, since MAb may be employed to screen expression libraries and to obtain pure enzyme for amino acid sequencing.

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CHAPTER 3

CYTOLOCALIZATION OF ZEATIN O-XYLOSYLTRANSFERASE IN *PHASEOLUS*

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Abstract

Zeatin O-xylosyltransferase mediates the formation of O-xylosylzeatin from trans-zeatin and UDP-xylose in immature seeds of *Phaseolus vulgaris*. Tissue printing with a monoclonal antibody specific to the enzyme demonstrated that the enzyme is primarily localized in the endosperm. Immunolocalization performed on monolayer endosperm at the free-nuclei stage and on EM sections demonstrated that the enzyme is associated with the nucleus as well as with the cytoplasm. Immuno-analysis of nuclear fractions revealed that the enzyme is retained in the nuclear pellet. Western analysis also showed that the enzyme is present in the nuclei of cotyledons and endosperm callus. The findings suggest that the enzyme may have additional functions other than the metabolism of zeatin.

Introduction

Cytokinins are plant hormones mediating cell division and differentiation (1). Zeatin is a naturally occurring cytokinin highly active in most plant systems (2). Genetic differences in cytokinin metabolism between species of *Phaseolus* were investigated by incubating immature seeds with radio-labeled zeatin (3). Exogenously supplied zeatin was rapidly converted to O-xylosylzeatin by immature seeds of *P. vulgaris* while O-glucosylzeatin was formed in *P. lunatus*. The species-specific formation of zeatin metabolites was related to the occurrence of a single, distinct enzyme in immature seeds of each of the two species; zeatin O-xylosyltransferase in *P. vulgaris* and O-glucosyltransferase in *P. lunatus*. Both enzymes are highly specific to trans-zeatin but differ in specificity for the donor of the glycosyl moiety (4,5).

UDP-xylose (UDPX) serves as the donor for O-xylosyltransferase from *P. vulgaris*, while the O-glucosyltransferase of *P. lunatus* can utilize both UDP-glucose (UDPG) and UDPX to mediate the formation of O-glucosylzeatin and O-xylosylzeatin respectively, but has ten times higher affinity for UDPG. The two glycosyl transferases have a similar molecular size (Mr 50,000) but can be separated by ion exchange chromatography (4).

Two additional zeatin metabolizing enzymes, a reductase and a *cis-trans* isomerase, have also been isolated from seeds of *Phaseolus* (6,7). Interestingly, the activities of all four enzymes are particularly pronounced in immature seeds. We are interested in characterizing the developmental regulation of these enzymes. Part of this study is focused on localizing enzymes in the developing seed.

Zeatin O-xylosyltransferase was chosen as the first enzyme for localization studies since monoclonal antibodies (MAbs) specific to the enzyme have been generated (8). In this paper we describe results obtained from tissue printing, using MAb (XZT-1) as a probe, immunolocalization performed on monolayer endosperm and EM sections, and Western analyses of cellular and nuclear fractions. The results indicate that the enzyme occurs predominantly in the endosperm nuclei and cytoplasm.

Materials and Methods

Plant Materials

Immature seeds of *P. vulgaris* cv. Great Northern (GN) were used for all experiments. Endosperm callus tissues were established from abnormal, over-grown endosperm of *P. coccineus* x *P. vulgaris* hybrids (9,10).

Tissue Printing

Protocols for tissue printing were those described by Cassab and Varner (11) with slight modifications. Nitrocellulose paper (pore size 0.45μ, Sigma) was soaked in 0.2 M $CaCl_2$ for 30 min and air dried. Cross or vertical sections of seeds 3-6 mm in length were cut with a razor blade, carefully transferred to the membrane, covered with Kimwipes, and pressed against the membrane for 15-30 sec. The prints were air dried overnight. For immunolocalization with the monospecific antibody (XZT-1), previously established protocols were followed (8). Secondary antibody (rabbit anti-mouse conjugated to alkaline phosphatase [RAM-AP], Jackson Laboratories) was used and antigenic regions were visualized using an AP Substrate Kit (Vector Laboratories). Control blots were treated in the same way, with the omission of the MAb XZT-1. The location of proteins on tissue prints was detected by staining membranes with 0.05% Coomassie Brilliant Blue R (Sigma) dissolved in 12.5% trichloroacetic acid and destaining with 1% acetic acid in methanol.

In Situ Immunolocalization Using Free-Nuclei Endosperm

The monolayer endosperm was dissected from seeds 2 mm in length and transferred directly to a gelatin-coated microscope slide. The slides were fixed in Sorensen's phosphate buffer containing 4% glutaraldehyde for 2 hr. RAM-AP antibody and goat anti-mouse (GAM) antibodies conjugated to colloidal gold (5 nm, BioCell Gold Conjugates) were used to visualize the location of the primary antibody. Treatments using GAM-gold antibodies included silver enhancement according to manufacturer's instructions (BioCell Research Laboratories). Control samples were incubated without the primary MAb XZT-1 or with an unrelated antibody (specific to viral coat proteins, a gift from R. Martin, Agricultural Canada).

Immunolocalization on EM Sections

Tissues were prepared essentially according to the methods of Spurr (12). Sections of 0.1 μm were prepared on a Sorvall Porter-Blum ultramicrotome. Grids were blocked with 0.5% BSA in TBS overnight at 4°C and incubated for 2 hr at room temperature with the primary antibody, XZT-1. They were then treated with goat anti-mouse antibody (1:50 dilution) conjugated to colloidal gold (20 nm) for 2 hr at room temperature, post stained with Reynold's lead citrate (13) and examined with a Phillips CM12 scanning transmission electron microscope.

Western Analysis and Enzyme Assays

Procedures for enzyme extraction, protein determination and immuno-detection have been reported earlier (4,8). The extracts obtained from cotyledons were purified by Blue Sepharose 6B chromatography while endosperm samples were used directly.

Isolation of Nuclei

Nuclei were isolated according to the methods of Cox and Goldberg (14). The nuclei were lysed with 0.5 M NaCl in Honda buffer for 45 min at 4°C and centrifuged. The supernatant and pellet were analysed by immunoblotting after SDS-PAGE as described previously (8).

Results

Tissue Printing

The locations of proteins and O-xylosyltransferase are illustrated in Figure 3-1. The direction of tissue sections and the location of endosperm are illustrated in column 1, the distribution of proteins in column 2, and the distribution of enzyme in column 3. In parallel sections (samples A and B), the endosperm was located at the lower side distal to the radicle. A strong immunogenic response was visible in the same region. In a perpendicular section (sample C) of the seed, the enzyme was visualized as surrounding the developing cotyledons and the radicle, coinciding with the location of the endosperm. The results demonstrate that the enzyme zeatin O-xylosyltransferase is localized primarily in the endosperm.

In Situ Immunolocalization Using Monolayer Endosperm

Endosperm at the free nuclei stage was used to determine the cellular location of O-xylosyltransferase. At this developmental stage, regions of the endosperm proximal to the radicle contain nuclei of varying sizes (as the result of endomitosis) sharing a cytoplasm. The nuclei are easily visualized with a light microscope (Figure3-2A).The presence of immunogenic proteins was determined with the MAb XZT-1 and secondary antibodies conjugated with colloidal gold or AP. The colloidal gold particles were found to be concentrated in the nuclei (Figure 3-2B). A sample of a nucleus in isolation (Figure 3-2C) illustrates the contrast between nucleus and the cytoplasm. Control samples with the omission of the primary antibody XZT-1 (Figure 3-2D) or with unrelated antibodies showed only faint images of the nuclei against equally tinged cytoplasmic background. Treatment with secondary antibodies linked with AP gave similar results. The nuclei were darkly stained in samples incubated with the primary antibody (Figure 3-2E) while control samples (Figure 3-3-2F) had similar coloration of nuclei and cytoplasm. These observations indicate that zeatin O-xylosyltransferase is associated with the nuclei as well as the cytoplasm of the endosperm.

Immunolocalization on EM Sections

The association of the enzyme with the nucleus was further examined by transmission electronmicroscopy. Regions of the endosperm distal to the radicle, containing highly mitotic cells, were used for immunolocalization on ultra-thin sections. After treatment with the MAb XZT-1, the location of the enzyme was

visualized with rabbit anti-mouse antibody conjugated to colloidal gold. In these samples, the gold particles were deposited on both the nucleus and the cytosol (Figure 3-3A and B). The distribution of the gold particles in the cytoplasm did not appear to be concentrated in particular locations. However, no deposits were observed in structurally intact organelles such as the vacuoles. Gold particles were associated with electron-dense regions such as the condensed chromatin (Figure 3-3C). A similar pattern was observed in endomitotic cells with more than one nucleus (Figure 3-3D). The nuclear envelope did not have higher concentrations of the gold particles (Figure 3-3E). Control sample (Figure 3-3F) incubated with unrelated primary antibody did not contain any colloidal gold. Similar results were obtained from sections of cotyledon tissues.

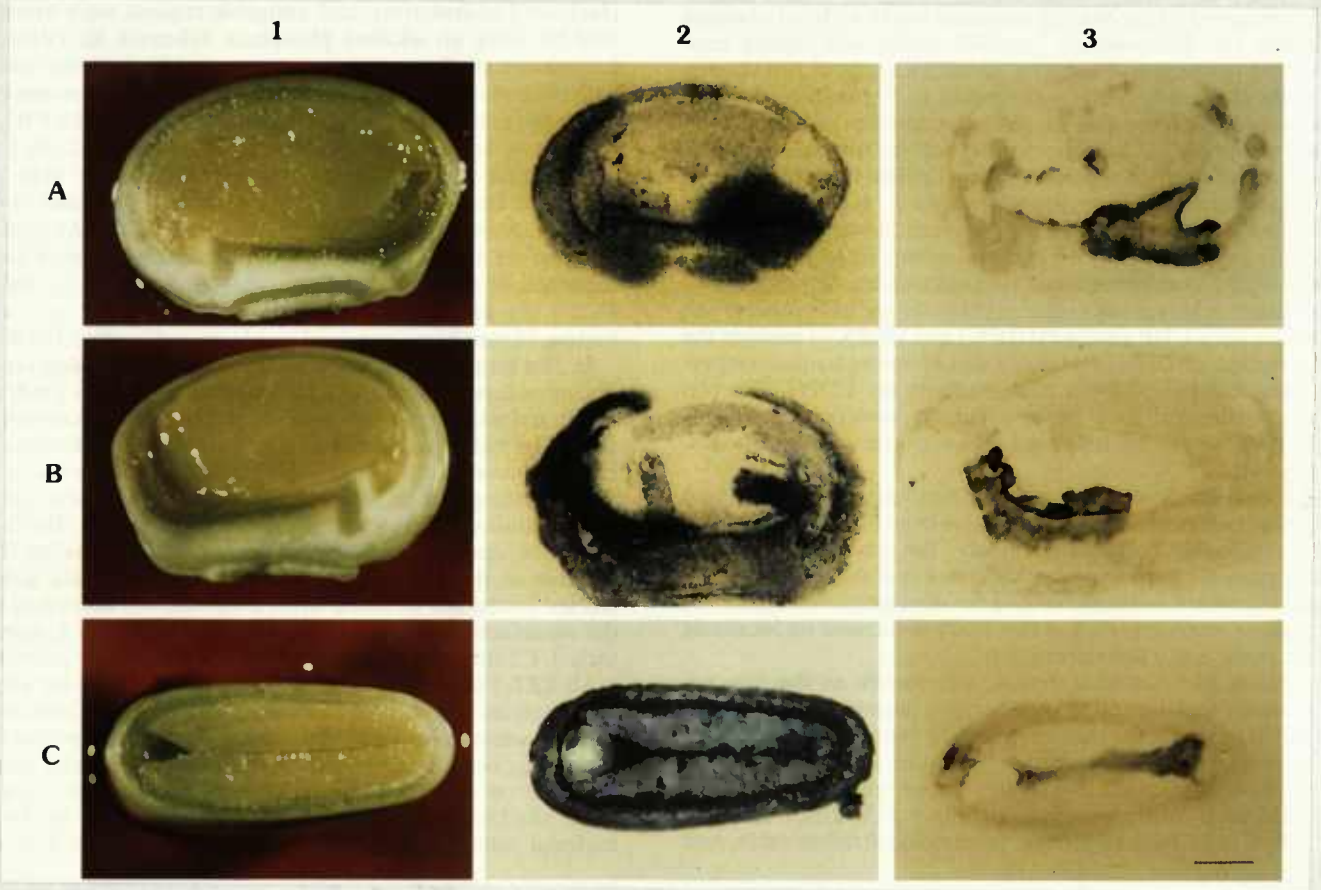

Figure 3-1. Sections and tissue prints of immature seeds.
(A) and (B) Parallel sections (to the plane of the cotyledons). (C) Perpendicular section (to the plane of the cotyledons). (1) Orientation of the sections. (2) Distribution of proteins visualized by Coomassie Brilliant Blue. (3) Distribution of zeatin O-xylosyltransferase detected by immunoblotting. The bar represents 1 mm.

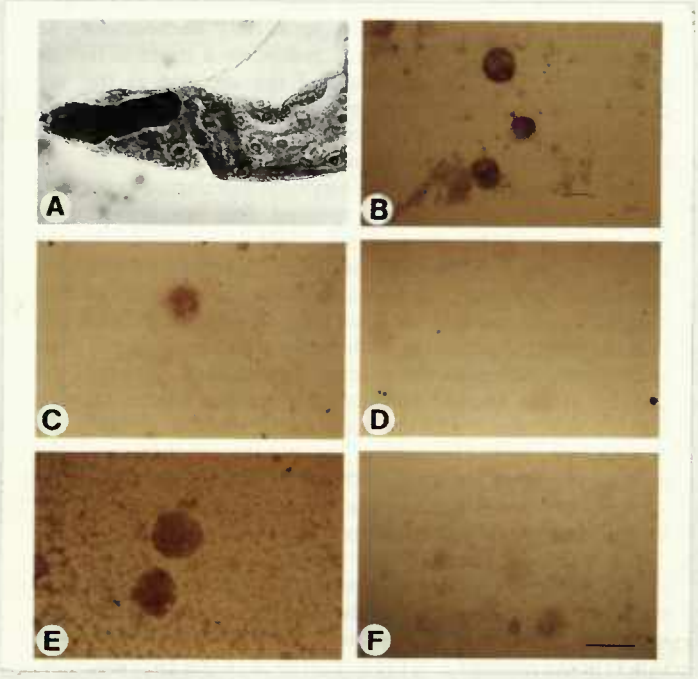

Figure 3-2. Representative sample of developing endosperm and in situ immunolocalization on endosperm at free-nuclei stage. (A) Embryo surrounded by sac-like endosperm at free-nuclei stage, 72 hr after fertilization. (B) Localization of O-xylosyltransferase in nuclei detected with GAM antibodies conjugated to colloidal gold and silver enhancement. (C) Same treatment as (B), showing a single nucleus and cytoplasm. (D) Control sample of treatment (B), incubated without primary antibody. (E) Localization of O-xylosyltransferase in nuclei detected with RAM antibodies conjugated to AP. (F) Control sample of treatment (E), incubated without primary antibody. The bar represents 0.5 μm.

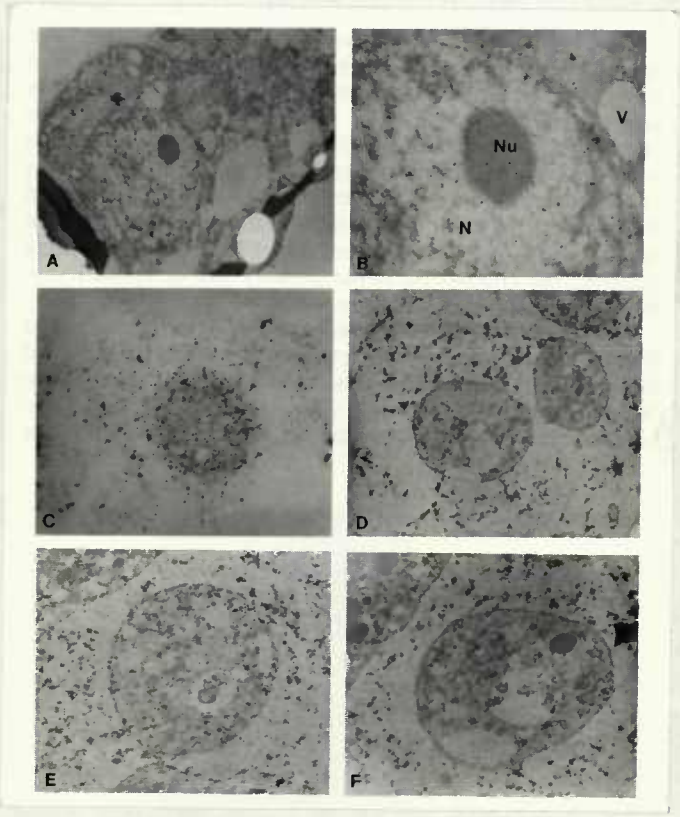

Figure 3-3. Immunolocalization of O-xylosyltransferase on EM sections of endosperm. (A) Section showing deposit of gold particles in cytoplasm and nucleus (x8,000). (B) Close-up of (A) showing nucleus (N) nucleolus (Nu) and vacuole (V) (x28,000). (C) Gold particles on condensed chromatin (x17,000). (D) Distribution of gold particles in cell containing two nuclei (x8,000). (E) Close-up of a nucleus with intact nuclear envelope (x10,000). (F) Control section treated with an unrelated primary antibody (x10,000).

Western Analyses of Nuclear Fractions From Reproductive and Vegetative Tissues

To confirm the association of the enzyme with the nucleus, nuclei were isolated from endosperm and cotyledons. After lysis, the soluble nuclear proteins and those remaining with the pellet were analysed by immuno-blotting after SDS-PAGE. Immunogenic bands were detected by the antibody in the pellet of lysed nuclei (Figure 3-4, lanes 1 and 2) but not in the supernatant containing nuclear proteins (Figure 3-4, lanes 4 and 5), suggesting that the enzyme may be associated with chromatin, nuclear matrix or the nuclear envelope.

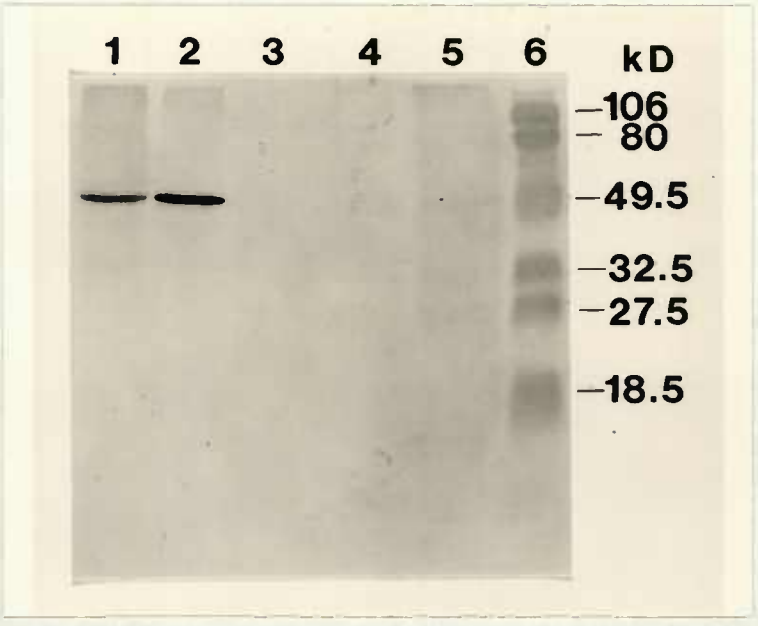

Figure 3-4. Immuno-blot of nuclear fractions.
Lanes 1 and 2: Proteins from pellet of lysed nuclei isolated from seeds and endosperm respectively. Lanes 4 and 5: Proteins from supernatant (nuclear protein) of lysed nuclei obtained from seeds and endosperm respectively. Lane 3: blank. Samples were treated with the MAb XZT-1 and visualized with GAM secondary antibodies conjugated to AP.

We have established in previous work (15) that O-xylosyltransferase occurs in soluble fractions of seeds but is not detectable in comparable fractions of vegetative tissues. In light of the findings described above, this difference could be the result of nuclear localization rather than the absence of the enzyme from vegetative tissues. To evaluate this possibility, cytosolic and nuclear fractions of roots, leaves, shoot meristems, and endosperm-derived callus were examined. As observed in earlier work, the 50 kD enzyme was not present in the cytosolic or nuclear fractions of vegetative tissues. However, an antigenic protein larger that 50 kD was detected in the nuclear fraction of endosperm-derived callus (data not shown) and root tissues (see Chapter 4).

Enzyme Levels in Endosperm and Cotyledons

The relative levels of the enzyme in the cytosolic fractions of the endosperm and cotyledon were determined by Western blotting and activity assays. For immuno--blotting, equal amounts (5 ug) of proteins (Figure 3-5, lanes 3 and 4) extracted from endosperm and cotyledons were separated by SDS-PAGE and detected with the MAb (Figure 3-5, lanes 1 and 2). A single antigenic band of 50 kD (which is the Mr of purified zeatin O-xylosyltransferase) occurred in both samples. However, the intensity of the antigenic band derived from the endosperm samples was much higher.

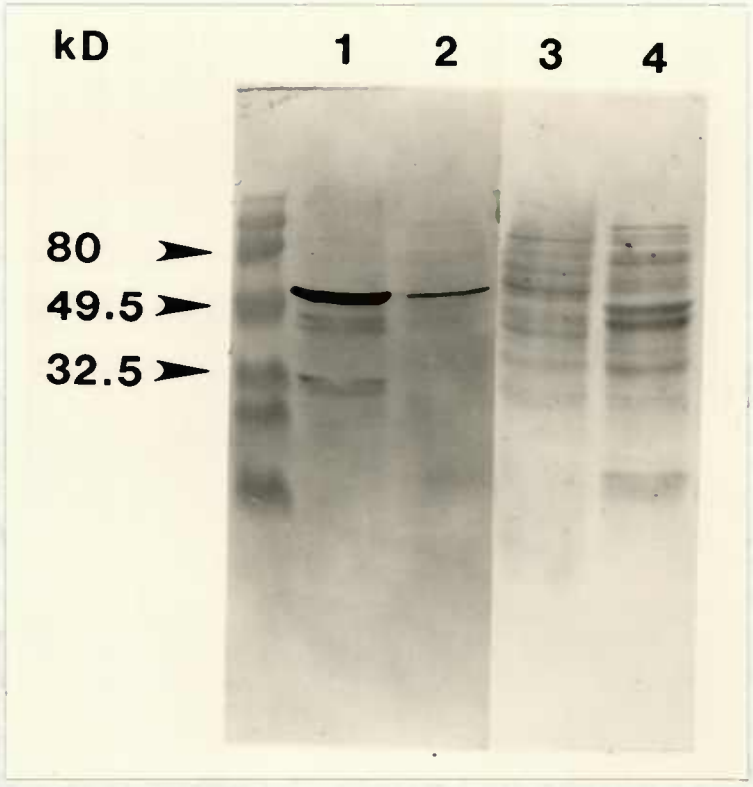

Figure 3-5. Immuno-blots of zeatin O-xylosyltransferase and proteins after SDS-PAGE. Lanes 1 and 2: Endosperm and cotyledon samples (5 ug of protein) respectively detected with MAb. Lanes 3 and 4: Endosperm and cotyledon samples respectively stained with Coomassie Blue.

Immunoblots of PAGE samples (on 10% gel at pH 8.5) also detected only a single band with the correct Rf of 0.5 in each sample (results not shown). Enzyme activity was determined as described earlier (4). The specific activity (cpm of O-xylosylzeatin formed/ ug protein) of the enzyme in the endosperm was about 200 times higher than that of the cotyledons (7264 vs. 39). This is probably an underestimate since the activity assays of the cotyledon samples were performed after

partial purification while endosperm samples were used directly. Samples obtained from seed coats did not exhibit enzyme activity. These results also substantiate observations obtained from tissue printing that endosperm is the primary site of zeatin O-xylosyltransferase in *P. vulgaris* seeds.

Enzyme Activity of the Endosperm Sap

Activity assays and Western analysis of the sap between the cotyledons revealed high enzyme activity. Samples of the liquid endosperm were withdrawn from intact seeds with a microsyringe and incubated directly with labeled zeatin, zeatin plus UDP-X or zeatin plus cotyledon extracts. Interestingly, O-xylosylzeatin was formed with zeatin in the presence of UDP-X or cotyledon extract but only trace amounts occurred with zeatin alone. These results suggest that the cotyledons may be the *in vivo* source of UDP-X.

Discussion

The results described in this chapter demonstrate that zeatin O-xylosyltransferase is primarily synthesized in the endosperm of the developing seed. The extractable (cytosolic) level of the enzyme in endosperm is about 200 fold higher than that of the cotyledons. In large-seeded legumes, to which *P. vulgaris* belongs, the endosperm is membranous and contains nuclei of various sizes (as the result of endomitosis). The sac-like endosperm surrounds the radicle and the cotyledons. Endosperm proximal to the radicle is highly metabolic with large (up to 0.5 μ) free nuclei while the distal portion (to the radicle) consists of mitotic cells. At later stages

of seed development, coincident with rapid expansion of the cotyledon, the endosperm gradually ceases to divide, desiccates, and remains as a dry membrane between the seedcoat and the cotyledons. The localization of the enzyme in the endosperm agrees with the tests of enzyme activity and with the earlier finding that enzyme activity decreases with seed maturation (15). Using immuno-assays, we have detected antigenic proteins in seeds of other plants including *Zea mays*, *Vicia faba*, and lupin (16). However, the proteins differ in mass from the O-glycosyltransferases of *Phaseolus*. We have not yet determined whether the immuno-reactive proteins in seeds of other species are also located in the endosperm.

In general, hormone activity is high during active seed growth (17). Elevated levels of cytokinins were found in endosperms of maize, rice and wheat during the highly mitotic phase (18,19,20). The high concentration of O-xylosyltransferase in *P. vulgaris* endosperm may be necessary to convert a less stable cytokinin, zeatin, to a more stable form (21). The presence of the enzyme in the sap between the endosperm and the cotyledons, the likely source of UDPX, allows the synthesis of O-xylosylzeatin.

The finding that the zeatin O-xylosyltransferase is located in the nuclei is somewhat unexpected. Our previous studies had established that the enzyme could be isolated from the soluble fraction of the seed, and was therefore presumably cytosolic. However, the immunolocalization studies of free nuclei (Figure 3-2) and EM sections (Figure 3-3) demonstrate clearly that the enzyme occurs in both the nucleus and cytoplasm of the endosperm. Although the precise subnuclear location

of the enzyme is not known, studies with isolated nuclei showed that it is retained with the pellet of the lysed nuclei (Figure 3-5). Association with chromatin, the nuclear matrix and the nuclear envelope are some of the possibilities. Results of EM studies, however, indicate that the enzyme is probably not located in the nuclear envelope.

The question arises whether the presence of the enzyme in the nucleus and cytoplasm is related to a dual function of the enzyme - a metabolic function in the cytoplasm and a yet unknown function in the nucleus - or that the primary function is in the nucleus and the occurrence of the enzyme in the cytoplasm is due to the rapid nuclear divisions and endomitotic nature of the endosperm. Proteins associated with the nucleus such as transcription factors, chromosomal proteins, and components of the nuclear envelope are dispersed in the cytoplasm during cell division and reassembled after the reformation of the nucleus. Examples of such proteins include transcription activators (22,23), the mitotic regulator protein RCC1 (24,25) and the lamin kinase (26). In slowly dividing tissues, the amount of nuclear proteins present in the cytoplasm is expected to be low and transient. However, in bean endosperm, the presence of such proteins may persist due to the rapid succession of dissolution and reassembly of the nuclei. This would lead to detection of the O-xylosyltransferase in the cytoplasm, but not in structurally intact bodies such as the vacuoles, which is in agreement with the results of the EM studies. The presence of the enzyme in the soluble fraction of the cotyledons (Figure 3-5), a rapidly dividing tissue, and its absence from the soluble fraction of endosperm-derived callus, a slowly dividing tissue, also support this interpretation. Although it is uncertain to what extent the

presence of the enzyme in the endosperm cytoplasm is related to the effect of nuclear division, it is clear that the presence of the enzyme in the nucleus is not the result of the reverse effect, i.e. inclusion in the nucleus of enzyme normally present in the cytoplasm due to rapid nuclear division. In that case the enzyme should have been found in the soluble nuclear protein fraction, whereas our results demonstrate that the protein is associated with the nuclear pellet.

Association of the enzyme with the nucleus implies that either the enzyme, its substrate, or product may interact directly with nuclear components. Possibly, the enzyme is involved in the transport and targeting of cytokinins or cytokinin-related molecules between the nucleus and cytoplasm. Alternatively, the enzyme may be part of the chromatin complex as structural protein or may have DNA-binding properties. Cellular proteins such as ras and nuclear lamins contain isoprenyl groups (farnesyl and geranylgeranyl) and attention has been directed at the possible role of isoprenoid derivatives in mammalian systems (27). Recently, antibodies against isopentenyladenosine (a plant cytokinin) were used to identify a protein (i^6A26) from Chinese hamster cell cultures, the level of which correlated with the rate of cell division (28). It was suggested that the i^6A26 protein may mediate DNA synthesis. As most naturally occurring cytokinins, i.e. isopentenyladenine and zeatin, are N^6-isoprenoid derivatives, it is conceivable that plant proteins with high affinity to cytokinins such as the O-xylosyltransferase may also interact with nuclear components related to the regulation of cell cycle. As the protein is intermediate in size (50 kDa), it is likely that an active transport mechanism is required to localize the protein to the nucleus (29). Studies of nuclear targeting in mammalians, yeast and amphibians revealed

active transport mechanisms for larger molecules (40 kDa and above) which have a nuclear localization signal (NLS) (30,31,32). Several plant proteins including the opaque-2 gene product and auxin-binding protein were reported to be localized in the nucleus (33,34) and the amino acid sequence responsible for nuclear targeting of plant transcription factors is being investigated (35). As the N-terminal sequence of the Agrobacterium VirD2 gene was able to target a fusion protein to the plant nucleus (36), the NLS is believed to operate across species boundaries. Sequence analyses of the enzyme and comparisons to known NLSs and DNA binding proteins may provide additional clues to the mechanism of nuclear targeting and the significance of nuclear localization of zeatin O-xylosyltransferase.

Genes involved in cytokinin biosynthesis and metabolism have not yet been isolated from higher plants. In prokaryotic systems, the only example is the ipt gene identified in Agrobacterium (37,38). Zeatin-specific enzymes of *Phaseolus* may be exploited for genetic and molecular studies of cytokinin metabolism in plant systems. Even for the zeatin O-glycosyltransferases, we have identified multiple but related transcripts in *P. vulgaris* immature embryos (unpublished results). The analyses of gene sequences, their expression, and localization of their product in relation to the function of the respective enzymes should further the understanding of the genetic regulation of cytokinin metabolism in higher plants.

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CHAPTER 4

CHARACTERIZATION OF PUTATIVE ZEATIN O-XYLOSYLTRANSFERASE cDNAs OF *PHASEOLUS VULGARIS*

Abstract

A monoclonal antibody specific to zeatin O-xylosyltransferase of *P. vulgaris* was used to screen a lambda cDNA expression library derived from mRNAs of *P. vulgaris* seeds. Two highly homologous clones of 2.2 and 2.4 kb with ORFs encoding proteins of approximately 67 and 69 kD, respectively, were selected for further analyses. Results of *in vitro* transcription/translation experiments indicated that the protein was processed by bean endosperm extracts and was associated with membranes. Transgenic tobacco plants containing the ORF under a constitutive promoter were generated. Analyses of selfed progeny of the transgenic plants confirmed the membrane association of the cDNA-encoded protein. Transgenic plants were sensitive to auxin in comparison to no-insert controls. The preprotein was processed in plants exhibiting auxin damage. The results suggest that the cDNAs encode preproteins requiring precise processing to give rise to active enzymes; and that the classical observation of opposing cytokinin-auxin effects may at least in part be mediated via auxin stimulation of cytokinin metabolic enzymes.

Introduction

Four zeatin metabolic enzymes have been identified and partially purified from immature seeds of *Phaseolus* (1). These enzymes provide useful tools for the isolation

of genes involved in the regulation of cytokinin metabolism. Monoclonal antibodies (MAbs) specific to O-glycosyltransferases of zeatin have been generated (see chapter 2) and used for localization studies (see chapter 3). This chapter presents the results of cDNA cloning and the effects of one cDNA in transgenic tobacco plants.

Materials and Methods

Plant Materials

mRNAs were isolated from immature seeds (less than 5 mm in length) of *P. vulgaris* cv. Great Northern (see below). Seeds were dissected, frozen immediately in liquid nitrogen and then stored at -80° C until use.

Construction of cDNA Library

Total RNA was isolated from immature seeds using the Phenol/SDS method (2). mRNA was further enriched with the PolyATract mRNA isolation system according to manufacturer's instructions (Promega, Madison, Wisc.). An expression library (directing the production of fusion proteins with β-Gal) was constructed using the SuperScript$_{TM}$ Lambda System (BRL Life Technologies, Inc., Figure 4-1). First strand synthesis was primed with a *Not*I-oligo-d-(T) primer-adapter. Following second strand synthesis *Sal*I adapters were ligated to the cDNA, which was subsequently digested with *Not*I. This allowed for directional cloning of cDNA into the λgt22 expression vector (BRL). The constructed λ cDNA library was packaged following manufacturer's instructions (BRL λ packaging system).

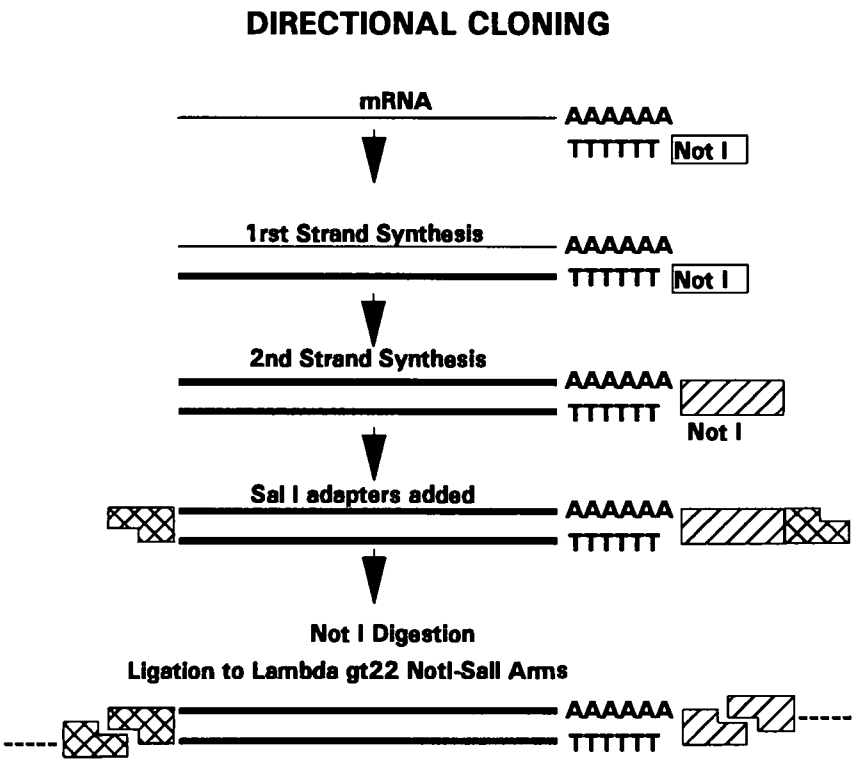

Figure 4-1. Construction of cDNA library via directional cloning.

Screening the cDNA Library With MAbs

The λgt22 cDNA library was plated with *E. coli* Y1090 on 1% top agar and incubated at 42° C for 3-4 hours. The plates were then overlaid with nitrocellulose filters (which had been previously soaked with 10 mM IPTG and dried) and then incubated at 37°C overnight. Filters were marked for orientation, removed from the plates, washed briefly in TBS (Tris-Buffered Saline) and immunoblotted following

standard protocols. The membranes were blocked in TBS containing 2% nonfat dry milk for 1-2 hours at room temperature with constant agitation. Filters were then incubated with blocking solution containing primary antibody for 1-2 hours at 37° C. Filters were washed three times (5 minutes each) in blocking solution. Secondary antibody (Rabbit anti-mouse conjugated to alkaline phosphatase, Jackson Laboratories) in blocking solution was applied to the filters for 1 hour at 37° C. Filters were washed and the positive clones were identified with an Alkaline Phosphatase Substrate Kit (Vector Laboratories). Immunopositive plaques were removed, replated and rescreened until pure plaques were obtained.

Subcloning and Sequencing of cDNA Clones

Selected clones were purified and the inserts were subcloned into pSport after digestion with the enzymes *Not*I and *Sal*I. The pSport plasmids were used for sequencing which was performed by the Central Services Lab (Center for Gene Research and Biotechnology, OSU) using an Applied Biosystems 370A DNA sequence analyzer. Selected cDNAs were also cloned into the pMal vector (New England BioLabs) to produce a fusion protein with the maltose binding protein (3). Depending on the clone, inserts in pSport were either digested with *Sal*I and *Hind*III and sub-cloned directly into pMal; or digested with *Bam*HI, blunt ended with T_4 DNA polymerase, released with *Sal*I, and ligated to a *Sal*I/blunt-ended pMal vector (if the clone contained an internal *Hind*III site). Competent DH5α cells were used as the host. The clones were also transfected in *E. coli* TB1 cells provided with the vector system. Induction with isopropyl-ß-D-thiogalactopyranoside (IPTG) and purification

of the fusion protein were performed according to the manufacturer's instructions. The ORFs of clones 30 and 1 were also subcloned into a pT7-7 vector (obtained from Dr. Walter Ream, OSU). This vector contains the bacteriophage T7 RNA polymerase promoter and translation start site for T7 gene 10 protein (4). Primers were designed to allow expression of the ORF with a histidine tag on the 3' end of the gene. The vector was transformed into BL21(DE3) cells. IPTG induced production of the T7 polymerase in these cells results in the subsequent production of the gene cloned into the pT7-7 vector (for details see Studier *et al*, 1990). Immunoanalysis of the proteins was performed as previously described (5).

For subcloning into Baculovirus vectors (6), the pSport clones 30 and 1 and the Baculovirus vector pVL1392 were digested with *Eco*RI and *Bam*HI. The inserts were ligated into pVL1392 overnight and transformed into DH5α competent *E. coli* cells. Infection of Sf9 (*Spodoptera frugiperda*) cells and identification and purification of positive plaques was performed by Jean Prahl at the University of Wisconsin-Madison. Purified virus was then used to infect Sf9 cells for expression and protein analysis following the manufacturer's instructions (Invitrogen, San Diego, CA.).

Northern Analysis

Total RNA (10 μg) or poly(A^+)-RNA (5 μg) was fractionated on a 1% agarose formaldehyde gel, blotted onto Hybond N^+ membranes (Amersham) and the blots were probed with a ^{32}P-labelled clone 30 cDNA insert (Random Prime, Amersham) following the manufacturer's instructions. The filters were hybridized overnight at

42°C, and then washed three times (30 min each) in 2 x SSC, 0.1% SDS at 42°C. Filters were then wrapped in Saran Wrap, exposed to film for several days at -80°C, and developed using a KODAK RR X-OMAT Processor, Model M6B.

Southern Analysis

Genomic DNA was isolated from bean leaves using the modified CTAB method of Doyle and Doyle (7). Approximately 10 μg of DNA was digested with several restriction enzymes (*Eco*RI, *Bam*HI, *Eco*RV, and *Spe*I) overnight. Restriction digested DNA was separated on a 1% agarose gel and transferred to a nylon membrane (Zeta Probe, BioRAD). The membrane was hybridized with ^{32}P-labelled probes (Random Prime, Amersham). The blots were prehybridized and hybridized following the manufacturer's protocols (BioRad). Hybridization was performed at 65°C overnight. Three washes were performed in 2 x SSC and 0.1% SDS at 65°C for 30 minutes each. Filters were then wrapped in saran wrap, exposed to film for several days at -80°C, and developed using a KODAK RR X-OMAT Processor, Model M6B.

In Vitro Transcription/Translation

pSport clones 1 and 30 were analyzed using a coupled transcription/translation Reticulocyte Lysate System following the manufacturer's instructions (TNT Coupled Reticulocyte Lysate System, Promega, Madison, Wisconsin). Purified plasmid DNA (1 μg) was linearized with *Bam*HI and transcribed *in vitro* from the T7 promoter. ^{35}S labelled methionine was included for the translation reactions. TNT analysis was also

performed in the presence of microsomes. The labelled products were incubated in the presence or absence of endosperm to assess the possibility of processing. The soluble and insoluble products were analyzed by 10% SDS PAGE. Following electrophoresis, the gels were dried and analyzed by autoradiography.

Transformation of Tobacco with Clone 30

A PCR-derived cassette containing the ORF of selected clones flanked by a *Bam*HI site at the 5' end and an *Eco*RI-*Bam*HI site at the 3' end was inserted into pPEV (8). This vector includes an enhanced 35S promoter, the 3' and 5' 35S untranslated sequences, a *Bam*HI cloning site, and the *npt*II gene conferring kanamycin resistance. The pPEV plasmids containing the inserts were transformed into DH5α cells. Plasmids containing the insert in the proper orientation were isolated and transferred into *Agrobacterium tumefaciens* EHA 105 (9) using a modified freeze thaw method (10). Tobacco *cv* W38 was transformed with the pPEV vector and the pPEV vector containing the inserts using standard protocols (11). Kanamycin (400 mg/L) was used to select for resistant plants and timentin (400 mg/L) was used to eliminate the *Agrobacteria* after the cocultivation stage. Regenerating shoots were placed on media with 400 mg/L kanamycin for rooting prior to transfer of plants to the greenhouse.

Analysis of Transgenic Tobacco

To detect the presence of antigenic proteins in transgenic plants, leaves from transformants were homogenized in a Tris EDTA (50 mM pH 8.0, 5 mM) buffer.

The pellet and the supernatant were analyzed by Western immunoblotting after SDS-PAGE. To test for solubility of proteins contained in the pellet portion, various detergents and organic solvents were used.

Seeds from primary transformants were harvested and transgenic seedlings were identified by their resistance to kanymycin. The possible effects of transgene expression on plant hormone interactions were examined by growing kanamycin resistant S_1 seedlings of transformants and no insert control plants on medium containing various hormones at multiple concentrations.

Results

Immunopositive Clones

Approximately 85 immunopositive plaques were detected from 1.5 million screened with the MAb, XZT-1. Initial selections were plaque purified and the antigenicity of the fusion proteins verified in lysogens (Figure 4-2). Inserts were then subcloned into pSport and sequenced. Two groups of cDNAs were identified. Group A, as illustrated by clone 30, is 2.4 kb in length with an ORF (bps 250 to 2130) encoding a protein of 69 kilodaltons (Figure 4-3). Group B cDNA is illustrated by clone 1 which is 2.2 kb in length (Fig 4-3). The ORF between bps 31-1857 codes for a protein of 67 kilodaltons. The homology between the nucleotide sequences of group A and B cDNAs is over 82%. The deduced amino acid sequences of the ORFs are 90% identical. However, clones belonging to group B can be distinguished by the presence of a *Hind*III site near the 3' terminal. (Four other immunopositive clones

were analyzed but were determined to be unrelated to zeatin O-xylosyltransferase, since transgenic tobacco harboring these inserts are not immunopositive to the MAb, XZT-1.)

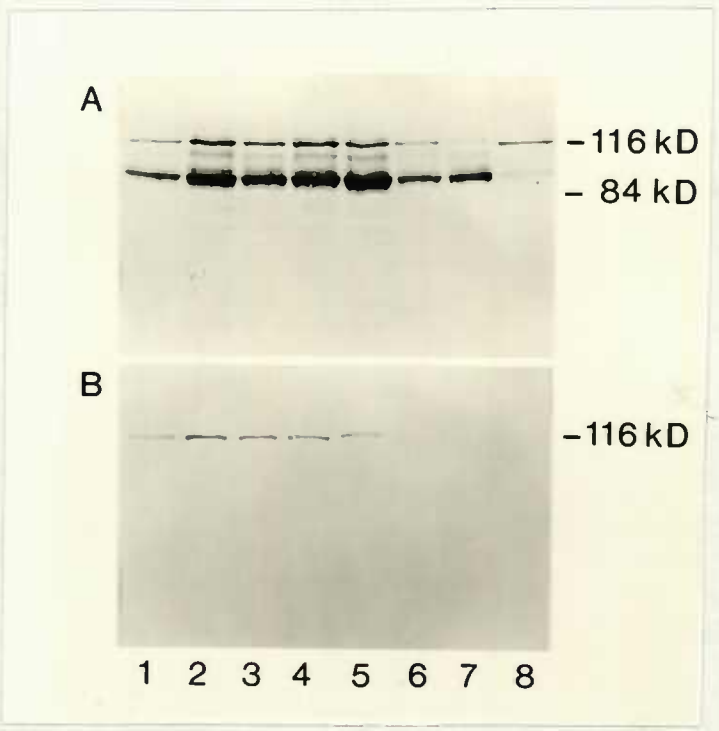

Figure 4-2. Immunoanalysis of fusion proteins obtained from lysogens of selected and control plaques. Proteins detected with antibody against ß-galactosidase (panel A) and antibodies against zeatin-O-glycosyltransferase (panel B). Lane 1-6, λ lysogen; Lane 7, control-truncated Lac Z; Lane 8, Control-Intact Lac Z.

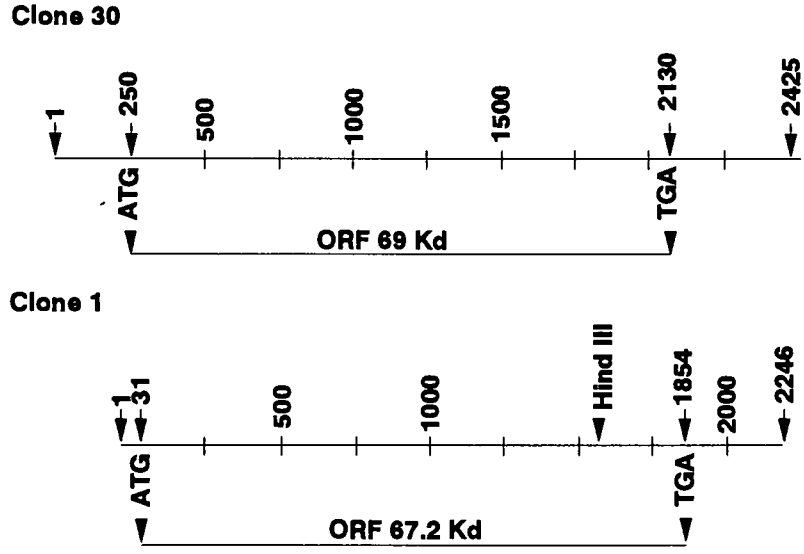

Figure 4-3. Diagramatic representation and comparison of cDNAs from groups A and B.

Northern and Southern Analyses

Northern hybridization using clone 30 as the probe detected a signal of 2.4 kb (Figure 4-4) in both total (lane 1) and polyA RNA (lane 2), suggesting that the cDNAs selected are full length. Results of Southern analyses (Figure 4-5) revealed three bands when genomic DNA was digested with both *Eco*RV and *Spe*I, suggesting that these cDNAs are derived from a small gene family of perhaps two to three genes.

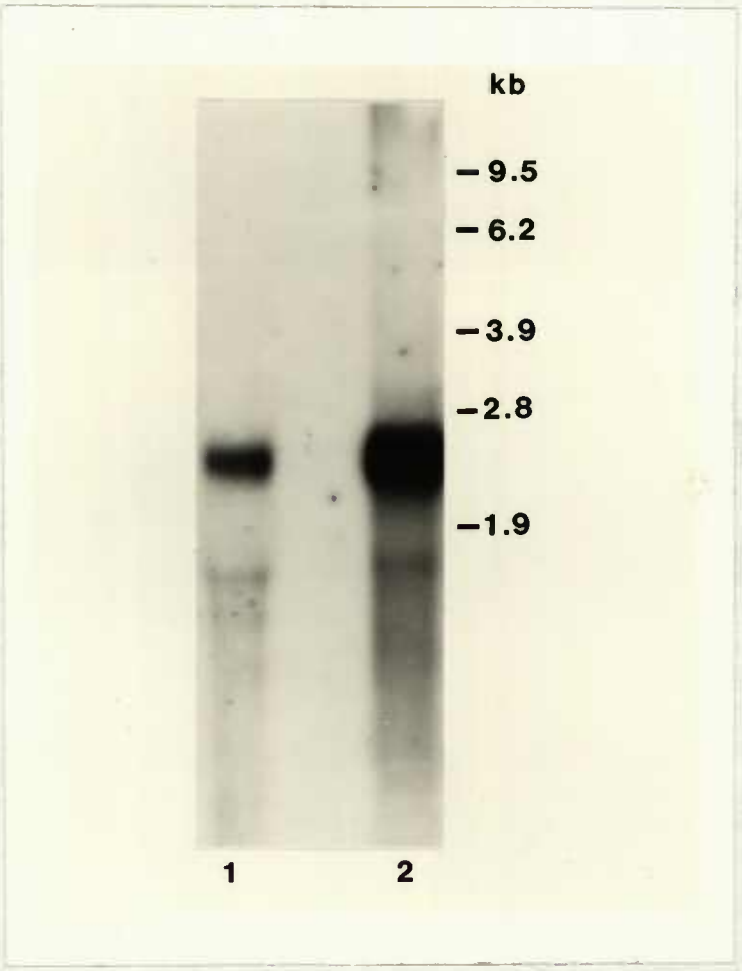

Figure 4-4. Northern hybridization of RNAs from immature seeds of *Phaseolus vulgarus* probed with clone 30. Lane 1: Total RNA, Lane 2: mRNA.

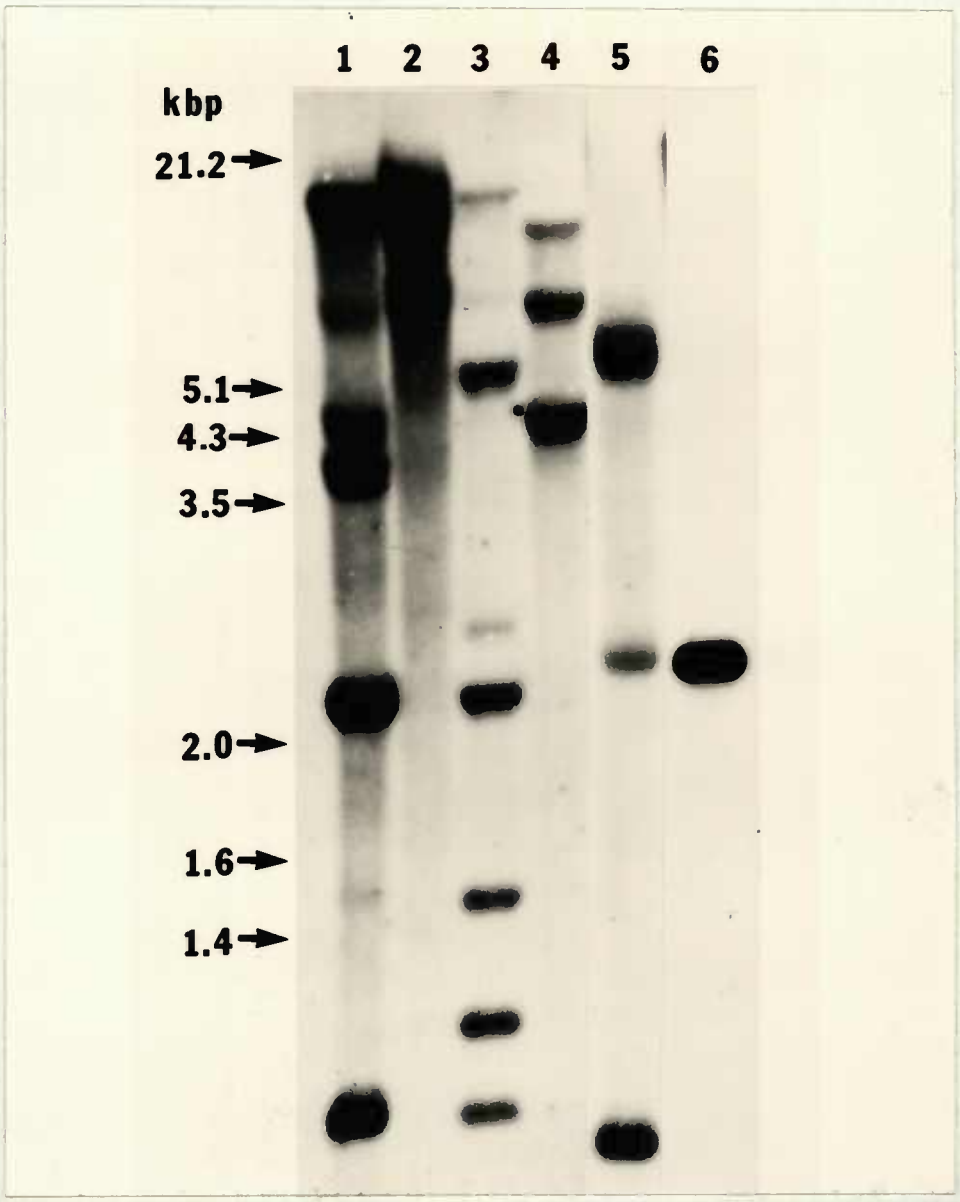

Figure 4-5. Southern hybridization of genomic DNA from *Phaseolus vulgaris* probed with clone 30. Genomic DNA digested with; *Eco*RI (Lane 1), *Bam*HI (lane 2), *Bam*HI and *Eco*RI (lane 3), *Eco*RV (lane 4), *Spe*I (lane 5); lane 6 has positive insert control DNA.

Sequence Analyses

Search of data banks (GENEBANK and EMBL) did not locate any sequence with significant homology to either clone 1 or 30. Based on the deduced amino acid sequence of the cDNAs, a unique motif (CKNTWGSYDCSC) is present in the C-terminal portion of the ORF of both clone 1 and 30. This motif (CxD/Nxxxx-F/YxCxC) occurs in the N-terminal portion of proteins such as low density lipoprotein (LDL) receptors and the epidermal growth factors (EGF), and is associated with the posttranslational hydroxylation of aspartic acid or asparagine (12). The significance of this motif in the cDNAs isolated is unknown at this time. Stretches of amino acid sequence homologous to poly (ADP-ribose)polymerase pseudogene from *Homo sapiens*, ADP-ribosyltransferase (NAD+) pseudogene from *Gorilla gorilla*, and UDP-glucuronosyltransferase from *Rattus norvegicus* were also detected (Figure 4-5). These similarities are of interest since they encompass a single region and could represent a dinucleotide binding site which is expected to be present in the zeatin O-xylosyltransferase protein.

Recombinant Proteins Produced in Prokaryotic and Eukaryotic Expression Systems

Fusion proteins, with β-Gal, of immunopositive clones produced in lambda phage were not aqueous soluble. Numerous growing and induction regimes as well as solvents were tested with no apparent effect. Subcloning into the pMal vector, which produces a smaller fusion protein with maltose binding protein, did not improve solubility. The ORFs of clones 30 and 1 were also ligated into the pT7-7 vector to

produce a free protein of 70 kD. Again, the antigenic proteins were not soluble in aqueous solutions and were associated with the pellet.

A.

```
                    CLONE 1   66 FDDSGISFKSKPGALSTVVLLDRGNCFFALKVWNA 100
                              |:|||||||||||||| |:|||||||:||||||||||
                   CLONE 30  159 FEDSGISFKSTPGALPTFVLLDRGSCFFALKVWNA 193
Poly(ADP-Ribose)              :::|::: :|:|: :|:  ||::||||| ::: :|
  Polymerase Pseudogene      2245 LATSSTPSTSSPSLFPS--LLTPGSCFFRVSLSSA 2277
ADP-ribosyltransferase        |||||||||||||||||  ||||||||||||||||
  (NAD+) pseudogene          1288 LATSSTPSTSSPSLFPS--LLTPGSCFFRVSLSSA 1320
```

B.

```
                          CLONE 1    79 ALSTVVLLDRGNCFFALKVWN  99
                                        ||||:|||||||:|||||||||
                          CLONE 30  172 ALPTFVLLDRGSCFFALKVWN 192
                                        :||: |||::|| ||:|:||| 
UDP-glucuronosyltransferase         371 GLPK-VLLXNGSHFFSLNVWN 390
```

Figure 4-6. Regions of amino acid sequences of cDNA with homology to proteins of interest.
A. Alignment of the predicted amino acid sequences of clone 1 and 30 with a region of the *Homo sapiens* poly(ADP-ribose) polymerase pseudogene and with a region of *Gorilla gorilla* ADP-ribosyltransferase (NAD+) pseudogene.
B. Alignment of the predicted amino acid sequence of clone 1 and 30 with a region of the UDP-glucuronosyltransferase from *Rattus norvegicus*. ¦ represents identical amino acids and : represents similar amino acids.

The ORFs of clones 1 and 30 were also cloned into baculovirus. Infected Sf9 cells produced abundant amounts of the antigenic protein of the expected size. However, the product was again tightly associated with the pellet fraction and denaturing agents (SDS or strong alkaline) were required to bring the antigenic proteins into solution. Proteins solubilized after such treatments did not exhibit enzyme activity.

In Vitro Transcription/Translation

As zeatin O-xylosyltransferase isolated *in vivo* is a soluble protein of 50 kD, the possibility of processing of the primary product was examined. A combined *in vitro* transcription/translation system (TNT system, Promega) with pSport clones 1 and 30 as templates was used. The ^{35}S methionine-labelled product of the reaction was detected by autoradiography (Figure 4-7). A soluble 70 kD protein was formed *in vitro* (Lane 1). However, when the reaction occurred in the presence of canine microsomes, the labelled product became insoluble and was pelleted with the microsomal fraction (Lane 3).

The soluble 70 kD protein produced *in vitro* was processed to a protein of approximately 50 kD when endosperm extracts of beans were added (Lane 2). This reaction was inhibited by PMSF, a serine protease inhibitor (data not shown). Moreover, the endosperm extracts failed to process the insoluble protein produced in the presence of microsomes (Lane 4). These results suggest that, *in vivo*, the primary products of the cDNA are most likely associated with membranes or at least with the

insoluble fraction, and that plant- or tissue- specific processing factors, perhaps protease(s), may be required to generate an active enzyme.

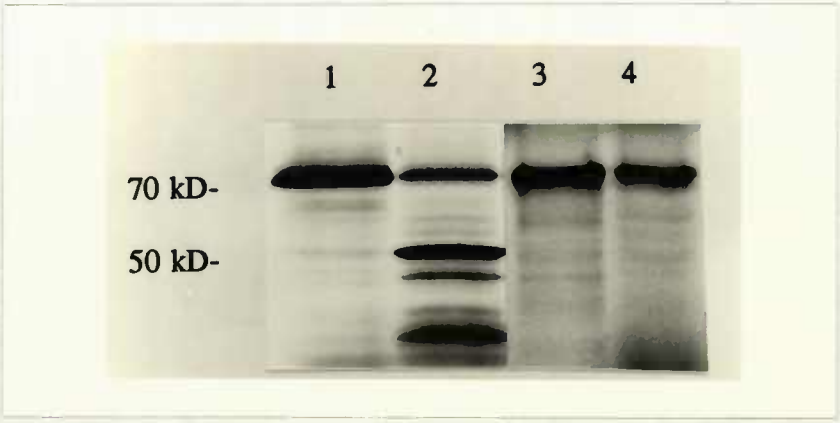

Figure 4-7. Autoradiograph of ^{35}S-labelled proteins obtained from in vitro transcription/translation reactions. Lane 1: Clone 30 TNT product. Lane 2: Clone 30 TNT product + endosperm (processing). Lane 3: Clone 30 TNT product with microsomes added, pellet fraction. Lane 4: Clone 30 TNT produced in the presence of microsomes + endosperm (no processing).

Transgenic Plants

As regeneration and transformation are not yet feasible in *Phaseolus*, tobacco plants were used in transgenic experiments. As clones 1 and 30 were 90% identical at the amino acid level, clone 30 was chosen for transgenic experiments. Tobacco W38 leaf discs were transformed with pPEV vectors with or without the ORF of clone 30. Numerous kanamycin resistant plantlets were recovered. Three primary transformants were selected for detailed analyses and selfed progeny from these plants were obtained.

Western analysis revealed a single antigenic protein in transgenic plants while no antigenic protein was detected in the plants transformed with pPEV without an insert. The deduced protein size of the ORF is 69 kD but the antigenic protein migrated near the 80 kD marker. The discrepancy in the expected and actual mobility may be due the vagaries of SDS-PAGE or plant-specific modifications. The protein occurs in the insoluble fraction. Of the various detergent and solvents tested, the nonionic detergent, octylthioglucoside, was able to solubilize the antigenic protein (Figure 4-8).

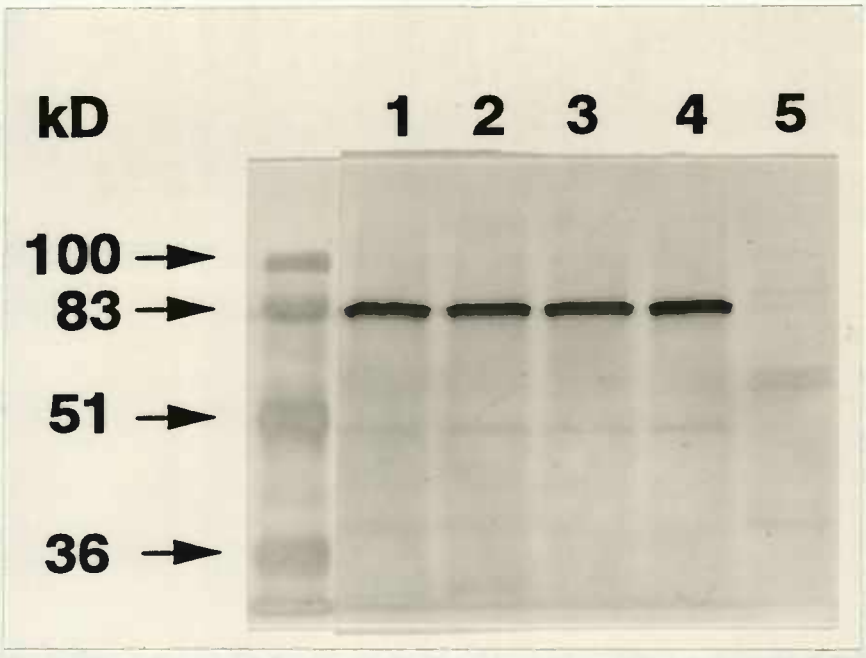

Figure 4-8. Immunoblots of proteins obtained from leaves of transgenic tobacco solubilized with octylthioglucoside. Lanes 1-4: Transgenic plants with insert 30. Lane 5: No-insert control plant.

S_1 seedlings from the primary transformants were obtained. The effect of transgenes on growth and development, especially their responses to exogenous growth regulators were examined. Sensitivity to auxin was one of the most dramatic properties of transgenic plants containing the ORF of clone 30. On medium containing NAA transgenic plants displayed damage at much lower concentrations in comparison with no insert controls. The symptoms included the loss of chlorophyll, inhibition of root elongation and general reduction in growth (Figure 4-9). Control plants were much more tolerant to low and moderate levels (up to 8 μM) of auxin (Figure 4-10). Only extremely high concentrations (such as 16 μM) of auxin induced death of both transgenic and control plants (Figure 4-11). On medium without hormones, both transgenic and control plants were normal (Figure 4-12). The auxin response was observed in three families of seedlings derived from independent primary transformants.

Figure 4-9. No insert control plant (left) and transgenic with ORF of clone 30 (right) grown on media with 2 μM NAA for 30 days.

Figure 4-10. No insert control plant (left) and transgenic with ORF of clone 30 grown on 8 μM NAA for 5 weeks.

Figure 4-11. No insert control plants (left) and transgenics with ORF 30 grown on 16 μM NAA for 5 weeks.

Figure 4-12. No insert control plants (left) and transgenics with ORF 30 grown on media without hormones for 5 weeks.

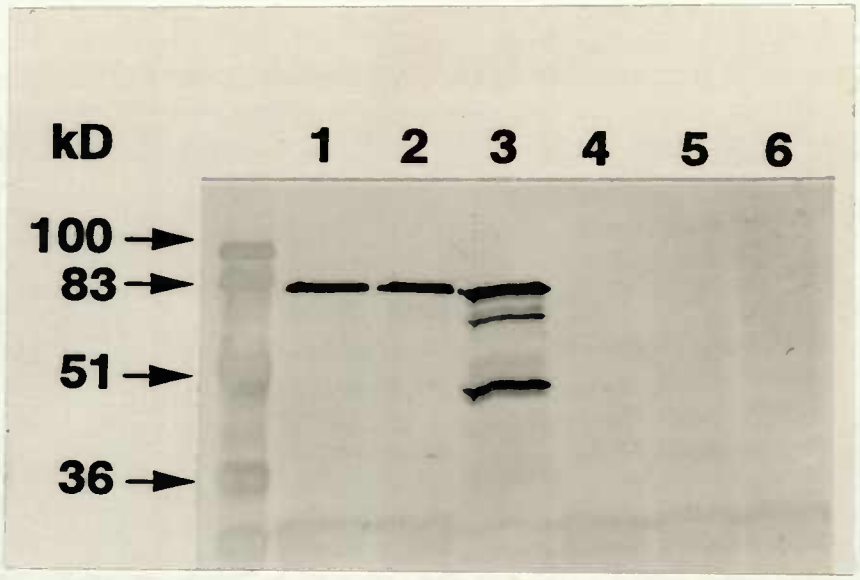

Figure 4-13. Immunoblots of proteins obtained from leaves of transgenic tobacco grown on hormones. Transgenics with ORF 30 grown on media with no hormones (lane 1), 10 μM Kinetin (lane 2), or 8 μM NAA (lane 3). No insert control plants grown on media with no hormones (lane 4), 10 μM kinetin (lane 5), or 8 μM NAA (lane 6).

More interestingly, western analysis of proteins from auxin-grown transgenic plants revealed that the preprotein was processed into smaller proteins (Figure 4-13, lane 3). Proteins from plants grown on media without NAA (lane 1) or on media with kinetin (lane 2) were not processed. Some of the the processed products had the same mobility as the native zeatin O-xylosyltransferase. As expected, no-insert control plants did not reveal any antigenic proteins (lanes 4-6).

The processing occurred in all seedlings after approximately two weeks on auxin medium (Figure 4-14).

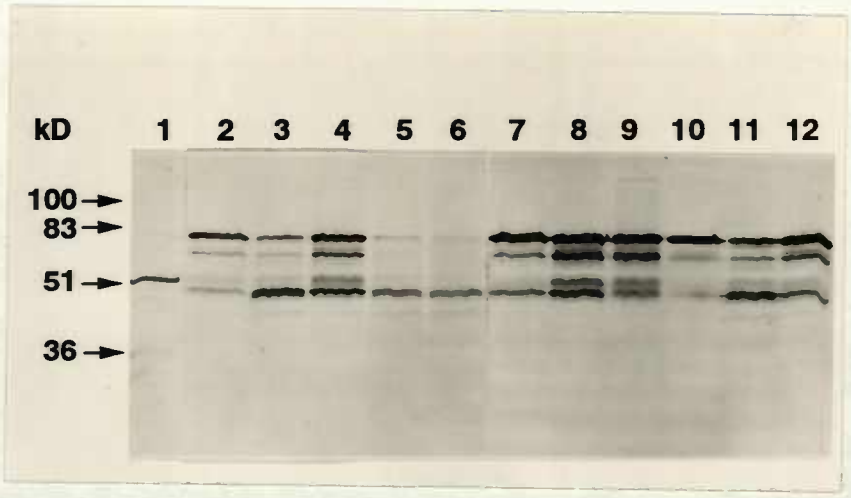

Figure 4-14. Immunoblots of proteins obtained from leaves of S_1 individuals grown on media containing 16 μM NAA for 2 weeks (lane 2-12). Lane 1: Native enzyme.

The accumulation of smaller antigenic protein was highest in plants after prolonged exposure to auxin. The gradual appearance of the smaller proteins seemed to correlate with the onset of auxin-induced damage. As the size of the smaller antigenic proteins was variable, it is reasoned that the processing factor(s) in tobacco leaves induced by auxin treatment are likely to be different from those present in bean endosperm. The effects of auxin on processing of the transgene product also appears to be gradual, since processing takes place only after at least two weeks on auxin medium.

The presence of zeatin O-xylosyltransferase activity in transgenic tobacco was assayed by incubating leaves with labeled zeatin. The conversion of zeatin to O-xylosylzeatin increased with increasing concentrations of NAA and with longer incubation periods. It appears that a certain threshold of NAA is essential for the

formation of active enzyme (See Table 4-1). Although the levels of O-xylosylzeatin were higher in transgenic plants than in no-insert control plants, the level of activity was much lower than native enzymes isolated from bean seeds.

Table 4-1. Percent OXZ formed after incubating leaves with ^{14}C-Zeatin.

	OXZ	Total cpm		OXZ	Total cpm
A-1. No-insert	0%	65K	B-1. No-insert	3%	59K
A-2. Transgenic	7%	69K	B-2. Transgenic	12%	51K
C-1. No-insert	0%	30K			
C-2. Transgenic	34%	62K			

A: Seedlings were grown on solid medium containing 2 μM NAA for two weeks. 1.5 g (fresh weight) of tissue was incubated with ^{14}C-zeatin.

B: Seedlings were grown on solid medium containing 8 μM NAA for three days and 0.5 g of leaf samples were taken and incubated with ^{14}C-zeatin.

C: Seedlings were grown on solid medium containing 10 μM NAA for three weeks and 0.5 g of leaf samples were taken and incubated with ^{14}C-zeatin.

Discussion

Two putative full-length cDNAs of zeatin O-xylosyltransferase were isolated based on the antigenicity of the gene product in prokaryotic systems and in transgenic plants. Proteins encoded by the cDNAs appear to be associated with membranes and may be strongly hydrophobic. The association of the protein with insoluble fractions is in agreement with previous immunolocalization studies (see chapter 2) detecting the protein in nucleus. Also, when canine microsomes were added to *in vitro* transcription/translation mixtures, the protein product was retained with the pellet after

centrifugation (see results, Figure 4-7 lane 3). Finally, the primary transgene product was detected in the pellet fractions of tobacco.

The formation of an active enzyme, the zeatin O-xylosyltransferase, as the result of tissue- (endosperm-) specific processing in *Phaseolus* is supported by the observation that bean endosperm was able to process the radio-labeled primary product of the cDNA *in vitro* (see figure 4-7 lane 2). The processing of the transgene product in transgenic tobacco lends additional support. It is possible that in beans, specific proteolytic enzymes occur in the endosperm giving rise to an active enzyme, while the processing induced by growing transgenic tobacco on auxin is not identical.

Examples of complex and unique post translational modification of plant proteins that are stage-specific have been observed, such as the processing of Concanavalin A (13). Another example of precise proteolytic processing of membrane-bound proteins in response to hormones is the case of the sterol regulatory binding protein I (SRBP-1) (14). This protein is synthesized as a 125 kD protein and is attached to the nuclear envelope and the endoplasmic reticulum. In the absence of sterol, the 125 kD protein is processed to a 69 kD protein (by a specific serine protease) which is translocated to the nucleus where it induces transcription of a low density liproprotein receptor (LDLR) and the enzyme 3-hydroxyl-3-methyl-glutaryl CoA (HMGCoA). When sterol is abundant, processing is inhibited resulting in reduced gene transcription. The involvement of hormone perception and processing and translocation of proteins are interesting features similar to the properties of the product of clone 30.

It is apparent that the greater sensitivity to auxin is caused by the over expression of the transgene since transgenic plants derived from three independent transformation events have similar response. The onset of auxin induced damage coincides with the occurrence of smaller, antigenic proteins. It is not yet certain that there is a cause-effect relationship between protein processing and auxin induced damage. It is clear, however, that protein processing is not associated with senescence *per se*, as naturally senescent (yellow) leaves of trangenic plants do not contain processed proteins. The mechanism(s) leading to lower auxin tolerance is also unknown but there are several possible explanations. The obvious one involves auxin stimulation of the synthesis of proteolytic enzymes required to process the preprotein. The processed products slowly conjugate cytokinins, albeit at a gradual rate, resulting in the development of symptoms. Less likely, but possible is that the transgene product may have auxin receptor-like properties, inducing toxic symptoms at auxin levels usually harmless to normal plants. There is, however no evidence suggesting such a possibility. In any event, the transgenic plants will be valuable in studying the classical cytokinin-auxin interaction or in analyzing auxin signal transduction.

The interesting properties of the O-xylosyltransferase discovered also brought on unexpected complexities in studying its regulation. There are at least two controls, transcriptional and post-translational processing. The transcription appears to be tissue specific in beans. Constitutive expression observed in transgenic tobacco resulted in auxin sensitivity. The post-translational control remains unexplored and will have to await the isolation of processing factors from bean endosperm.

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CHAPTER 5

SUMMARY

The studies described in this thesis support the idea that cytokinin metabolic enzymes are useful in dissecting the regulatory steps involved in cytokinin metabolism. The work was centered on one of the four enzymes, zeatin O-xylosyltransferase, isolated from *Phaseolus*. The findings indicate that activity of the enzyme is regulated by at least two controls, transcription and/or translation and processing. The enzyme is derived from a larger preprotein which requires precise processing, which occurs in endosperm of beans. The subcellular location(s) of the pre- and processed-protein may be different. Overproduction of the preprotein in transgenic tobacco leads to auxin sensitivity which coincides with processing of the preprotein. Auxin-stimulated processing factor(s) in tobacco is not identical to those in beans. The unusual and potentially important observations are the possible alternative location (and function) of proteins before and after processing and the induction of processing in response to external signals. Of greater relevance to plant hormone physiology, the results suggest that cytokinin-auxin balance may be affected by auxin stimulation of cytokinin metabolic enzymes.

A similar approach can be utilized to study the other zeatin metabolic enzymes. Comparing the regulatory steps and the gene sequences of several such enzymes may generate sufficient information to formulate useful models regarding active cytokinin levels and interactions with other hormones.

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