AN ABSTRACT OF THE THESIS OF

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Title: Partial Purification and Characterization of a Zeatin *Cis-Trans* Isomerase from *Phaseolus vulgaris* L.

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Conversion of exogenous *cis*-zeatin to *trans*-zeatin in immature seeds of *Phaseolus vulgaris* L. led to the isolation of a *cis-trans* isomerase from the endosperm. The enzyme was purified over 2000-fold by chromatography on a series of FPLC (anion exchange, gel filtration, and hydrophobic interaction) and Concanavalin A columns. Non-enzymatic isomerization occurred under standard assay conditions but the presence of the enzyme enhanced conversion significantly. The enzymatic reaction favors conversion from the *cis* to the *trans* form and requires flavin, blue light, and DTT. Optimal conditions for isomerization were identified using a Phenyl Superose-purified enzyme extract. Concentrations of 0.1-0.316 mM FAD and 0.5 mM DTT resulted in high conversion. The pH optimum for the reaction was 7.5. Although the isomerase was heat stable, a temperature of 35°C was chosen for the assay reaction in order to minimize non-enzymatic thermal isomerization. Retention on the Concanavalin A column as well as shifting in mobility of all visible protein bands on SDS-PAGE following N-glycosidase-F treatment indicated that the enzyme is a glycoprotein. The enzyme was stable for at least 8 weeks when stored at -80°C. The isomerase is capable of converting *cis*-zeatin riboside and *trans*-zeatin riboside. Changing the isoprenoid side

chain location from N^6 in zeatin to the 9-position of the adenine ring in 9-(4-hydroxy-3-methylbut-2-enyl)adenine also resulted in isomerization. The occurrence of *cis-trans* isomerization suggests that *cis*-zeatin and *cis*-zeatin riboside formed by tRNA degradation could be precursors of biologically active cytokinins.

Partial Purification and Characterization of a Zeatin *Cis-Trans* Isomerase from *Phaseolus vulgaris* L.

By

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This thesis is dedicated

to my husband,

to my son

and

to my parents

with all my love

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ABBREVIATIONS

AMP	adenosine monophosphate
Δ^2iPP	Δ^2-isopentenyl pyrophosphate
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
DTT	dithiothreitol
DPU	N-N'-diphenylurea
b^{6}Ade	N^6-benzyladenine
i^{6}Ade	N^6-(Δ^2-isopentenyl)adenine
i^{6}Ado	N^6-(Δ^2-isopentenyl)adenosine
i^{6}AMP	N^6-(Δ^2-isopentenyl)adenosine monophosphate
trans-io^{6}Ade	*trans*-zeatin, zeatin
cis-io^{6}Ade	*cis*-zeatin
io^{6}Ade	zeatin, *trans*-zeatin
io^{6}Ado	*trans*-zeatin riboside, zeatin riboside
io^{6}AMP	zeatin riboside monophosphate
UDPX	uridine diphosphate xylose
UDPG	uridine diphosphate glucose
BSTFA	N,O,-bis(trimethylsilyl)trifluoroacetamide
Pi	orthophosphate
PPi	pyrophosphate
FPLC	fast protein liquid chromatography

ABBREVIATIONS, Continued

HPLC	high performance liquid chromatography
TEA	triethylamine
TLC	thin layer chromatography
GC/MS	gas chromatography/mass spectrometry

Partial Purification and Characterization of a Zeatin *Cis-Trans* Isomerase from *Phaseolus vulgaris* L.

INTRODUCTION

Cytokinins are a group of plant growth substances which were first described as cell division factors (Skoog and Miller, 1957). They have since been implicated in various aspects of plant growth and development. These include seed germination, release of buds from apical dominance, *de novo* bud formation, leaf expansion, chloroplast development, flowering, delay of leaf senescence and seed formation (reviewed by Mok, 1994).

The naturally-occuring cytokinins are isoprenoid derivatives of adenine, of which *trans*-zeatin and derivatives are the most prevalent forms. *Trans*-zeatin is highly active in most cytokinin bioassays (Letham et al., 1983). *Cis*-zeatin and its riboside have also been found in plant tissues (Watanabe et al., 1978, 1981; Mauk and Langille, 1978; Dauphin et al., 1979; Yokota et al., 1981; Hashizumi et al., 1982; Murofushi et al., 1983; Takagi et al., 1985; and Parker et al., 1989), but they are insignificant components of the total cytokinins in plant tissues. Moreover, *cis*-zeatin is only very weakly active (Schmitz et al., 1972; Vreman et al., 1974; and Mok et al., 1978). However, in many plant species analyzed, *cis*-zeatin is the predominant modified base in t-RNA where it is located adjacent to the 3' end of the anticodon (Taller, 1994). The significance of this modified base is unknown.

Two pathways have been suggested for biosynthesis of free cytokinins in plants. The first one, *de novo* synthesis, consists of condensation of adenosine monophoshate (AMP) and Δ^2-isopentenyl pyrophosphate (Δ^2iPP) followed by hydroxylation of the side chain. The enzyme responsible for the condensation step was first isolated from *Dictyostelium* (Taya et al., 1978) and then it was partially purified from cytokinin autonomous tobacco callus cultures (Chen and Melitz, 1979; Chen, 1994). The hydroxylase enzyme has not been purified but hydroxylase activity has been reported in various plant tissues (Miura and Hall, 1973; Chen and Leisner, 1984; Einset 1984). The second pathway proposed for cytokinin biosynthesis involves breakdown of t-RNA (Skoog and Armstrong, 1970; Maaß and Klambt, 1979). A problem with this pathway is that plant t-RNAs contain mainly the weakly active *cis*-zeatin riboside, which exhibits at least 100-fold lower activity than its isomer, *trans*-zeatin, in the tobacco callus bioassay (Schmitz et al., 1972). Critical support for this pathway would require the identification of mechanisms capable of converting the biologically inactive *cis* isomers to their active *trans* counterparts.

In the course of studying cytokinin metabolism, we have discovered isomerase activity in immature seeds of bean (*Phaseolus vulgaris*) capable of interconverting *cis*- and *trans*-zeatin. The isomerization reaction favors *cis* to *trans* conversion. Both enzymatic as well as non-enzymatic isomerization occur but the enzymatic reaction by far exceeds the non-enzymatic one under most conditions. The reaction requires a flavin (FAD, FMN or riboflavin), a thiol (DTT) and blue light. This thesis describes the purification of the zeatin isomerase and the characterization of the enzymatic reaction.

LITERATURE REVIEW

Discovery of Cytokinins

It has long been known that plants contain chemical factors that can promote cell division (Wiesner, 1892; Haberlandt, 1913). The actual discovery of cytokinins occurred in 1955 by Skoog and Miller (reviewed by Skoog, 1994) when attempting to isolate the purine-containing compound responsible for stimulating cell division in tobacco pith tissue. However, the name cytokinin for plant cell division promoters was proposed by Skoog in 1965 as a replacement for the original term kinin, used in animal biochemistry (Skoog et al., 1965). The term cytokinin has since been adopted to describe compounds which stimulate cell division of an appropriate plant tissue grown on a well defined medium (Horgan, 1984).

The active ingredient causing cell division in tobacco pith tissue placed on basal medium was named kinetin even before its structure was elucidated. Purification of kinetin to homogeneity was achieved in 1955 from autoclaved herring sperm DNA (Miller et al., 1955, 1956). The structure was subsequently identified to be 6-(furfurylamino) purine by chemical synthesis. Though active, kinetin is an artefactual product of heated DNA and is not present in plant tissues.

An active kinetin-like factor was first isolated and partially characterized from maize endosperm by Miller in 1963 (Miller, 1963). This first naturally-occuring cytokinin, zeatin, was purified in crystalline form also from immature kernels of *Zea mays* in 1963 by Letham (Letham, 1963). The structure of zeatin was tentatively

proposed as 6-(4-hydroxy-3-methylbut-*trans*-2-enylamino)purine by Letham (Letham et al., 1964). Confirmation of this correct structure by chemical synthesis quickly followed (Shaw and Wilson, 1964). Zeatin as well as several new cytokinins were later isolated from different plant sources. Most naturally-occuring cytokinins were identified as N^6-substituted adenine derivatives.

N-N'-Diphenylurea (DPU) was first isolated from coconut milk for its cytokinin-like activity in stimulating cell division in carrot tissue cultures (Shantz and Steward, 1955). DPU however, exhibited mostly weak cytokinin activity in various bioassays. Furthermore, Letham (1968) reported that the high cytokinin activity associated with coconut milk resulted from the presence of the highly active adenine-type cytokinins zeatin and its ribonucleoside. Still, high biological activity has been attributed to many phenylurea-type compounds by Bruce and Zwar (Bruce et al., 1965; Bruce and Zwar, 1966). In addition, Mok et al., (1982) have demonstrated the ability of thidiazuron (N-phenyl-N'-1,2,3-thiadiazol-5-ylurea) and N-phenyl-N'-4-pyridylurea to substitute for the highly active zeatin in the *Phaseolus lunatus* callus culture bioassay. Therefore, cytokinin activity is generated by two types of compounds: N^6-substituted adenine derivatives and substituted phenylureas.

Sites of Cytokinin Biosynthesis

Cytokinins have been found in roots, stems, leaves, flowers, fruits and seeds. They are probably present in all living cells of intact higher plants. Relatively higher levels of cytokinins in a particular tissue do not necessarily indicate a site of

biosynthesis, and the tissue may only represent a storage or sink site. Determination of sites of cytokinin biosynthesis has been plagued by two major problems: the low levels of endogenous cytokinins in plant tissues and the crucial role of their precursors in cellular metabolism.

Biosynthesis in the Root and Shoot

The root has been established unequivocally as a site of cytokinin biosynthesis (Letham, 1994). For example, cytokinins were released into the culture medium during aseptic culture of rice root tips (Yoshida and Oritani, 1974), as well as maize and tomato roots (Van Staden and Smith, 1978). Also, marked increases in cytokinin production in the laminae followed adventitious root formation on petioles of excised bean leaves (Engelbrecht, 1972). Furthermore, formation of lateral root primordia in response to decapitation of pea roots resulted in a large increase in root cytokinin level (Forsyth and Van Staden, 1981). Moreover, a decrease in cytokinin levels was detected in xylem exudates of roots of many plants when subjected to unfavorable growth conditions such as high heat, low temperature, flooding, water deficiency and salinity (Skene, 1975; Letham, 1978). In addition, studies of the maintenance of cytokinin production by root systems of detopped plants indicate that the plant root is a cytokinin producing organ (Kende and Sitton, 1967; Wagner and Michael, 1971; Henson and Wareing, 1976). Finally, in seedlings and cuttings, exogenous cytokinins often substitute for roots in inducing or maintaining growth and/or physiological responses in the shoot (Goodwin et al., 1978).

Studies of cytokinin levels in the root indicate that synthesis occurs in the tip. A basipetally decreasing gradient of cytokinin was observed in an immunocytochemical study of cytokinins in the tip region of tomato roots (Sossountzov et al., 1988). Also, decapping of *Zea mays* roots led to a decrease in cytokinin level in the terminal millimeter of the decapped root (Feldman, 1975). Cytokinin synthesis seems to result from an interaction between the quiescent center and the proximal meristem. Removal of the root cap plus the quiescent center from *Zea mays* roots was reported to result in a considerable reduction in the cytokinin level of the tissue of the proximal meristem (Feldman 1979a). Furthermore, when a capless root tip section containing both the proximal meristem and the quiescent center were cultured, a new root evolved in the absence of exogenous cytokinin. However, when either the proximal meristem or the quiescent center were cultured separately, cytokinin had to be added for root regeneration to occur (Feldman, 1979b).

Intact shoot tissues appear to be largely dependent on root-produced cytokinin for development and retardation of leaf senescence. Many studies indicate that excision of roots results in a decline of cytokinin levels in the shoot (Henson and Wareing, 1977; Carmi and Van Staden, 1983; Chailakhyan and Khrianin, 1987). Such derooting significantly suppresses several aspects of shoot development, which can be offset by the application of exogenous cytokinin (Letham, 1994).

The vegetative organs of the shoot including the cambial tissue and the shoot apex in addition to young leaves are probable sites of biosynthesis (Letham, 1993). Cambial extracts of poplar exhibit strong cytokinin activity (Nitsch and Nitsch, 1965) and cambium can be cultured in cytokinin-free medium (Gautheret, 1959). When

cultured *in vitro*, cambial tissue of *Salix babylonica* bark exhibited rapid cell division and a marked increase in cytokinin level (Van Staden and Choveaux, 1980). The meristematic cells of the apex and young leaf primordia of tomato and maize contain high cytokinin levels relative to the adjacent tissues (Sossountzov et al., 1988; Hansen et al., 1984). When asparagus shoot apices were cultured, they diffused constant amounts of cytokinins into the agar medium during five subcultures while maintaining the same cytokinin content throughout the culture period (Koda and Okazawa, 1980). Evidence of cytokinin production by young leaves comes from cytokinin analysis of root xylem exudates and leaf exudates (phloem sap) from *Perilla* (Grayling and Hanke, 1992). While root exudates contained a relatively large amount of zeatin-type cytokinin, leaf exudates, however, exhibited predominantly isopentenyl-type cytokinins. Assuming the *de novo* pathway of cytokinin biosynthesis is valid and zeatin-type cytokinins are derived from isopentenyl-type cytokinins, Letham (1994) concludes that cytokinin synthesis must have occurred in the *Perilla* leaves. Furthermore, incorporation of ^{14}C-adenine into cytokinins in pea leaves (Chen et al., 1985) and in young developing tobacco leaves (Singh et al., 1992) provides more evidence that leaves can synthesize cytokinins.

Biosynthesis in the seed

The high cytokinin activities in developing seeds of apple (Letham and Williams, 1969) and avocado plants (Blumenfeld and Gazit, 1970; Gazit and Blumenfeld, 1970) indicate that the developing seed is a site of active cytokinin biosynthesis. The

cytokinin activities found in the above developing seeds are considerably greater than those detected in the receptacles and mesocarp respectively. Further evidence of cytokinin synthesis by the developing seed comes from the ability to culture avocado cotyledon tissue (Blumenfeld and Gazit, 1971) and very immature soybean seeds and intact pods (Obendorf et al. 1983; Hsu and Obendorf, 1982) on hormone-free media. Blumenfeld and Gazit (1971) found that cotyledon tissue derived from avocado seeds can be cultured *in vitro* without exogenous cytokinin and that in fact it produces cytokinins. When immature soybean seeds and pods were cultured on hormone-free media, they produced normal mature seeds and pods respectively (Obendorf et al., 1983; Hsu and Obendorf, 1982). Genetically parthenocarpic fruits contain less cytokinin when compared to seeded fruits, thus indicating that seeds produce cytokinins (Mapeli, 1981). Recently, following incubation of developing seeds and pod walls of pea fruit with ^{14}C-adenine for 6 h., Van Staden and Drewes (1993) recovered a compound that co-chromatographed with authentic zeatin after semi-preparative HPLC and TLC. When treated with $KMnO_4$, 98 % of the radioactivity shifted from zeatin's retention time, indicating the presence of an unsaturated side chain.

Germinating seed appears to possess the ability to synthesize cytokinins based on studies of cytokinin levels in cotyledons cultured with the axis attached or detached (Ilan and Gepstein, 1981). Axis excision resulted in a considerable reduction of cytokinin level in cotyledons of beans (Van Onckelen et al., 1977; Hutton et al., 1982), chickpeas (Munoz et al., 1990), and yellow lupin (Nandi et al., 1988). Also exogenous cytokinins can replace the embryonic axis of several species of dicot seeds including bean (Gepstein and Ilan, 1979) and yellow lupin (Nandi, 1988) in inducing amylolytic

and proteolytic enzyme activities in the cotyledons (Ilan and Gepstein, 1981). Exogenous N^6-benzyladenine (b^6Ade) or dihydrozeatin substituted the axis in inducing cotyledon expansion and chlorophyll formation in yellow lupin cotyledons (Nandi et al., 1988). Further evidence that the embryonic axis may act as a site of biosynthesis is provided by studies of ^{14}C-adenine incorporation into cytokinins in germinating seeds of yellow lupin (Nandi et al., 1988) and maize (Hocart and Letham, 1990; Van Staden and Drewes, 1994). Following incubation with ^{14}C-adenine, Nandi et al. (1988) reported the incorporation of ^{14}C into dihydrozeatin riboside and its nucleotide in intact cotyledons, intact embryos as well as excised embryos of yellow lupin. The identity of the products was established using HPLC, TLC and chemical derivatization. Different compounds were recovered after incubating germinating maize seeds with ^{14}C-adenine. Hocart and Letham (1990) found incorporation into *trans*-zeatin nucleotide while Van Staden and Drewes (1994) reported ^{14}C-labelling of *trans*-zeatin. Hence, germinating seeds are capable of cytokinin synthesis but the discrepancies uncovered in feeding studies must be resolved in order to elucidate the various steps in cytokinin biosynthesis.

Cytokinin Biosynthesis

In plants, cytokinins occur as free compounds or as components of t-RNA species corresponding to codons with the initial letter U (i.e. isoacceptors for cysteine, leucine, phenylalanine, serine, tryptophan and tyrosine) (Armstrong et al., 1969). When present, cytokinins occur only once per t-RNA molecule where they are located

adjacent to the 3' end of the anticodon, at position 37. The *trans* isomers of zeatin and its derivatives are the predominant form of free cytokinins in plants. The *cis* isomers of zeatin and its riboside have been isolated from hydrolysates of t-RNA in plants and microorganisms (Hall, 1973; Watanabe et al., 1982; Letham and Palni, 1983). However, several studies report the occurrence of the *cis* isomers as free cytokinins. *Cis*-zeatin riboside has been found in cones of the hop plant (Watanabe et al., 1978, 1981), in above-ground and below-ground tissues of *Solanum tuberosum* L. (Mauk and Langille, 1978), in shoot apices of monoecious *Mercurialis ambigua* (Dauphin et al., 1979), in immature seeds of *Dolichos lablab* (Yokota et al., 1981) and in tubers of sweet potato (Hashizumi et al., 1982). Both *cis*-zeatin and its riboside were reported in shoots, roots and ears of rice (Takagi et al., 1985) as well as in root exudates of rice plants (Murofushi et al., 1983) and in xylem sap samples of oat and wheat (Parker et al., 1989).

Biosynthesis of Free Cytokinins (Fig. 1)

A. *De Novo* (Direct) Pathway

The direct pathway of cytokinin biosynthesis, *de novo* biosynthesis from AMP and and Δ^2iPP, was first demonstrated using a cell-free preparation from the slime mold *Dictyostelium discoideum*. A crude enzyme preparation which converted AMP and Δ^2iPP to N^6-(Δ^2-isopentenyl)adenosine monophosphate (i^6AMP) was isolated from *Dictyostelium* (Taya and Nishimura, 1978) (Fig. 2). Purification of the enzyme to near

Figure 1. Pathways of biosynthesis of free cytokinins in plants. Abbreviations: AMP, adenosine monophosphate; Δ^2iPP, Δ^2-isopentenyl pyrophosphate; PPi, pyrophosphate; Pi, orthophosphate; i^{6}AMP, N^6-(Δ^2-isopentenyl)adenosine monophosphate; io^{6}AMP, zeatin riboside monophosphate; i^{6}Ado, N^6-(Δ^2-isopentenyl)adenosine; io^{6}Ado, zeatin riboside; i^{6}Ade, N^6-(Δ^2-isopentenyl)adenine; io^{6}Ade, zeatin.

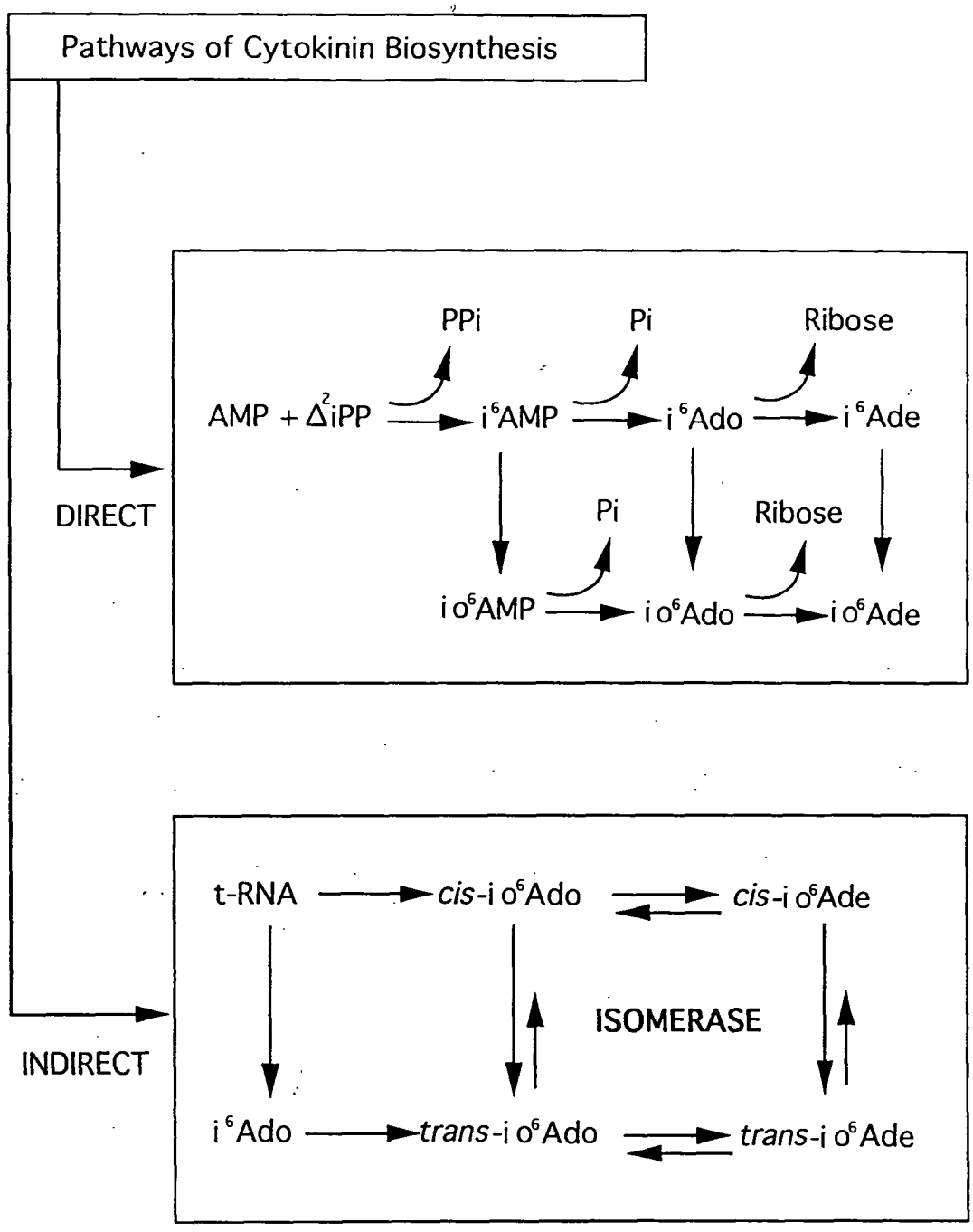
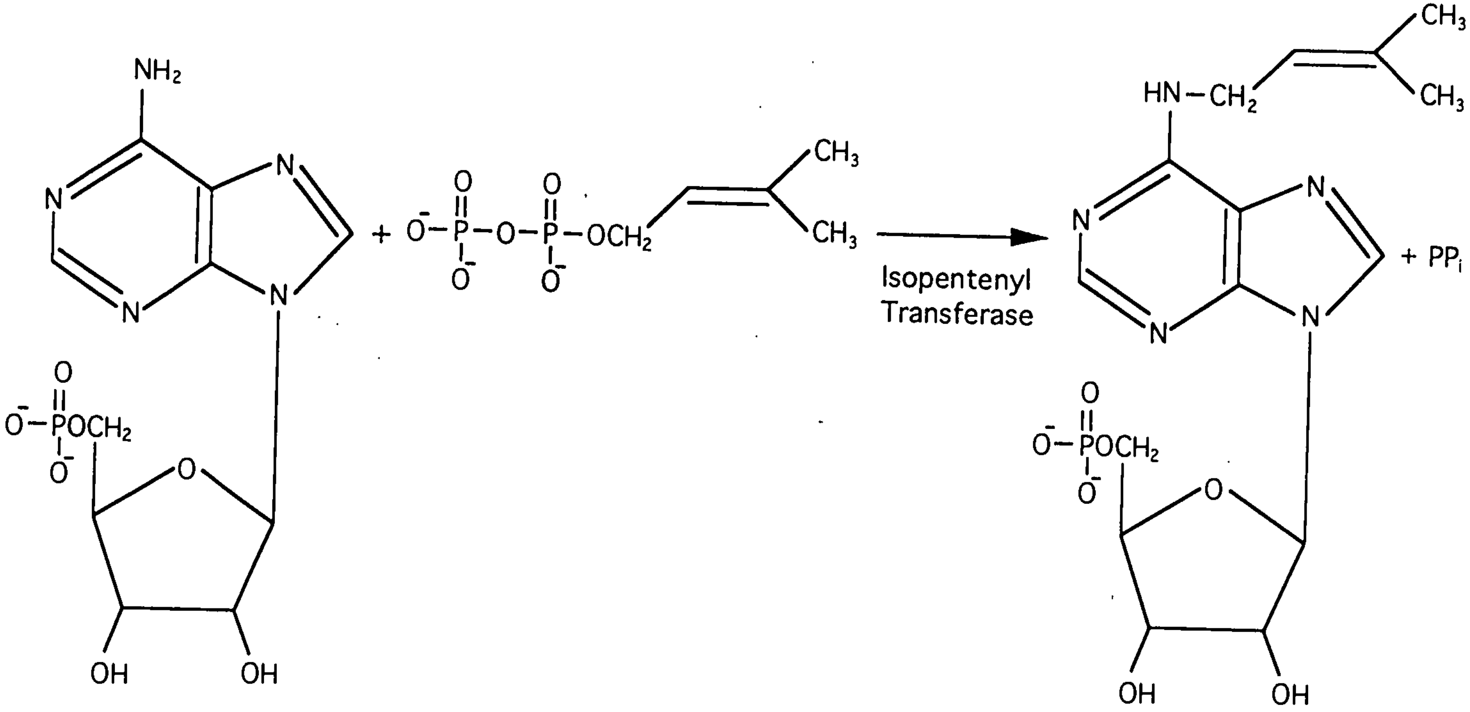

Pathways of Cytokinin Biosynthesis
DIRECT
PPi
Pi
Ribose
AMP + Δ²iPP
i⁶AMP
i⁶Ado
i⁶Ade
Pi
Ribose
i o⁶AMP
i o⁶Ado
i o⁶Ade
INDIRECT
t-RNA
cis-i o⁶Ado
cis-i o⁶Ade
ISOMERASE
i⁶Ado
trans-i o⁶Ado
trans-i o⁶Ade

Figure 1.

Figure 2. Isopentenyl transferase-catalyzed condensation of AMP and Δ^2iPP to i^{6}AMP. Abbreviations: AMP, adenosine monophosphate; Δ^2iPP, Δ^2-isopentenyl pyrophosphate; PPi, pyrophosphate; i^{6}AMP, N^6-(Δ^2-isopentenyl)adenosine monophosphate.



AMP Δ^2i PP i^{6}AMP

Figure 2.

homogeneity has not been achieved due to its reported instability. However, the slime mold Δ^2iPP transferase was later purified 6687-fold using ammonium sulfate precipitation and various chromatography procedures including ion exchange, gel filtration, 5'AMP^{ox-red}-Sepharose 4B affinity and DEAE cellulose. Based on gel filtration chromatography, the molecular weight of the enzyme was determined to be 40 kD (Ihara et al., 1984). An AMP-Δ^2iPP transferase has been purified about 50-fold from cytokinin-autonomous tobacco callus (Chen and Melitz, 1979). The tobacco enzyme has a molecular weight of 52 $\pm$ 2 kD and utilized AMP (but not adenine and adenosine) and Δ^2iPP as substrates. The isopentenyl transferase was also found in tobacco strain XD6S cells (Nishinaru and Syono, 1980) and developing corn kernels (Reinecke et al., 1991).

An initial study on *Vinca rosa* crown-gall tumor tissue demonstrated the incorporation of labeled adenine into zeatin and zeatin riboside (Peterson and Miller, 1976). The isopentenyl transferase gene (*ipt* or *tmr*), located on the T-region of the Ti plasmid of *Agrobacterium tumefaciens*, has been cloned and expressed in *E. coli* (Barry et al., 1984). It encodes a protein product of 27 kD. An antibody raised against a synthetic decapeptide derived from the *tmr* coding sequence, crossreacted with both the enzyme contained in crown gall tumors and the protein resulting from *ipt* gene expression in *E. coli* (Buchmann et al., 1985). Nopaline strains of *Agrobacterium* contain another gene, *tzs* (*trans*-zeatin secreting), which also encodes an isopentenyl transferase (Beaty et al., 1986). The nucleotide sequence of *tzs* was found to be extensively homologous to that of *tmr*. However, *tzs* mapped outside the T-DNA

region of the Ti plasmid but close to the *vir* region. Cytokinin production in *Pseudomonas savastanoi* was caused by a gene, *ptz* (Powel and Morris, 1986). This *ptz* has also been cloned and expressed a 26.6 kD protein in *E. coli*. Overall there is 48% identity between *tzs* and *ptz*, while *tmr* and *ptz* contain perfect matches at 44% of the amino acid positions. The extent of homology is highest within the N-terminal region of the genes.

Interestingly, no homology to the *ipt* gene sequence could be detected in plants (Morris, 1987; Smigochi and Owens, 1988). Also, antibody to a decapeptide of the *ipt* gene sequence did not recognize any protein in non-transformed tissues (Buchmann et al., 1985). Therefore, the plant and the bacterial *ipt* gene could have different gene and protein sequences but still share the same function. Such similarity in function might result from similarities in binding sites or protein conformation.

The second step of *de novo* synthesis of zeatin, *trans*-hydroxylation of the isopentenyl side chain, has been demonstrated in *Rhizopogon rosealus* (Miura and Miller, 1969; Miura and Hall, 1973), *Zea mays* endosperm (Miura and Hall, 1973), *Vinca rosa* crown-gall tissues (Palni et al., 1983), *Actinidia* (Einset, 1984) and cauliflower microsomes (Chen and Leisner, 1984). However, the enzyme mediating this hydroxylation has not been isolated.

B. t-RNA Hydrolysis (Indirect Pathway)

Since t-RNA contains cytokinins, its hydrolysis could lead to the release of free cytokinins in plant tissues. The cytokinin nucleosides present in plant t-RNA are N^6-

(Δ^2-isopentenyl)adenosine (i^6Ado), *cis*-zeatin riboside, *trans*-zeatin riboside, and the 2-methylthio derivatives of these nucleosides. Although the exact composition varies with tissue source, the weakly active *cis*-zeatin riboside is by far the most abundant constituent in plant tissues, whereas generally only traces of the *trans* isomer are found. For example, the *cis/trans* ratio is 40 in *Pisum sativum* (Vreman et al., 1974) and 50 in *Spinacea oleracea* (Vreman et al., 1978). This predominance of the weak cytokinin *cis*-zeatin riboside in t-RNAs led to the conclusion that an indirect pathway would not lead to high cytokinin activity (Letham and Palni, 1983). Further evidence against this pathway include: first, the t-RNA of cytokinin requiring tissues contains cytokinins (Chen and Hall, 1969) and second, the t-RNA turnover in many plant tissues is low (Hall, 1973). In addition, the level of incorporation of ^{14}C-adenine into free cytokinins was judged too high to allow for a contribution by t-RNA turnover (Stuchbury et al., 1979). On the other hand, evidence of selective turnover of t-RNA subpopulations rich in cytokinins was presented in animal tumor tissues (Borek et al., 1977). Furthermore, Maaß and Klambt (1979) favored the indirect pathway based on a combination of experimental data and mathematical models. The kinetics of t-RNA and oligonucleotide degradation was determined in *Phaseolus vulgaris* var. Saxa roots by pulse labeling with ^{14}C-adenine for 6 h followed by a chase with 1000-fold concentration of ^{12}C-adenine for another 6 h. The turnover rate of cytokinins was also studied in leaves after feeding a single leaf with ^{3}H-N^6-Δ^2-isopentenyl adenine and ^{14}C-zeatin for 3 h and following their recovery and activity in the leaf lamina. A mathematical formula was subsequently used to determine the half-lives of t-RNA, oligonucleotides and cytokinins. After comparing the experimental data to the theoretical models, Maaß and Klambt

concluded that the amount of cytokinins and the half-life of the oligonucleotide fraction was probably underestimated. Therefore, 40-50% of cytokinin biosynthesis in *Phaseolus* was considered to depend on t-RNA breakdown while the other 50-60% could come from mRNA and oligonucleotide breakdown. Also in potato tissue, 40% of cytokinin biosynthesis was reported to result from the breakdown of t-RNA (Barnes et al., 1980). Still, the predominance of the weakly active *cis*-zeatin riboside in t-RNA presents a major argument against the indirect pathway for cytokinin biosynthesis. Hence, the presence of a *cis-trans* isomerase system which can convert the t-RNA derived *cis*-zeatin riboside into its *trans* form, would make the t-RNA hydrolysis pathway more plausible.

Biosynthesis of Cytokinins in t-RNA

The biosynthesis of t-RNA cytokinins seems to occur at the polymer level during post-transcriptional processing (Hall, 1973). Incorporation of ^{14}C-mevalonic acid into i^6Ado moieties in t-RNA has been demonstrated in tobacco callus tissue (Chen and Hall, 1969). The isopentenyl side chain is derived from mevalonic acid pyrophosphate (Chen and Hall, 1969) which undergoes decarboxylation, dehydration and isomerization to give Δ^2iPP. The latter then condenses with the adenosine residue in the t-RNA to give the i^6Ado moiety. The enzyme responsible for this condensation reaction, a Δ^2-iPP:t-RNA-Δ^2-isopentenyl transferase, has been partially purified from *E. coli* (Bartz and Soll, 1977). It utilizes t-RNA and Δ^2iPP as substrates and enzyme activity is enhanced by the presence of Mg^{2+} and mercaptoethanol. The molecular weight of this *E. coli*

isopentenyl transferase was estimated to be 55 kD. Holtz and Klambt (1978) have purified another isopentenyl transferase from *Zea mays* which was able to isopentenylate t-RNA, polyadenylic acids and adenosine. The isopentenyl transferase has also been partially purified from *Lactobacillus* (Holtz and Klambt, 1975) and yeast (Kline et al., 1969) and was shown to occur in animal tissues (Fittler et al., 1968). Both the prokaryotic (*E. coli*, *Salmonella typhimurium* and *A. tumefaciens*) isopentenyl transferase gene, *miaA* (*trpX*) (Caillet and Droogmans, 1988; Dihanich et al., 1987), and the eukaryotic (yeast) *mod5* gene have been cloned and sequenced (Connolly and Winkler, 1991; Najarian et al., 1987). They are localized in the cytoplasm (Gillman et al., 1991). In *E. coli*, *miaA* is part of a complex operon (Connolly and Winkler, 1989). In yeast however, *mod5* is a nuclear gene that has a minimum of two translation initiation sites and codes for at least two proteins (Najarian et al., 1987; Gillman et al., 1991). *Mod5* is responsible for the isopentenylation of both cytoplasmic and mitochondrial t-RNA (Dihanich et al., 1987). When prokaryotic and yeast t-RNA:isopentenyl transferase gene and protein sequences were compared, significant homology was found at both levels and four highly conserved regions were discovered in these proteins (Connolly and Winkler, 1991; Gray et al., 1992). Some homology was also found between *A. tumefaciens* t-RNA isopentenyl transferase and *tzs* and *ipt* genes and proteins responsible for biosynthesis of free cytokinins (Gray et al., 1992).

The attachment of the methylthio group seems to occur after the isopentenylation of the adenosine residue in t-RNA (Gefter and Russell, 1969). Thiolation precedes methylation (Agris et al., 1975). Cysteine is the donor of the sulphur atom and S-adenosyl methionine provides the methyl group (Gefter, 1969).

Cytokinin Metabolism in Plants

Cytokinin metabolism can be divided into four broad categories: conjugation, hydrolysis, reduction and oxidation (recently reviewed by Mok and Martin, 1994; and Jameson, 1994). Since the exact function of cytokinin derivatives is obscure, they are discussed in terms of our knowledge of their enzymology and the possible roles they could play in the regulation and expression of cytokinin activity.

Conjugation includes: (1) formation of 9-ribosides and their 5' mono-, di- and tri-phosphates, (2) 3-, 7- and 9-glucosides, (3) O-glycosides and (4) alanine conjugates. The ribosides and their aglycones [i.e. zeatin and N^6-(Δ^2-isopentenyl)adenine (i^6Ade)] are extremely active in bioassays (Letham et al., 1983). Ribosides seem to be important translocation forms of cytokinin in the xylem (Goodwin et al., 1978; Letham, 1978). There is a rapid interconversion of the cytokinin bases, ribosides and ribotides. The enzymes responsible for these interconversions were characterized using preparations from wheat germ (Chen and Eckert, 1977; Chen and Pestchow, 1978). N-glucosylation may play an important role in the regulation of cytokinin activity levels. N-glucosides are generally considered detoxification or deactivation products since they are biologically inactive but extremely stable (Letham et al., 1983). N-glucosylation is the predominant fate of externally applied cytokinins in radish cotyledons where two glucosyl transferases have been partially purified (Entsch and Letham, 1979). O-glucosides however, are biologically very active compounds in bioassays (Letham et al., 1983), and are readily converted to aglycones under certain conditions, which indicates that they may be cytokinin storage forms. Two enzymes responsible for the O-

glycosylation of zeatin have been purified from immature seeds of bean (Turner et al., 1987; Dixon et al., 1989). The O-xylosyl transferase was isolated from *P. vulgaris* embryos and uses UDP-xylose (UDPX) for the O-xylosylation of both *trans*-zeatin and dihydrozeatin. The O-glycosyl transferase was isolated from *P. lunatus* embryos and utilizes both UDP-glucose (UDPG) and UDPX as glycosyl donors for the enzyme, but has much higher affinity for UDPG. Alanine conjugation consists of attachment of alanine to the 9-position of the purine ring to form lupinic acid (Summons et al., 1981). The enzyme responsible for alanine conjugation, a ß-(9-cytokinin) alanine synthase, has been partially purified from lupin seeds (Entsch et al., 1983). Alanine conjugates are biologically inactive and extremely stable. Observation of free zeatin release from lupinic acid indicates that alanine conjugation could lead to a potential role in storage of cytokinins (Palni et al., 1984).

The hydrolysis of cytokinin ribosides and ribotides happens very readily to externally applied cytokinins. O-glucosides are readily hydrolyzed by almond ß-glucosidase (emulsin). It is of interest to note that the *rolC* locus of the Ri plasmid of *Agrobacterium rhizogenes* has been suggested to code for a ß-glucosidase capable of cleaving cytokinin N-glucosides (Estruch et al., 1991).

Reduced forms of zeatin (or dihydrozeatin derivatives) are commonly found in plant tissues. The reduction of zeatin has been reported in embryos of *P. vulgaris* (Sondheimer and Tzou, 1971; Mok and Mok, 1987). A reductase capable of converting zeatin to dihydrozeatin has been partially purified from *Phaseolus coccineus* embryos (Martin et al., 1989). In the *P. lunatus* callus bioassay, dihydrozeatin derivatives are less active than their zeatin analogues (Mok et al., 1978). However, in the *P. vulgaris*

callus bioassay, due to the high breakdown of cytokinins with unsaturated side chains (Mok et al., 1982) caused by the presence of oxidases (Armstrong, 1994), zeatin derivatives exhibit higher activity than their zeatin counterparts. Therefore, dihydrozeatin derivatives may be important in the maintenance of cytokinin activity levels in an oxidative environment.

Oxidative side chain cleavage is the predominant fate of externally applied cytokinins (zeatin, i^6Ade, and their corresponding ribosides) to yield adenine, adenosine and adenine nucleotides (reviewed by Armstrong, 1994). Side chain cleavage leads to the irreversible loss of cytokinin activity and may be important in the regulation of cytokinin activity levels. Enzymes that catalyze this removal of the isoprenoid side chain have been isolated and partially purified from tobacco tissue cultures (Paces et al., 1971), maize kernels (Whitty and Hall, 1974), *Vinca rosea* crown gall tissue (Scott et al., 1982; McGaw and Horgan, 1983) and *Phaseolus* (Chatfield and Armstrong, 1986, 1987).

Transformation Using the *Ipt* Gene

Manipulation of cytokinin levels through exogenous application to plant tissues has been plagued by problems of uptake, transport and metabolism. However, generation of transgenic plants expressing the *ipt* gene through transformation allows precise temporal and spatial manipulation of cytokinin levels (recently reviewed by Klee, 1994). Constitutive expression of the *ipt* transgene in transformed plant tissues resulted in hormone-autonomous growth and extensive shoot formation (Binns et al.,

1987; Beinsberger et al., 1987; Smigocki and Owens, 1989). However, these transgenic shoots were unable to root despite the addition of exogenous auxins. An interesting approach to solve such problem of *ipt* gene expression during regeneration was undertaken by Estruch et al. (1991) who inserted the maize transposon *Ac* into the untranslated leader sequence of the 35S-*ipt* gene, thus inactivating the *ipt* gene. Transposition of the *Ac* element reactivated the 35S-*ipt* gene construct. Tobacco plants transformed with this gene were somatic genetic mosaics composed of cells expressing the *ipt* gene intermixed with cells that did not express it. Leaf areas expressing the *ipt* gene contained high levels of *trans*-zeatin riboside and were viviparous. The transgenic adventitious shoots formed were derived from parenchyma cells which do not normally divide. Therefore, cytokinins induced cell division in nondividing parenchyma cells, thus overriding the developmental fate of those cells.

The use of an inducible promoter can also circumvent the inhibitory effect of high cytokinin levels on root initiation. Hence, heat shock promoters have been widely used to drive *ipt* expression in transgenic tobacco (Medford et al., 1989; Schmulling et al., 1989; Smart et al., 1991; Smigocki, 1991) and *Arabidopsis* plants (Medford et al., 1989). These *ipt*-containing plants share many morphological and biochemical "cytokinin effects". They all exhibit reduced stature by up to 50%. Such reduction is caused by a decrease in the internode length rather than the node number. Tobacco plants containing the *hsp*70-*ipt* gene construct were dark green and remained green for extended periods of time when compared to untransformed control plants (Smart et al., 1991). Transgenic plants were also observed to have smaller leaves, a greatly reduced root system, a less developed vascular system, reduced xylem content and reduced

apical dominance. Both cytokinin overexpression and auxin reduction in transgenic plants expressing the *ipt* gene (Medford et al., 1989; Smart et al., 1991; Smigocki, 1991) and the indoleacetic acid lysine synthetase gene (*iaaL*) (Romano et al., 1991), result in extensive growth of lateral shoots. Therefore, apical dominance seems to be controlled by the ratio of cytokinins and auxins, whereby a high cytokinin:auxin ratio results in growth (Klee, 1994).

It is of interest to note that these "cytokinin effects" were expressed in most *ipt*-containing plants irrespective of heat shock, presumably due to the stress-induced or low basal expression of heat shock promoters (Medford et al., 1989; Smart et al., 1991; Smigocki, 1991). Smigocki (1991) also suggested that the altered phenotype of non-heat shocked transgenic plants could have resulted from expression of the *ipt* gene in tissues that are particularly responsive to changes in endogenous cytokinin levels. Cytokinin levels in non-heat shocked transgenic tobacco and *Arabidopsis* plants were high when compared to control untransformed plants (Medford et al., 1989). However, no *ipt* RNA was detected in the absence of heat shock (Medford et al., 1989; Smart et al., 1991; Smigocki, 1991). Induction of the *ipt* gene by heat treatment incited some plants to exhibit a darker green pigment and continued growth of lateral buds (Smart et al., 1991; Smigocki, 1991). Accordingly, cytokinin levels increased substantially following heat shock. In transgenic plants expressing the *ipt* gene, i^6Ado levels increased 3-fold while *trans*-zeatin and *trans*-zeatin riboside levels increased 20 to 200-fold, suggesting the presence of a hydroxylase activity in the plant capable of converting i^6Ado cytokinins to their more active zeatin counterparts (Klee, 1994). Furthermore, the high

level of zeatin-7-glucoside 4 h after heat shock, indicates metabolic activity causing cytokinin inactivation (Medford et al., 1989; Klee, 1994).

Transformation Using the *RolC* gene

The *rolC* gene from *A. rhizogenes* T_L-DNA has been used to transform tobacco and potato plants, under the control of its own promoter and that of the heterologous 35S promoter (Oono et al., 1987; Schmulling et al., 1988; Fladung, 1990; Fladung, 1993; Martin-Tanguy et al., 1993; Nilsson et al., 1993). Transgenic plants display varying amount of phenotypic alterations descriptive of the "RolC phenotype" (Nilsson et al., 1993), characterized by dwarf plants, short internodes, a large number of internodes formed before flowering, thin and light green lanceolate leaves, small male-sterile flowers, and an increase in root growth *in vitro* (Schmulling et al., 1988).

It has been suggested that the phenotypic changes associated with expression of the *rolC* gene were caused by a decrease in auxin activity or an increase in cytokinin activity (Schmulling et al., 1988; Spena et al., 1989). Estruch et al., (1991) proposed that the *rolC* gene codes for a cytokinin-ß-glucosidase capable of hydrolyzing the inactive 7- and 9-cytokinin glucosides, thus raising the level of active cytokinins. It is of interest to note here that a ß-glucosidase, Zm-p60, originally identified through photoaffinity labeling with an azido derivative of indoleacetic acid (Campos et al., 1992), was found to hydrolyze indoxyl-O-glucoside (Campos et al., 1992) and cytokinin-O-glucosides (Bretislav et al., 1993). Surprisingly, kinetin-N^3-glucoside, but not cytokinin-N^7 or N^9-glucoside was hydrolyzed by Zm-p60 (Bretislav et al., 1993).

No overall sequence homology was detected between a *Zm-p60* complementary DNA clone, *Zm-p60.1* and *rolC*. Further studies must be undertaken to test the proposed ß-glucosidase activity of rolC and the significance of Zm-p60 in maize.

Cytokinin levels were measured in pale green (*rolC* expressing) and dark green (not expressing) sectors of leaves from tobacco plants mosaic for somatic expression of the *rolC* gene (Estruch et al., 1991). A 2- to 5-fold increase in i^6Ado was observed in tissue expressing *rolC*. In contrast, Nilsson et al. (1993) recently reported a drastic reduction of i^6Ado in *rolC* expressing tobacco plants, suggesting a possible reduction in the biosynthetic rate of cytokinins. In addition, compared to wild-type tobacco plants, a 3-fold decrease in GA_1 coupled to a 5- to 6-fold increase in GA_{19} was observed in these transgenic plants, indicating a reduction in GA_{19} oxidase activity. No changes were detected in the indole-3-acetic acid pool size or its rate of turnover. Therefore, *rolC* gene expression could be associated with reduction in the rate of synthesis of both cytokinins and gibberellins. However, it is not clear if such effects result from primary or secondary effects of the RolC enzymatic activity (Nilsson et al., 1993).

Recently, *rolC* transformation was shown to alter ethylene production and in accumulation of di- and polyamines, tyramine and their derivatives (Martin-Tanguy et al., 1993). Male-sterile flowers of 35S-*rolC* transformed tobacco plants exhibited a reduction in ethylene production, accompanied by a depression in the accumulation of water-insoluble polyamine and tyramine conjugates and by an increase in free water-soluble amines in transformed flowers, thus indicating that *rolC* could affect flowering and fertility through its effect on ethylene production and on the pool of polyamines and their derivatives.

In conclusion, the function of the *rolC* gene remains unclear. *RolC* expression has been associated with hydrolysis of cytokinin N^7- and N^9-glucosides on the one hand (Estruch et al., 1991), and with a possible reduction in cytokinin biosynthesis on the other hand (Nilsson et al., 1993). Such conflicting roles for the *rolC* gene are compounded by its inhibitory effect on GA_{19} oxidase activity (Nilsson et al., 1993), on ethylene production as well as on the accumulation of water-insoluble polyamines (Martin-Tanguy et al., 1993).

Cis-trans Isomerization

Enzymatic as well as non-enzymatic *cis-trans* isomerization about carbon-carbon double bonds have been reported. A limited number of *cis-trans* isomerases have been described including maleate isomerase (Scher and Jakoby, 1969), maleyl isomerases (Angaw-Ouguma et al., 1992), linoleate isomerase (Kepler and Tove, 1967) and aconitate isomerase (Klinman and Rose, 1971). All the maleyl isomerases isolated thus far require a sulfhydryl compound, glutathione, as a cofactor. Shine and Loomis (1974) reported the isolation of cell-free extracts from carrot and peppermint which catalyzed the enzymatic *trans-cis* isomerization of geraniol and geranyl phosphate to nerol and neryl phosphate respectively. This enzyme system requires the presence of a flavin (FMN or FAD), a thiol or sulfide, and blue light. Under the same conditons, non-enzymatic isomerisation took place. Recently, Engeland and Kindl (1991) described the purification and characterization of a plant peroxisomal Δ^2,Δ^3-enoyl-CoA isomerase from fat-degrading cotyledons of cucumber seedlings. The isomerase was purified

17,000 fold to yield a protein of apparent homogeneity. It is a homodimer with a molecular weight of 50 kD and exhibits optimal activity at pH 9. Furthermore, a retinal-binding protein, retinal photoisomerase has been purified from honey bee eyes (Schwemer et al., 1984; Smith and Goldsmith, 1991). The retinal photoisomerase catalyzes the blue light-induced isomerization of all-*trans*-retinal to 11-*cis*-retinal and its molecular weight was determined to be 27 kD.

Many different catalysts are capable of causing non-enzymatic *cis-trans* isomerization. Flavins were first demonstrated to promote non-enzymatic photoisomerisation reactions by Posthuma and Berends (1966). Riboflavin was observed to sensitize the photoisomerisation of both *cis*- and *trans*-stilbene-4 carboxylic acids. Flavins also catalyzed the photoisomerisation of both all-*trans*-retinol and 13-*cis*-retinol (Gordon-Walker and Radda, 1967). Nucleophiles, including dihydroflavins and dithiothreitol (DTT), catalyze the geometrical dark isomerisation of all-*trans*-retinal to 9-*cis*-retinal and 13-*cis*-retinal (Futterman and Rollins, 1973). In addition, the iodine-catalyzed isomerization of 11-*cis*-retinal to all-*trans* retinal has also been described (Hubbard, 1966). A high concentration of reduced glutathione was required for non-enzymatic isomerization of maleylacetoacetate. The rate of the reaction was however many times slower than that of the isomerase-catalyzed reaction (Knox and Edwards, 1955). In conclusion, both enzymatic as well as non-enzymatic dark- or blue light-requiring *cis-trans* isomerization are known to occur.

MATERIALS AND METHODS

Plant Material

Immature seeds (3 to 10 mm long) of *P. vulgaris* L. cv. Great Northern were used for enzyme isolation.

Chemicals

trans-Zeatin, *trans*-zeatin riboside, *cis*-zeatin, *cis*-zeatin riboside, FAD, FMN, and α-D-mannopyranoside were obtained from Sigma. *cis*-[8-^{14}C]Zeatin was synthesized from 6-Cl-[8-^{14}C]purine (24 mCi/mmol) by the procedures reported previously (Kadir et al., 1984). BSTFA was obtained from Pierce. N-Glycosidase F was purchased from Boehringer Mannheim corporation. The substrates 9-(4-hydroxy-3-methylbut-trans-2-enyl)adenine, crotyladenine and tertiary-butylzeatin were generously provided by Professor Gordon Shaw (University of Bradford, United Kingdom).

Enzyme Isolation

Preliminary experiments comparing the enzyme activities in extracts of various components of the seeds indicated that endosperm was a rich source of the enzyme. Liquid endosperm removed from seeds with a microsyringe was mixed with an equal volume of buffer A (55 mM Tris-HCl, pH 7.5, and 4 mM DTT). Proteins precipitating between 30 to 60% ammonium sulfate saturation were collected after centrifugation at

12,000*g* for 30 min., desalted on an Econo-Pac 10DG column (Bio-Rad), and concentrated by centrifugation at 4,000*g* in Centricon 30 ultrafiltration tubes (Amicon).

Enzyme Purification

Anion Exchange Chromatography

The enzyme extract was applied to a Mono Q HR 5/5 anion exchange FPLC column equilibrated with buffer A and fractionated with a linear gradient of 0 to 500 mM KCl in buffer A over 20 min. at a flow rate of 1 ml/min. Fractions of 0.5 ml were collected. Individual fractions were assayed for enzyme activity, and the positive fractions pooled, and concentrated by Centricon-30 ultrafiltration.

Gel Filtration FPLC

The active fractions obtained after Mono Q FPLC were loaded on a Superose 12 FPLC column equilibrated with buffer A plus 150 mM NaCl. Proteins were eluted with this buffer at a flow rate of 0.5 ml/min. Fractions of 0.25 ml were collected and assayed for enzyme activity. The M_r of the enzyme was estimated by its elution relative to bovine serum albumin (M_r 66,000), carbonic anhydrase (M_r 29,000), cytochrome C (M_r 12,400), and aprotinin (M_r 6,500).

Phenyl Superose FPLC

The positive fractions from gel filtration FPLC were brought to 30% ammonium sulfate saturation and applied to a Phenyl Superose HR 5/5 FPLC column (Pharmacia) equilibrated with buffer B (55 mM Tris-HCl, pH 7.5, 2 mM DTT, and 30% ammonium sulfate). Enzyme was eluted with a linear gradient of 30% to 0% ammonium sulfate over 30 min. The flow rate was 0.5 ml/min and the fraction size 0.5 ml.

PAGE

Proteins purified by the FPLC steps were concentrated and separated by PAGE on 10% acrylamide gels, 1.5 mm thick. The running buffer consisted of 5 mM Tris-HCl and 3.8 mM glycine. Electrophoresis was performed overnight at constant voltage (90 V) at 4°C. Gel sections (5 mm) were transferred to dialysis tubes (MW cut-off of 14,000) containing 1.5 ml buffer (55 mM Tris-HCl, pH 7.5, and 1 mM DTT) and proteins were eluted in an electrophoresis chamber (200 V for 1 h).

Concanavalin A - Sepharose 4B Chromatography

Concanavalin A - Sepharose 4B was washed with 50 bed volumes of 50 mM bis Tris-HCl, pH 6.5, containing 0.25 M ammonium sulfate, 0.1 M methylmannose, 1 mM $CaCl_2$, and 1 mM $MnCl_2$, followed by 50 bed volumes of 50 mM bis Tris-HCl, pH 6.5, with 0.25 M ammonium sulfate. Column material was packed in a Poly-Prep Column

(Biorad) and equilibrated with 50 mM bis Tris-HCl buffer containing 0.25 M ammonium sulfate and 2 mM DTT. The Phenyl Superose-purified enzyme preparation was loaded onto the column, which was then washed with 10 bed volumes of equilibration buffer. Enzyme was eluted with five bed volumes of the same buffer containing 0.1 M methyl α-D-mannopyranoside.

Enzyme Assay

The assay mixture consisted of 70 μl Tris-HCl buffer (55mM; pH 7.5) with or without enzyme extract, 10 μl *cis*-[8-^{14}C]zeatin (0.01 μCi; 0.4 nmol), 10 μl FAD (1 mM), and 10 μl $MgCl_2$ (0.4 M). DTT concentrations ranged from 2 mM to 0.15 mM depending on the purity of the enzyme (see Results). The assay mixture was incubated at 35°C under cool white fluorescent light (Philips, 2A, F42T12/CW/HO, High Output, 110 μmol.m^{-2}.s^{-1}) for 1 h. Ice-cold methanol (100 μl) was added to terminate the reaction. The mixture was centrifuged at 27,000*g* for 10 min and the supernatant was stored at -80°C until analyzed. For all the characterization studies however, the assay mixture without the substrate *cis*-[8-^{14}C]zeatin was first exposed to light at 35°C for 30 min. Labelled zeatin was then added and the mixture was further incubated for another 30 min.

Cytokinin Analysis

To detect and quantify cytokinins resulting from the reaction, a Beckman model 110A dual pump HPLC system with a reversed-phase C_{18} column (Ultrasphere ODS,

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 3.5 by triethylamine (TEA). Samples were eluted with a linear gradient of methanol (5 to 50% over 90 min.). The flow rate was 1 ml/min and 0.5 ml fractions were collected. Radioactivity was determined in Ready-Gel scintillation fluid with a Beckman LS 7000 scintillation counter. The enzyme activity was calculated after subtracting the background conversion which was determined in control samples without the enzyme.

Protein Determination

The amount of protein in each fraction was determined with a Bio-Rad protein assay kit and procedures recommended by the manufacturer, with BSA as the standard. The protein composition of various fractions was determined by SDS-PAGE (Laemmli, 1970); for details see Martin et al. (1990).

N-Glycosidase F Treatment

A Phenyl Superose-purified enzyme preparation (10 μg in 10 μl buffer) was denatured in 1% SDS (w/v) by boiling for 2 min. Incubation buffer (90 μl of 0.1 M sodium phosphate containing 0.5% octylglucoside) was then added and the mixture was again boiled for 2 min. After allowing the mixture to cool down to 37°C, 1 unit of N-glycosidase F was added. Incubation was then carried out at 37°C overnight. Deglycosylation was determined after comparing the mobility of the enzyme on SDS-PAGE before and after N-Glycosidase F treatment.

Mass Spectrometry

The product of the enzymatic conversion was separated from the original *cis*-zeatin substrate by HPLC, dried, and derivatized in 5 μl pyridine and 5 μl BSTFA for 15 min at 60°C. GC/MS analyses of the trimethylsilylated product and standards (*cis*- and *trans*-zeatin) were performed with a Finnigan Model 4023 instrument in the electron impact (EI) mode. Samples were injected in a 30 m x 0.25 mm SE-54 column and eluted at a 25 cm/sec linear velocity of the carrier gas (purified helium). The temperature was increased from 100°C to 300°C over 10 min.

Enzyme Characterization

The enzyme preparation used for all the characterization studies was first purified by ammonium sulfate precipitation followed by heating at 42°C for 30 min. The enzyme extract was centrifuged at 12,000 g for 30 min and the supernatant was further purified by Mono-Q anion exchange, Concanavalin A, and Phenyl Superose chromatography. About 30 μl equivalent of endosperm was used per assay and the DTT concentration was 0.5 mM. The assay mixture without *cis*-^{14}C-zeatin was preincubated in cool white fluorescent light (110 μmol m^{-2} s^{-1}) at 35°C for 30 min. *cis*-^{14}C-Zeatin was then added and the mixture was incubated under the same light conditions at 35°C for another 30 min.

The optimum FAD concentration was first determined. Different amounts of FAD (0, 0.01, 0.0316, 0.1, 0.316, 1 and 3.16 mM) were tested without enzyme and in the presence of 30, 60 and 120 μl-equivalent of endosperm.

The optimum pH was determined using 55 mM Tris-HCl at pH 7.25 to 8.75 with 0.25 increments. The buffer was adjusted to the desired pH following incubation in a 35°C water bath for 1 h.

The enzymatic as well as the non-enzymatic reaction were carried out at different temperature settings (25°C to 75°C with 5°C increments). The preincubation and incubation temperatures were identical.

The light quality was studied using a red acrylic filter (Shinkolite 102, Argo Plastics Co., Los Angeles, CA.) and a blue acrylic filter (No. 2424, Denco Sales Co., Portland, OR.). The effect of light quality on both enzymatic as well as non-enzymatic isomerization was determined.

Isomerization was tested with and without enzyme at different DTT concentrations (0.05, 0.25, 0.5, 0.75, 1.0 and 2.0 mM). In this case, experiments were performed following Phenyl Superose purification.

The time course of the reaction was studied by incubating the assay mixture for 0, 15, 30, 45 and 60 min. after the addition of the substrate. Preincubation time was set at 30 min. for all time points tested.

The compounds tested as substrates for the enzyme included *trans*-zeatin, tertiary-butylzeatin, crotyladenine, *trans*-zeatin riboside, *cis*-zeatin riboside and 9-(4-hydroxy-3-methylbut-trans-2-enyl)adenine. About 20 nmol of these compounds was added to the assay mixture which was incubated at 35°C for 1 h after the addition of the substrate. The reaction was terminated as for *cis*-zeatin. Substrates and products were separated on a reversed-phase HPLC column.

The interaction between all 4 components making up the isomerization reaction (i.e. the substrate *cis*-zeatin, the cofactor FAD, the enzyme and light) was studied using preincubation assays. In the preincubation mixture, 1 or 2 components of the reaction were exposed to light or dark conditions in a 35°C water bath for 30 min. followed by the addition of the remaining component(s) and the subsequent incubation of the whole mixture for another 30 min. at 35°C under light or darkness. These preincubation combinations included: FAD ± enzyme followed by *cis*-zeatin; substrate ± enzyme followed by FAD; ± enzyme followed by FAD + *cis*-zeatin; FAD followed by substrate ± enzyme; and *cis*-zeatin followed by FAD ± enzyme.

RESULTS

Isomerization Reaction and Identification of Reaction Products

Incubation of radiolabeled *cis*-zeatin under both assay conditions (standard and preincubation), with or without enzyme, resulted in formation of a product which co-chromatographed with *trans*-zeatin (Fig. 3). The non-enzymatic conversion ranged from 9-12%, whereas enzymatic conversion could be as high as 70%. When the enzyme extract was boiled for 10 min., conversion was the same as without enzyme. Incubation with radiolabeled *trans*-zeatin under the same conditions indicated that the reverse reaction could also take place. However, conversion in this direction was only 2% in the absence of enzyme and up to 25% in the presence of enzyme. Thus, the conversion from *cis*- to *trans*-zeatin is favored and is the only reaction that was further characterized.

The occurrence of isomerization was confirmed by characterization of products by GC/MS. Derivatization with BSTFA led to formation of two products, the mono- and di-trimethylsilyl derivatives, which were separated by GC. The spectra of the mono-derivatives are shown in Fig. 4. The product resulting from incubation of *cis*-zeatin with enzyme (Fig. 4C) had the characteristic MS pattern of *trans*-zeatin (Fig. 4B). Specifically, the relative intensity of the m/e 188 ion was higher for the product and *trans*-zeatin than for *cis*-zeatin, while that of the m/e 192 ion was lower.

Figure 3. Separation by HPLC on reversed-phase C_{18} of the radiolabeled product (*trans*-zeatin; t-z) and substrate (*cis*-zeatin; c-z) after incubation with (**A**) or without (**B**) isomerase.

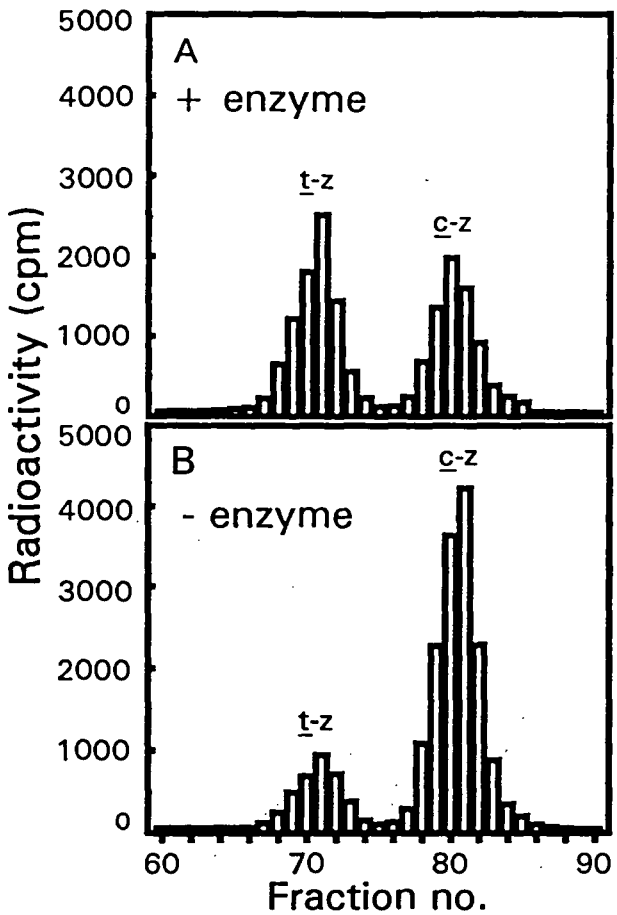

5000
4000
3000
2000
1000
0
A
+ enzyme
t-z
c-z
Radioactivity (cpm)
5000
4000
3000
2000
1000
0
B
- enzyme
c-z
t-z
60
70
80
90
Fraction no.

Figure 3.

Figure 4. Mass spectra of *cis*-zeatin (**A**), *trans*-zeatin (**B**), and the product of enzymatic conversion of *cis*-zeatin (**C**) after derivatization with BSTFA.

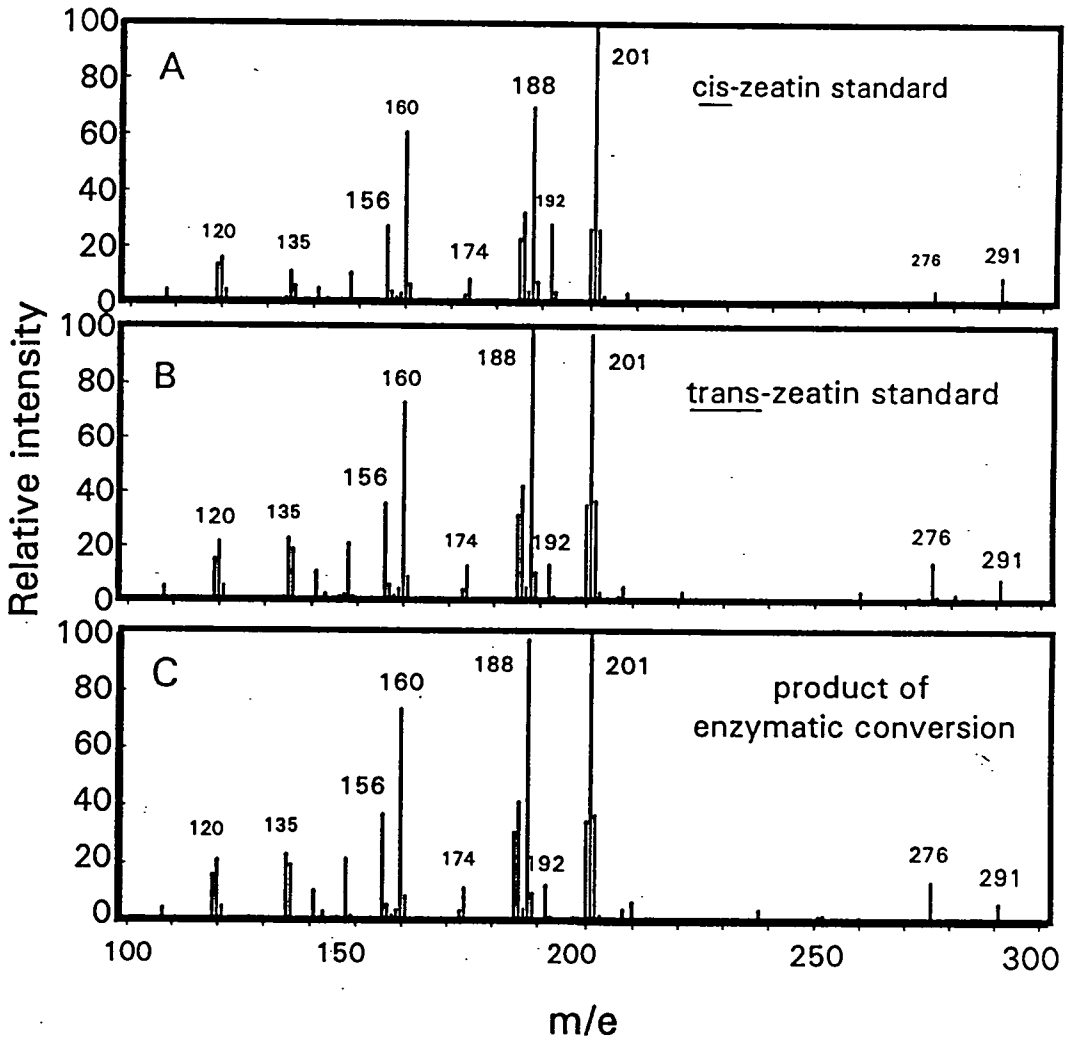

A
cis-zeatin standard
B
trans-zeatin standard
C
product of
enzymatic conversion
Relative intensity
m/e
100
80
60
40
20
0
120
135
156
160
174
188
192
201
276
291
100
150
200
250
300

Figure 4.

Enzyme Purification

It was difficult to determine enzyme activity in crude endosperm preparations, due to the large volume. Proteins were concentrated by precipitation with ammonium sulfate (30 to 60% saturation), and this fraction was used as the initial extract from which further purification of the enzyme was calculated. The standard assay of 1 h incubation at 35°C under cool-white fluorescent light (110 μmol m^{-2} s^{-1}) was used for all the purification studies. To compare activity in different fractions, conversion was kept under 40%.

Anion exchange FPLC on a Mono Q column separated proteins into three major regions with the enzyme eluting with the third peak (Fig. 5); six-fold purification of the enzyme was obtained (Table 1). Gel filtration on a Superose 12 FPLC column (Fig. 6) increased the purity three-fold. Based on the elution volume relative to standards, the molecular mass was estimated to be 68$\pm$4 kD. However, this estimate may not reflect the true molecular size since the enzyme seems to be a glycoprotein. Evidence for the glycoprotein nature of the isomerase comes from the ability of the enzyme to bind to a Concanavalin A column from which it was eluted with α-D-mannopyranoside. In addition, a shift in mobility of all visible protein bands was observed on SDS-PAGE following treatment of the partially purified enzyme extract with N-glycosidase F. Hydrophobic interaction FPLC was effective in separating the enzyme from other proteins (Fig. 7), resulting in about nine-fold purification over the active fraction from gel filtration. In addition to the major peak at 15 ml, two smaller peaks were present

Figure 5. Elution profile of zeatin *cis-trans* isomerase after Mono Q HR 5/5 FPLC. Protein from 5 ml endosperm was applied to the column and eluted with a linear gradient of 0 to 500 mM KCl in 55 mM Tris-HCl buffer (pH 7.5) with 4 mM DTT over 20 min at a flow rate of 1 ml/min. Enzyme activity was determined with 10 μl from each 0.5 ml fraction.

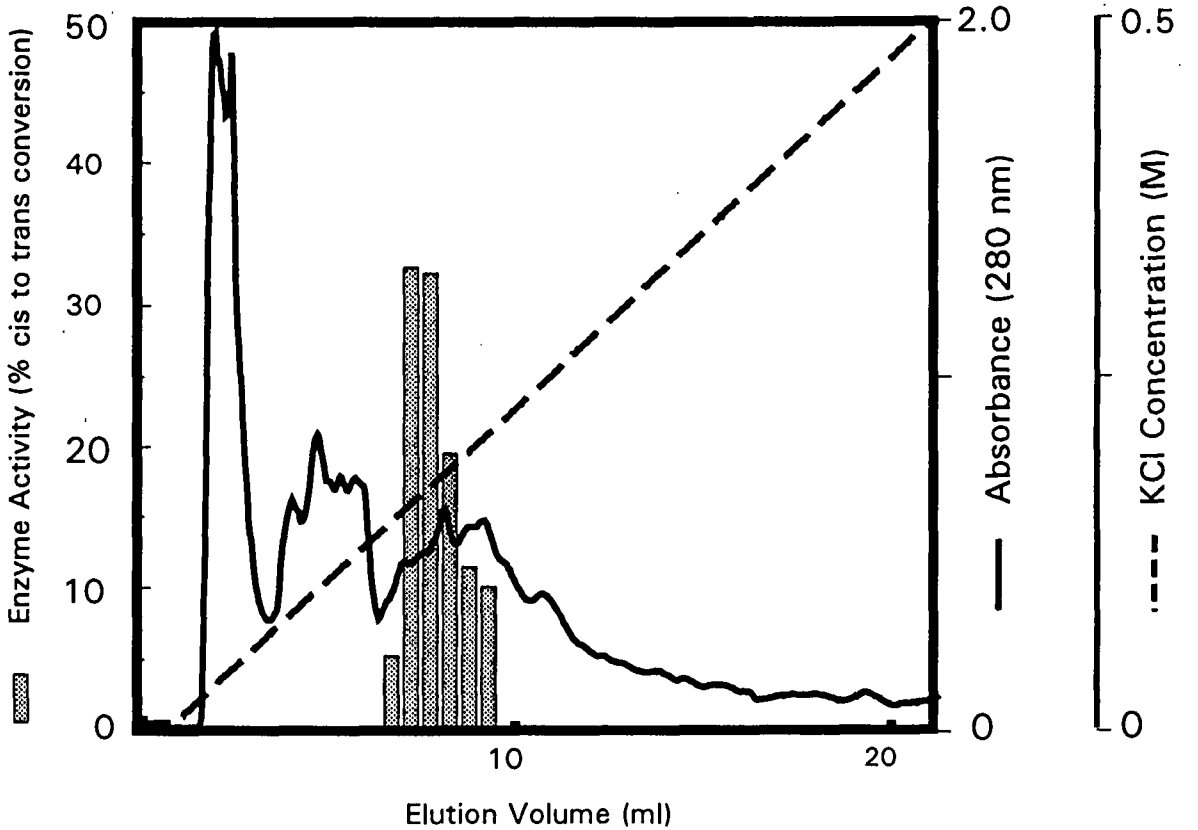

Enzyme Activity (% cis to trans conversion)
50
40
30
20
10
0
10
20
Elution Volume (ml)
2.0
0
Absorbance (280 nm)
0.5
0
KCl Concentration (M)

Figure 5.

Figure 6. Elution profile of zeatin *cis-trans* isomerase after gel filtration FPLC on Superose 12. The column was equilibrated and developed with 55 mM Tris-HCl buffer (pH 7.5) containing 4 mM DTT and 150 mM NaCl. Active fractions from Mono Q FPLC (equivalent to 10 ml endosperm) were applied to the column and eluted with the same buffer at a flow rate of 0.5 ml/min over 60 min. Enzyme activity was determined with 2.5 μl from each 0.25 ml fraction.

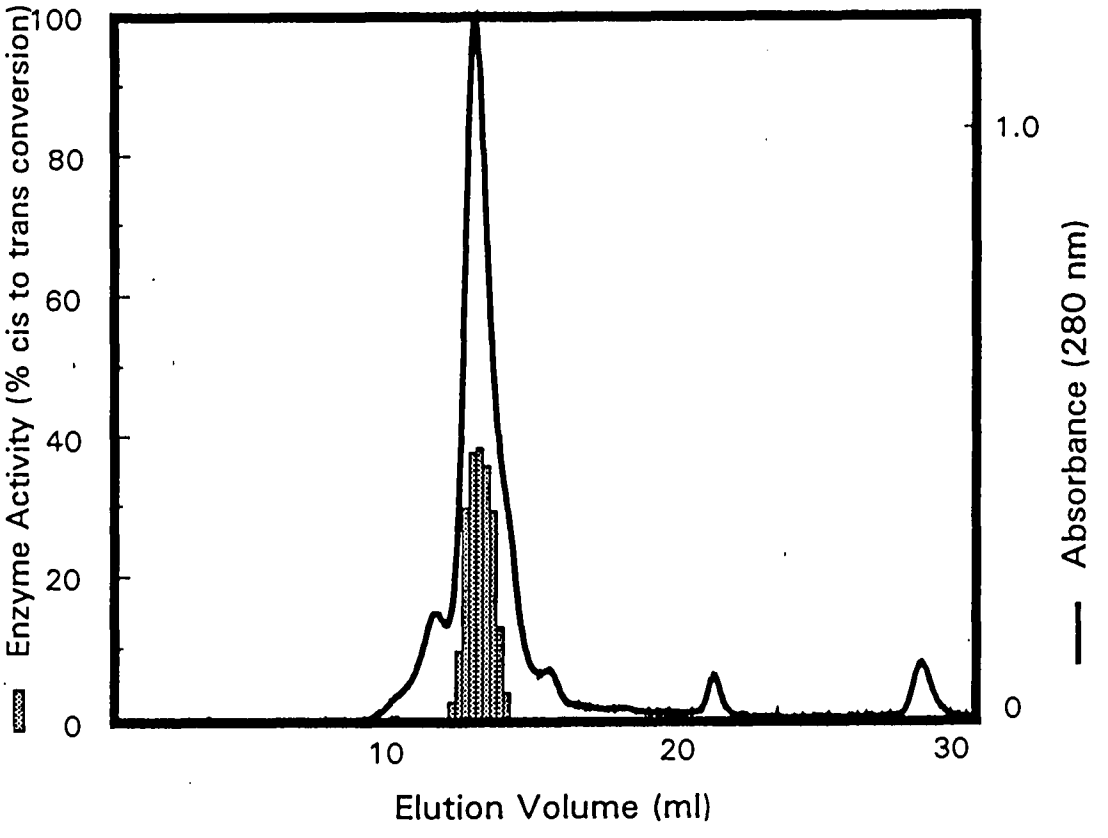

Enzyme Activity (% cis to trans conversion)
100
80
60
40
20
0
10
20
30
Elution Volume (ml)
1.0
0
Absorbance (280 nm)

Figure 6.

Figure 7. Elution profile of zeatin *cis-trans* isomerase after Phenyl Superose HR 5/5 FPLC. Active fractions from Superose 12 FPLC (equivalent to 10 ml endosperm) were applied to the column equilibrated with 55 mM Tris-HCl (pH 7.5) containing 2 mM DTT and 30% ammonium sulfate. Enzyme was eluted with a linear gradient of 30 to 0% ammonium sulfate at a flow rate of 0.5 ml/min over 30 min. Enzyme activity was determined with 5 μl from each 0.5 ml fraction.

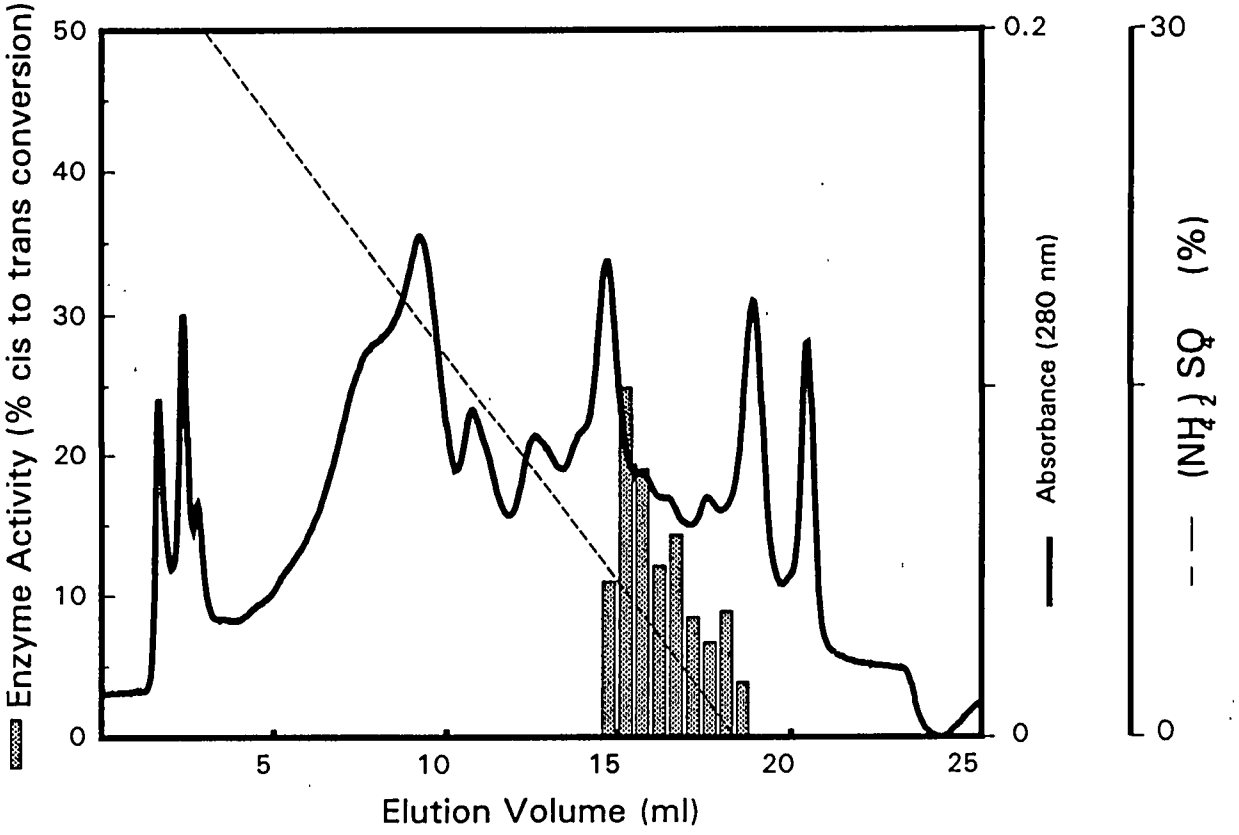

Enzyme Activity (% cis to trans conversion)
50
40
30
20
10
0
0.2
0
Absorbance (280 nm)
30
0
(%) δS (½HN)
5
10
15
20
25
Elution Volume (ml)

Figure 7.

Purification Step	Total Activity (*pmol/h*)	Protein (*μg*)	Specific Activity (*pmol/μg/h*)	Purification (*fold*)	Recovery (%)
1. 30-60% $(NH_4)_2SO_4$	39187	26823	1.5	1	100
2. Mono Q	25769	2763	9.3	6	66
3. Superose 12	33089	1313	25.2	17	84
4. Phenyl Superose	24329	105	232.7	155	62
5. PAGE	3668	3	1079.0	719	9
5. Concanavalin A	17030	5	3406.0	2271	44

Table I. Purification of zeatin *cis-trans* isomerase from *P. vulgaris* endosperm. For details of purification, see "Materials and Methods." Total activity and protein are for 10 ml endosperm equivalent.

in the activity region. It is not clear whether this signifies the presence of isozymes; all other purification methods resolved only a single peak of activity. Either PAGE or Concanavalin A chromatography was the final purification step. Although PAGE resulted in increased specific activity, it also caused substantial loss of the enzyme (Table 1). When substituted by a Concanavalin A column, loss of enzyme was minimal and purification was 15-fold. The enzyme can be stored at -80°C for at least 8 weeks without loss of activity.

The sequential purification steps resulted in more than 2000-fold purification of the enzyme (Table 1). As the calculation was based on the sample obtained after ammonium sulfate fractionation, the actual extent of purification was probably higher. Enzyme recovery was 44% after Concanavalin A chromatography. The protein composition after each purification step was analyzed by SDS-PAGE (Fig. 8). As expected, the complexity of the protein profiles decreased with each purification step. After PAGE, the gel slice with the highest activity contained a prominent protein band with estimated mass of about 64 kD.

Enzyme and Reaction Characterization

All characterization experiments were repeated at least twice. However, I chose to report here the results of experiments which contained a more complete set of data, and which were performed at the same time and using the same enzyme extract. In all cases, the assay mixture minus the substrate were preincubated together for 30 min. at 35°C, in light. The isomerization reaction was initiated by the addition of the substrate,

Figure 8. Coomassie brilliant blue-stained SDS-PAGE gel of pooled active fractions after each purification step. Protein in each lane is from 10 μl endosperm, or multiple if indicated. Lanes: **1** Molecular markers, **2** 30-60% ammonium sulfate precipitation, **3** Anion exchange chromatography, **4** Superose 12 FPLC, **5** Superose 12 FPLC (10x), **6** Phenyl Superose FPLC, **7** Phenyl Superose FPLC (10x), **8** PAGE, **9** PAGE (50x)

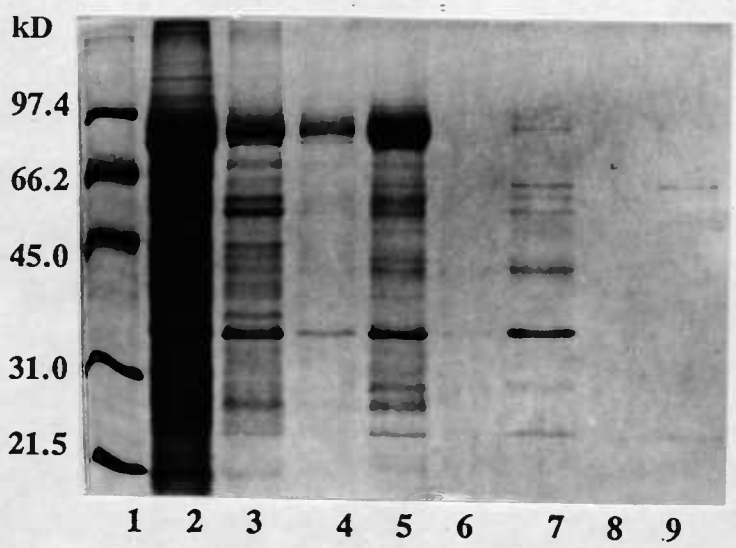


Figure 8.

NEENAH Bond
25% Cotton Fiber

cis-^{14}C-zeatin. Incubation then occurred under the same conditions for another 30 min. About 0.48 nmol (25,000 cpm) of *cis*-^{14}C-zeatin was added to all assays. Enzymatic isomerization was obtained by subtracting *trans*-^{14}C-zeatin formed in the presence of the enzyme from that formed in the absence of the enzyme. Since *cis-trans* isomerization is a reversible reaction, product formation may not be proportional to the amount of enzyme, especially at high enzyme concentration. However, we have established that conversion over 30 min. (following the 30 min. of preincubation time) was proportional to the amount of enzyme used if less than 40% of the *cis*-zeatin was converted to *trans*-zeatin. Therefore, the assay time was kept at 30 min. and whenever conversion was higher than 40% at 35°C in any assay, it was repeated with less enzyme.

Enzymatic and non-enzymatic formation of *trans*-zeatin were determined after 0, 15, 30, 45 and 60 min. of incubation in a 35°C water bath under cool white fluorescent light (110 μmol m^{-2} s^{-1}) (Fig. 9). Enzymatic isomerization by far exceeded nonenzymatic isomerization at every time point tested. *trans*-Zeatin formation increased over time. In the presence of the isomerase, formation of *trans*-zeatin appeared linear over the first 45 min. but decreased after that. Determination of linearity necessitates regression analysis of the data, which was not done in this case. However, some deviation from linearity is not surprising since the isomerase mediates the reverse reaction under the same conditions. Non-enzymatic formation of *trans*-zeatin, however, seemed to increase linearly over time to attain a maximum of about 11% after 60 min. of incubation time. The reverse reaction, from *trans*- to *cis*-zeatin, in the absence of the enzyme, is too insignificant (maximum 2%) to affect the time course of non-enzymatic *cis*- to *trans*-zeatin isomerization.

Figure 9. Time course of the enzymatic and the non-enzymatic isomerization reaction. The preincubation mixture contained: 10 μl of FAD (1 mM), 10 μl of $MgCl_2$ (0.4 M), 70 μl of Tris-HCl buffer (55 mM) with 30 μl-equivalent of Phenyl Superose-purified endosperm or without enzyme. The pH was 7.5 and DTT concentration was 0.5 mM. Preincubation was carried out at 35°C under cool-white fluorescent light (110 μmol m^{-2} s^{-1}) for 30 min. 10 μl of *cis*-^{14}C-zeatin (0.48 nmol) was subsequently added and the assay mixture was then incubated under the same conditions. Substrate and product were separated by HPLC on a reversed-phase C_{18} column and quantified by determining radioactivity using a Beckman LS 7000 scintillation counter. Enzymatic formation of *trans*-zeatin was determined by subtracting the amount of ^{14}C-*trans*-zeatin formed in the absence of the enzyme from that obtained in the presence of the isomerase.

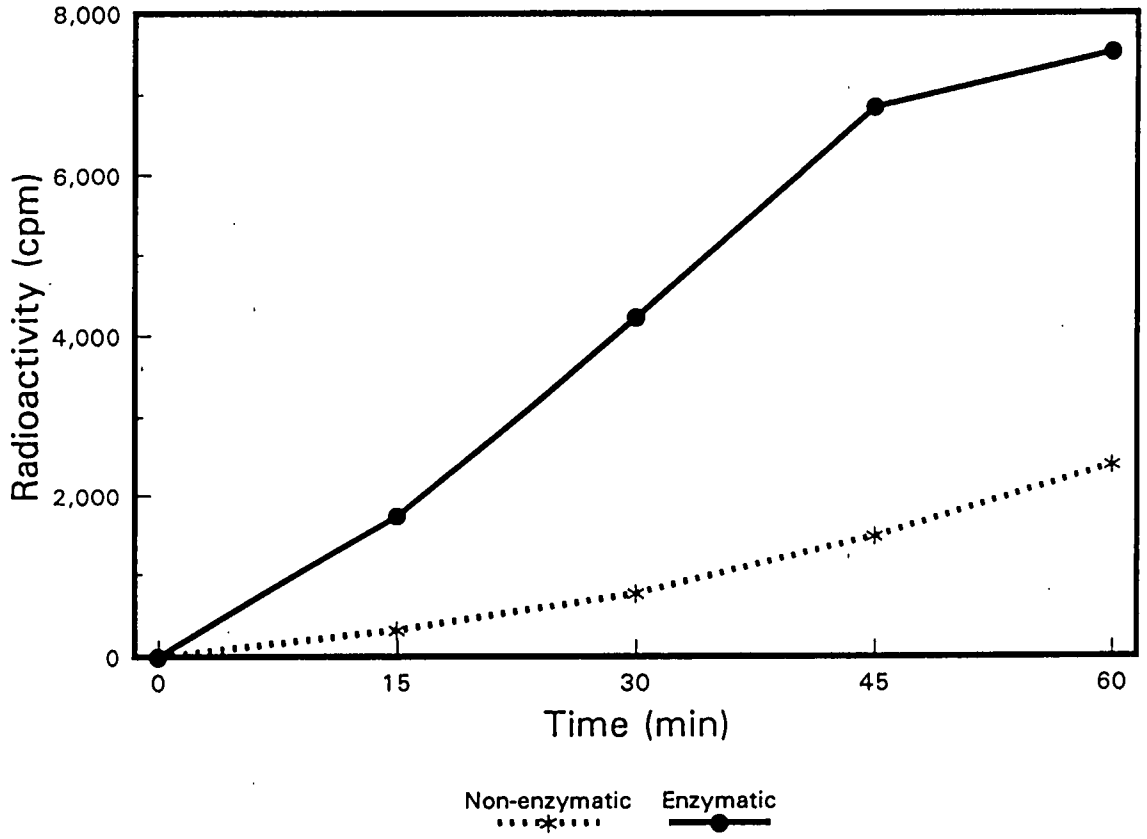

8,000
6,000
4,000
2,000
0
Radioactivity (cpm)
0
15
30
45
60
Time (min)
Non-enzymatic
Enzymatic

Figure 9.

Light, FAD and DTT were necessary for *cis-trans* isomerization. Enzymatic as well as non-enzymatic formation of *trans*-zeatin occurred when a blue acrylic filter was used during the assay. No isomerization was found, however, when a red acrylic filter was utilized under the same assay conditions. Therefore, the light needed for *cis-trans* isomerization of zeatin is in the blue wavelength range of 400-500 nm.

The optimum FAD concentration was determined without enzyme and in the presence of 30, 60 and 120 μl-equivalent of endosperm (Fig. 10). No isomerization was found in the absence of the flavin cofactor, FAD. In the presence of all three concentrations of the enzyme, 0.1-0.316 mM FAD resulted in the highest conversion of *cis* to *trans*-zeatin. A sharp decline in enzymatic isomerization was observed at FAD concentrations exceeding 3.16 mM. A high concentration of FAD (3.16 mM) resulted in equally low enzymatic and non-enzymatic conversion of *cis* to *trans*-zeatin. Non-enzymatic isomerization, however, increased slowly with increasing FAD concentrations up to 1 mM after which a slight decline was noted.

The sulfhydryl reagent DTT was required for the isomerization reaction and to limit the breakdown of zeatin (to adenine) under the assay conditions. However, the optimal DTT concentration for isomerization decreased as the purity of the enzyme increased. For instance, during purification of the enzyme, 2 mM DTT was optimal after ammonium sulfate precipitation and after anion exchange FPLC, 1 mM after gel filtration and Phenyl Superose FPLC, 0.5 mM after Concanavalin A chromatography, and 0.15 mM after PAGE. For all the characterization studies, gel filtration and PAGE were omitted from the purification scheme and Concanavalin A chromatography preceeded hydrophobic interaction chromatography. Phenyl Superose-purified enzyme

Figure 10. Effect of FAD concentration on isomerization in the absence of the enzyme and in the presence of 30 (1x), 60 (2x) and 90 (4x) μl-equivalent of Phenyl Superose-purified endosperm. The preincubation mixture contained: 10 μl of FAD (1 mM), 10 μl of $MgCl_2$ (0.4 M), 70 μl of Tris-HCl buffer (55 mM) with 30, 60 or 90 μl-equivalent of Phenyl Superose-purified endosperm or without enzyme. Preincubation was carried out at 35°C under cool-white fluorescent light (110 μmol m^{-2} s^{-1}) for 30 min. 10 μl of *cis*-^{14}C-zeatin (0.48 nmol) was subsequently added and the assay mixture was then incubated under the same conditions for 30 min. Substrate and product were separated by HPLC on a reversed-phase C_{18} column and quantified by determining radioactivity using a Beckman LS 7000 scintillation counter. Enzymatic formation of *trans*-zeatin was determined by subtracting the amount of ^{14}C-*trans*-zeatin formed in the absence of the enzyme from that obtained in the presence of the isomerase.

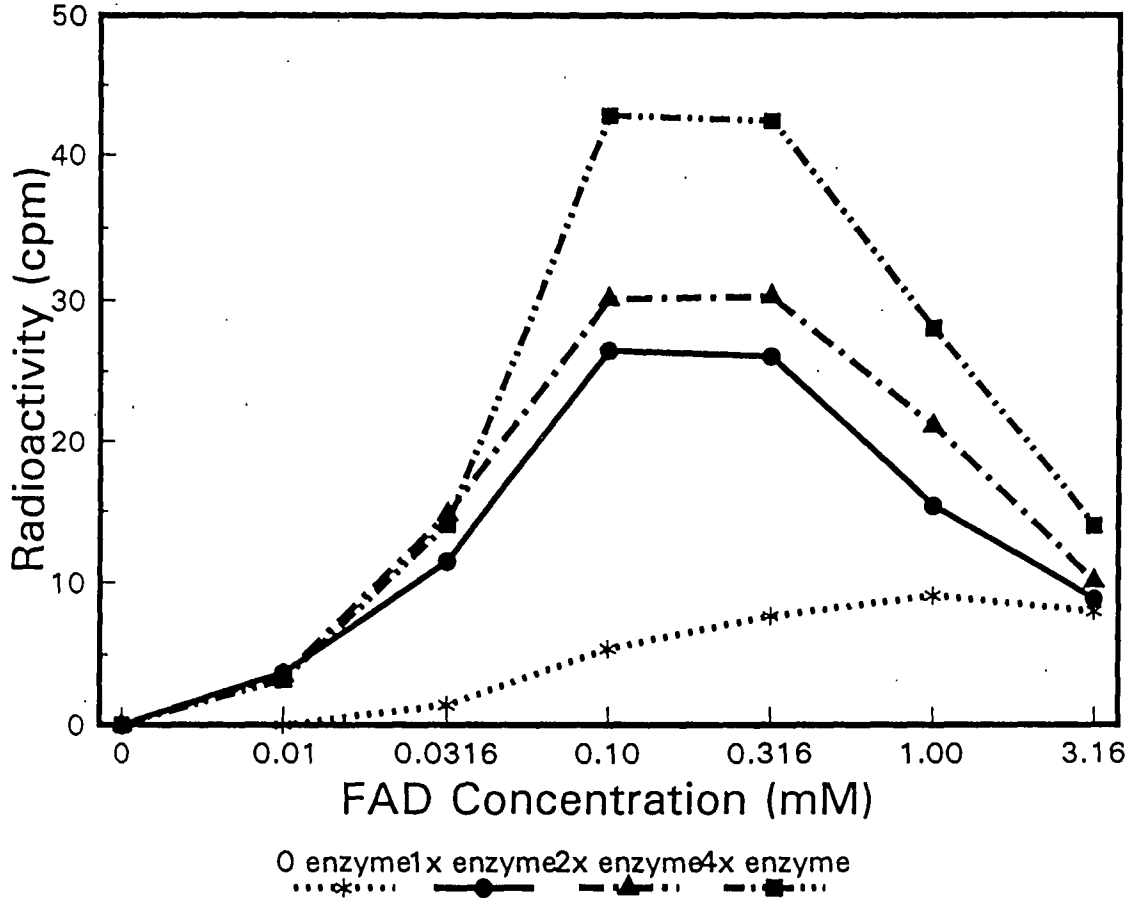

50
40
30
20
10
0
Radioactivity (cpm)
0
0.01
0.0316
0.10
0.316
1.00
3.16
FAD Concentration (mM)
0 enzyme1x enzyme2x enzyme4x enzyme

Figure 10.

was used for determining the optimal DTT concentration for isomerization (Fig. 11B). Among the seven DTT concentrations tested (0.05, 0.25, 0.5, 0.75, 1.0 and 2.0), 0.5 mM resulted in the highest enzymatic conversion of *cis* to *trans*-zeatin (Fig. 11B). During enzymatic isomerization, zeatin breakdown (side chain cleavage) was highest (about 4600 cpm) at the lowest DTT concentration tested, 0.05 mM. As the DTT concentration increased, zeatin breakdown steadily decreased to a low of 280 cpm when 2.0 mM DTT was used. Non-enzymatic isomerization, however, was not affected by DTT concentrations and zeatin breakdown was constant (around 2000 cpm) (Fig. 11A).

The optimum pH for isomerization was determined using Tris-HCl buffer (55 mM) that was brought to the assay temperature of 35°C and then adjusted to the desired pH (7.25 to 8.75 with 0.25 increment) (Fig. 12). The highest amount of enzymatic *cis* to *trans*-zeatin conversion was obtained when the pH was 7.5. Non-enzymatic isomerization was not significantly affected by pH (Fig. 12).

Recovery of high isomerase activity following heat treatment (42°C for 30 min.) caused us to believe that the enzyme was heat stable. To determine the optimum temperature for the isomerization reaction, enzymatic as well as non-enzymatic assays were carried out at temperature settings ranging from 25°C to 75°C with 5°C increments (Fig. 13). Enzymatic isomerization was low between 25°C and 30°C and increased sharply between 30°C and 35°C. A slow increase in enzymatic conversion of *cis*- to *trans*-zeatin was observed at temperatures that exceeded 35°C until at 65°C a maximum was reached. Above 65°C enzymatic isomerization declined sharply. This sharp decline in enzymatic isomerization may have been caused by a possible increase in non-enzymatic thermal isomerization above 65°C. In conclusion, the *cis-trans* isomerase

Figure 11. Effect of DTT concentration on non-enzymatic (**A**) and enzymatic isomerization of *cis*-zeatin (**B**) and on breakdown of zeatin to adenine. The preincubation mixture contained: 10 μl of FAD (1 mM), 10 μl of $MgCl_2$ (0.4 M), 70 μl of Tris-HCl buffer (55 mM) with 30 μl-equivalent of Phenyl Superose-purified endosperm or without enzyme. The pH was 7.5 and DTT concentrations ranged from 0.05 to 2.0 mM. Preincubation was carried out at 35°C under cool-white fluorescent light (110 μmol m^{-2} s^{-1}) for 30 min. 10 μl of *cis*-^{14}C-zeatin (0.48 nmol) was subsequently added and the assay mixture was then incubated under the same conditions for 30 min. The breakdown products as well as the substrate and product formed upon isomerization were separated by HPLC on a reversed-phase C_{18} column and quantified by determining radioactivity using a Beckman LS 7000 scintillation counter. Enzymatic formation of *trans*-zeatin as well as the breakdown of *cis*-zeatin to adenine were determined by subtracting the amount of *trans*-^{14}C-zeatin and ^{14}C-adenine formed in the absence of the enzyme from those obtained in the presence of the isomerase.

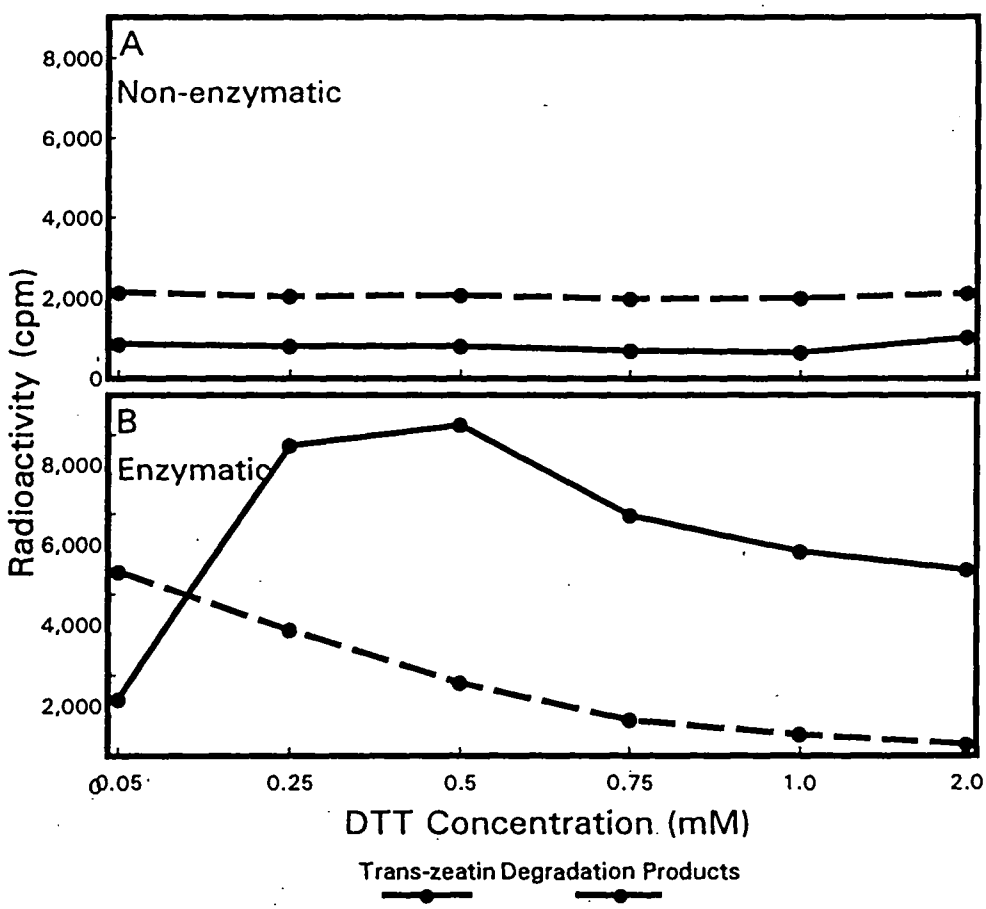

A
Non-enzymatic
8,000
6,000
4,000
2,000
0
B
Enzymatic
8,000
6,000
4,000
2,000
Radioactivity (cpm)
0.05
0.25
0.5
0.75
1.0
2.0
DTT Concentration (mM)
Trans-zeatin Degradation Products

Figure 11.

Figure 12. Effect of pH on the enzymatic and the non-enzymatic conversion of *cis*- to *trans*-zeatin. The preincubation mixture consisted of: 10 μl of FAD (1 mM), 10 μl of $MgCl_2$ (0.4 M), 70 μl of Tris-HCl buffer (55 mM) adjusted to the desired pH after incubation at the assay temperature of 35°C for 1 h, 30 μl-equivalent of Phenyl Superose-purified endosperm or without enzyme. DTT concentration was 0.5 mM. Preincubation was carried out at 35°C under cool-white fluorescent light (110 μmol m^{-2} s^{-1}) for 30 min. 10 μl of *cis*-^{14}C-zeatin (0.48 nmol) was subsequently added and the assay mixture was then incubated under the same conditions for 30 min. Substrate and product formed upon isomerization were separated by HPLC on a reversed-phase C_{18} column and quantified by determining radioactivity using a Beckman LS 7000 scintillation counter. Enzymatic formation of *trans*-zeatin was determined by subtracting the amount of *trans*-^{14}C-zeatin formed in the absence of the enzyme from that obtained in the presence of the isomerase.

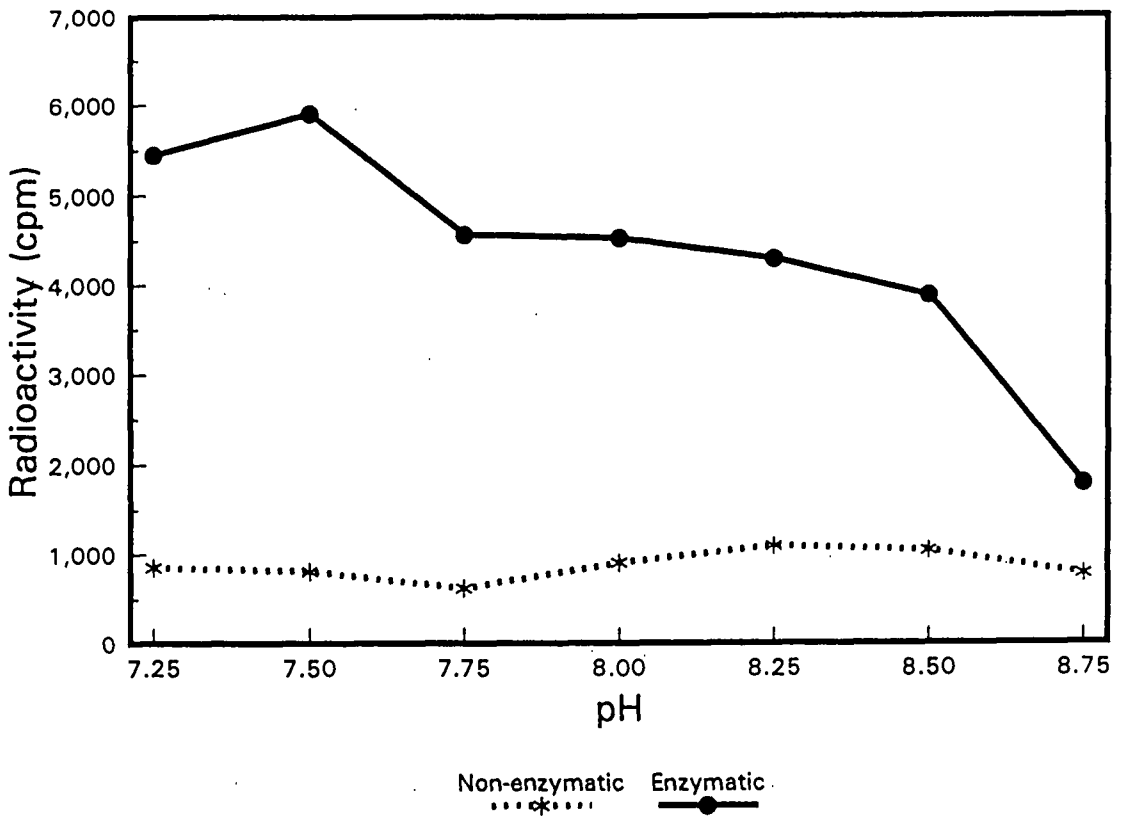

7,000
6,000
5,000
4,000
3,000
2,000
1,000
0
Radioactivity (cpm)
7.25
7.50
7.75
8.00
8.25
8.50
8.75
pH
Non-enzymatic
Enzymatic

Figure 12.

Figure 13. Effect of temperature on enzymatic and non-enzymatic isomerization of *cis*-zeatin. The preincubation mixture contained: 10 μl of FAD (1 mM), 10 μl of $MgCl_2$ (0.4 M), 70 μl of Tris-HCl buffer (55 mM) with 30 μl-equivalent of Phenyl Superose-purified endosperm or without enzyme. The pH was 7.5 and DTT concentration was 0.5 mM. Preincubation was carried out at temperatures ranging from 25°C to 75°C with 5°C increments under cool-white fluorescent light (110 μmol m^{-2} s^{-1}) for 30 min. 10 μl of *cis*-^{14}C-zeatin (0.48 nmol) was subsequently added and the assay mixture was then incubated under the same conditions for 30 min. Substrate and product were separated by HPLC on a reversed-phase C_{18} column and quantified by determining radioactivity using a Beckman LS 7000 scintillation counter. Enzymatic formation of *trans*-zeatin was determined by subtracting the amount of *trans*-^{14}C-zeatin formed in the absence of the enzyme from that obtained in the presence of the isomerase.

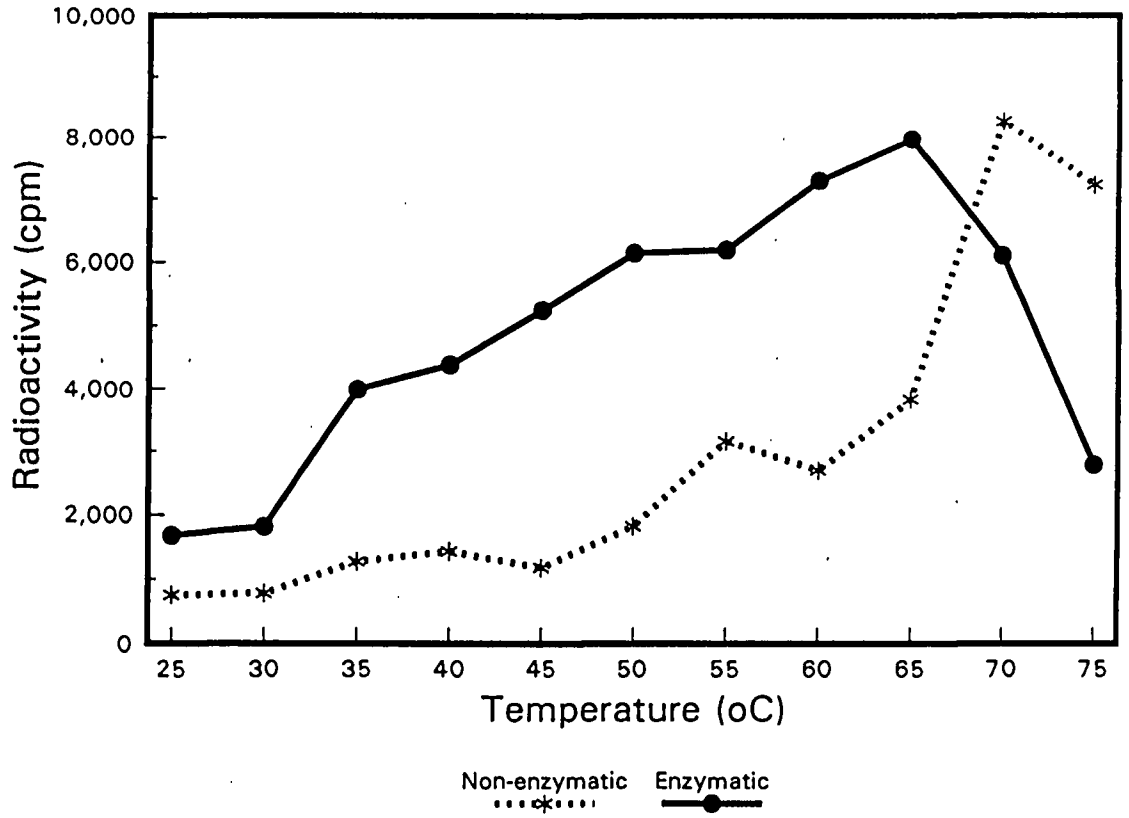

10,000
8,000
6,000
4,000
2,000
0
Radioactivity (cpm)
25
30
35
40
45
50
55
60
65
70
75
Temperature (oC)
Non-enzymatic
Enzymatic

Figure 13.

is heat stable but it is difficult to separate enzymatic isomerization from thermal non-enzymatic isomerization at high temperature.

To determine the substrate specificity of the isomerase, 20 nmol of the following compounds: 9-(4-hydroxy-3-methylbut-2-enyl)adenine, *trans*-zeatin riboside, *cis*-zeatin riboside, tertiary-butylzeatin and crotyladenine replaced *cis*-^{14}C-zeatin in the assay mixture. The assays were incubated at 35°C for 1 h. Isomerization of *trans*-zeatin riboside, *cis*-zeatin riboside and 9-(4-hydroxy-3-methylbut-2-enyl)adenine (Fig. 14) was indicated by the appearance of the isomer peak (UV absorption) following incubation. Enzymatic as well as non-enzymatic isomerization of the above mentioned substrates appeared to have occurred and conversion in the presence of the enzyme exceeded that obtained in the absence of the isomerase. Unfortunately, we did not possess the *cis* isomer of 9-(4-hydroxy-3-methylbut-2-enyl)adenine or and the small amount of product formed from the *trans* isomer precluded unequivocal identification of the products. Crotyladenine and tertiary-butylzeatin, however, were not isomerized.

Preincubation studies were undertaken to elucidate the interaction between *cis*-zeatin, FAD, the isomerase and light (Fig. 15). Dark preincubation followed by dark incubation after the addition of the substrate did not give isomerization. However, when followed by exposure to light for 30 min. after the addition of *cis*-^{14}C-zeatin, isomerization occurred irrespective of preincubation light conditions, in all the combinations tested. Preincubating FAD and the enzyme for 30 min. in light resulted in the highest enzymatic formation of *trans*-zeatin (8,600 cpm). Still, when FAD and the enzyme were preincubated in darkness, enzymatic formation of *trans*-zeatin was also high (5,600 cpm). The remaining preincubation combinations resulted in formation of

Figure 14. HPLC analysis on reversed-phase C_{18} of isomerization products of 9-(4-hydroxy-3-methylbut-2-enyl)adenine following incubation for 1 h at 30°C in the absence (**A**) or in the presence of the enzyme (**B**). Preincubation was carried out for 30 min. at 35°C. Incubation occurred after the addition of substrate 9-(4-hydroxy-3-methylbut-2-enyl)adenine. All the components of the assay were used at the following standard concentrations: 10 μl of FAD (1 mM), 10 μl of $MgCl_2$ (0.4 M), 70 μl of Tris-HCl buffer (55 mM, pH 7.5, containing 0.5 mM DTT) with 30 μl-equivalent of Phenyl Superose-purified endosperm or without enzyme, and 10 μl of 9-(4-hydroxy-3-methylbut-2-enyl)adenine (20 nmol).

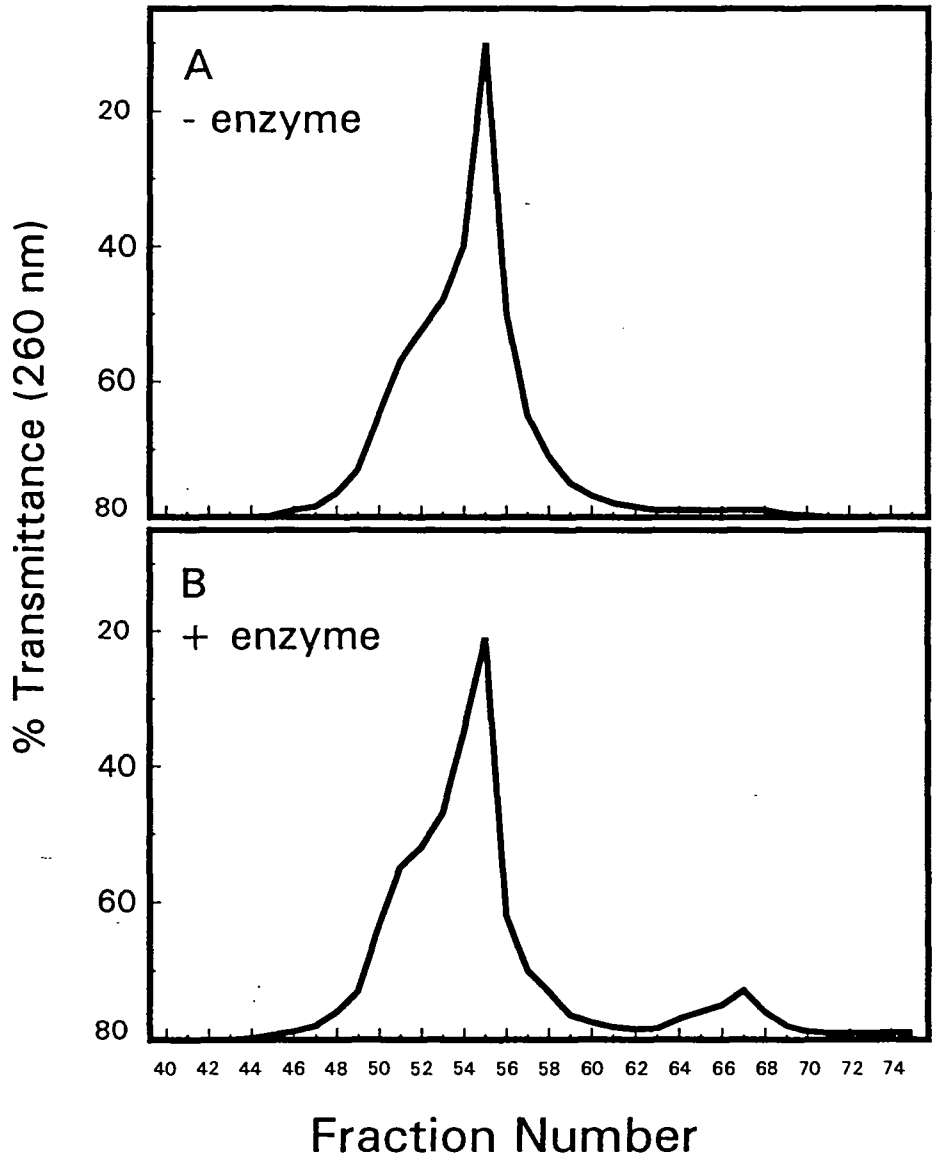

A
- enzyme
B
+ enzyme
% Transmittance (260 nm)
20
40
60
80
40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70 72 74
Fraction Number

Figure 14.

Figure 15. Effect of light or dark preincubation of enzyme+FAD (EF), enzyme+substrate (ES), enzyme (E), FAD (F), and substrate (S) on the extent of *cis-trans* isomerization of zeatin following incubation of the complete assay mixture in light (cool-white fluorescent light). Preincubation and incubation were carried out for 30 min. at 35°C. Incubation occurred after the addition of substrate, FAD, FAD+substrate, enzyme+substrate and enzyme+FAD respectively. All the components of the assay were used at the following standard concentrations: 10 μl of FAD (1 mM), 10 μl of $MgCl_2$ (0.4 M), 70 μl of Tris-HCl buffer (55 mM, pH 7.5, containing 0.5 mM DTT) with 30 μl-equivalent of Phenyl Superose-purified endosperm or without enzyme, and 10 μl of *cis*-^{14}C-zeatin (0.48 nmol). Substrate and product were separated by HPLC on a reversed-phase C_{18} column and quantified by determining radioactivity using a Beckman LS 7000 scintillation counter. Enzymatic formation of *trans*-zeatin was determined by subtracting the amount of *trans*-^{14}C-zeatin formed in the absence of the enzyme from that obtained in the presence of the isomerase.

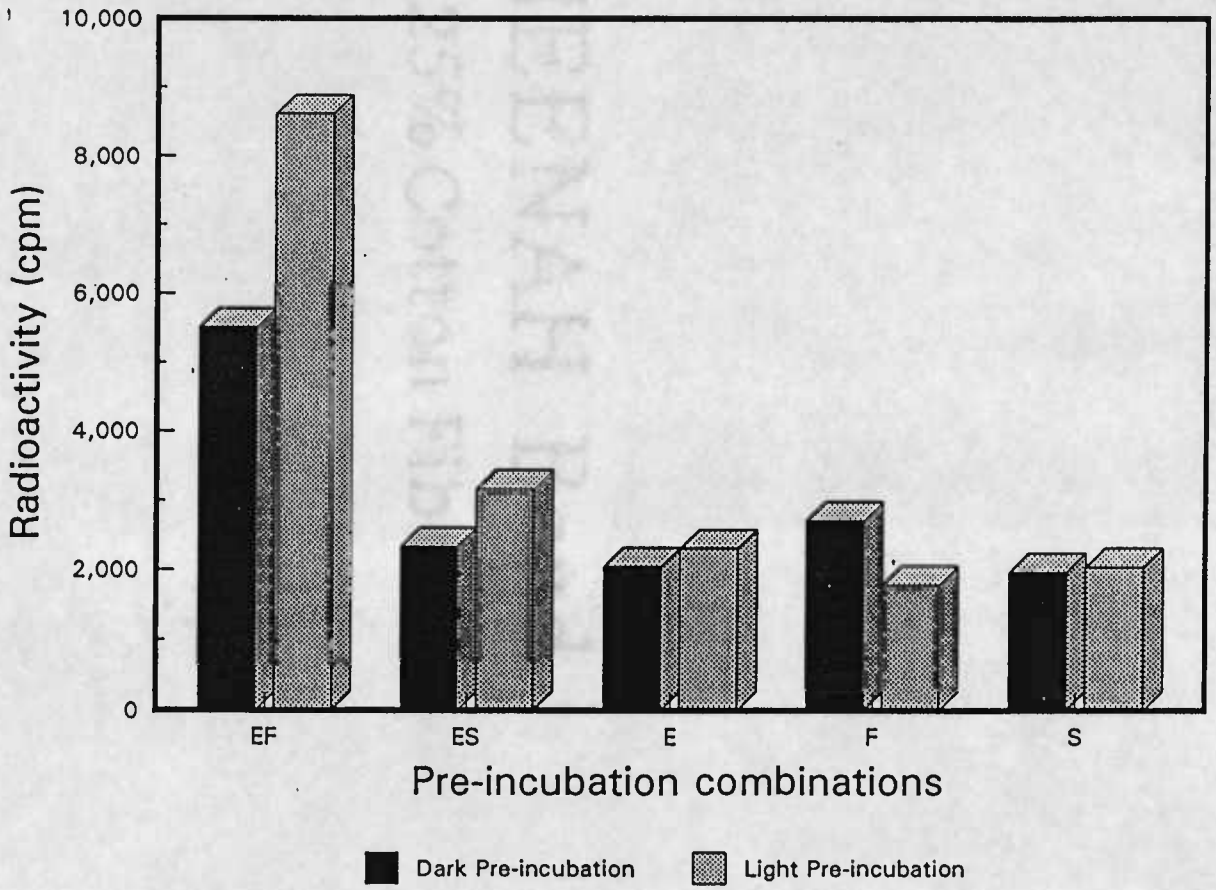

10,000
8,000
6,000
4,000
2,000
0
Radioactivity (cpm)
EF
ES
E
F
S
Pre-incubation combinations
Dark Pre-incubation
Light Pre-incubation

Figure 15.

low amount of *trans*-zeatin (only about 2,000 cpm).

DISCUSSION

The isolation and partial purification of the *cis-trans* zeatin isomerase confirmed our earlier observation that interconversion of the two forms of zeatin occurs in immature seeds of *Phaseolus* (Mok et al., 1992). Conversion from the *cis* to *trans* form is, however, favored. Presence of all the cofactors results in low non-enzymatic conversion. Similarly, under favorable conditions, non-enzymatic isomerization is common to many compounds including geraniol and geranyl phosphate (Shine and Loomis, 1974), *cis* and *trans*-stilbene-4 carboxylic acid (Posthuma and Berends, 1966), all-*trans*-retinol and 13-*cis*-retinol (Gordon-Walker and Radda, 1967) as well as all *trans*-retinal (Futterman and Rollins, 1973). The presence of the *cis-trans* zeatin isomerase, however, enhances conversion significantly. Similar enhancement in geraniol and geranyl isomerization to their respective nerol isomers has been observed in the presence of enzyme extracts from carrot and peppermint (Shine and Loomis, 1974).

The three essential requirements for isomerization are flavin, light and DTT. The flavin FAD seems to bind to the enzyme since preincubating them together, irrespective of light conditions, seems to enhance *cis-trans* isomerization. In photosensitized reactions such as the zeatin *cis-trans* isomerization, photons are absorbed by the sensitizer (FAD in this case). The resulting energy rich state(s) of FAD (triplet or semiquinone radicals) then undergoes reactions that ultimately result in the chemical alteration of another molecule in the system (ie., substrate or enzyme). Loomis and Shine (1974) observed that extensive reduction of the flavin greatly

inhibited isomerization, therefore indicating the possible involvement of the flavin semiquinone in geraniol isomerization.

It is a well known fact that flavins might serve as blue light photoreceptors. Zeatin isomerase, a flavin-requiring protein, needs blue light of wavelengths ranging from 400-500 nm for *cis* to *trans* conversion. Such range indicates the possible involvement of the 450 nm flavin absorption band. The light requirement for isomerization could stem from its direct role in the formation of the flavin triplet state and/or flavin semiquinone radicals.

The third necessary factor for zeatin *cis-trans* isomerization, DTT, is a sulfhydryl containing compound. Another sulfhydryl reagent, glutathione, is required for conversion of maleylacetoacetate to fumarylacetoacetate by maleylacetoacetate *cis-trans* isomerase (Angaw-Duguma, 1992). Studies using maleylacetone as an analogue indicated that substrate and coenzyme bind to the enzyme. The glutathiyl group adds to C2 of the substrate to form a diendiol intermediate. Internal rotation is then followed by expulsion of glutathione to yield the *trans* isomer (Angaw-Duguma et al., 1992). The zeatin *cis-trans* isomerase, however, requires the presence of FAD and light in addition to DTT. Therefore, DTT's presence could be important for stabilizing FAD in a particularly reactive state. It appears that this stabilization might be achieved through an association between the flavin and the sulfur compound (Muller and Massey, 1969). DTT could also activate a cysteine residue in the enzyme or reduce an essential disulfide thus freeing a sulfhydryl group(s) on the isomerase which could then interact with the flavin. Alternatively, DTT might be involved in the association/dissociation between the substrate and FAD semiquinone as was suggested by Shine and Loomis

(1974) who also reported that the thiol reagent was not simply acting as a reducing agent since DTT could not be replaced by the reducing agents sodium ascorbate, NADH, partially reduced flavin or EDTA. In fact, EDTA was inhibitory to both zeatin and geraniol isomerization. The zeatin assay mixture contained $MgCl_2$, a source of Mg^{2+}, which seemed to stimulate isomerization. Therefore, the inhibitory action of EDTA could stem from its ability to chelate Mg^{2+}. On the other hand, excessive photereduction of the flavin may occur in the presence of EDTA and light (Shine, 1973).

Studies of substrate specificity indicated that the enzyme is capable of not only converting *trans*-zeatin but also both *cis*- and *trans*-zeatin ribosides. However, the reaction favored formation of *cis*-zeatin riboside, the isomer predominantly found in t-RNAs, and only traces of the *trans* isomer were formed. The possibility that cytokinins may be derived from degradation of tRNA through the indirect pathway, in addition to being synthesized *de novo* through the direct pathway, has been debated for many years. The direct pathway consists of condensation of AMP and Δ^2iPP by an isopentenyl transferase followed by hydroxylation of the corresponding side chain. Evidence for *de novo* biosynthesis of cytokinins in plants comes from the partial purification of the isopentenyl transferase from slime mold (Ihara et al., 1984) and from cytokinin-autotrophic tobacco tissue cultures (Chen and Melitz, 1979; Chen, 1982). Further support for the direct pathway is evident from the recovery of ^{14}C-labelled-zeatin or dihydrozeatin derivatives after feeding ^{14}C-adenine to developing seeds and pod walls of peas (Van Staden and Drewes, 1993) as well as to germinating seeds of lupin (Nandi et al., 1988) and maize (Hocart and Letham, 1990; Van Staden and

Drewes, 1994). No isopentenyl derivatives were ever recovered in the feeding studies undertaken so far, presumably due to their rapid conversion to their zeatin counterparts. However, hydroxylase activity has been reported in various plant tissues (Miura and Miller, 1969; Miura and Hall, 1973; Palni et al., 1983; Einset, 1984; Chen and Leisner, 1984) but the enzyme responsible for such hydroxylation has not been isolated. The importance of the indirect pathway as a cytokinin biosynthetic route has been disputed by many scientists (Letham and Palni, 1983). A problem often cited is the low turnover rates of cytokinin containing t-RNAs. However, selective turnover of cytokinin rich t-RNAs occurs in animal tissues (Borek et al., 1977). Furthermore, according to the calculations of Barnes et al. (1980) and Maaß and Klämbt (1981), cytokinins in tRNA could contribute 40-50% of the total cytokinin pool. Therefore, the reported low turnover rates of these t-RNAs could have been underestimated. Still, the major problem with this pathway is the predominance of inactive *cis*-zeatin riboside in tRNA while the free cytokinins are the *trans* isomers. Thus, our discovery of a *cis-trans* isomerase forges a critical link between biologically inactive and active pools of cytokinins. However, we can not predict at this point the extent of the contribution of isomerization to the overall biosynthesis of cytokinins.

Even though the *trans* form of zeatin is the predominant form of free cytokinins in plants, *cis*-zeatin and/or its riboside have been reported for a number of plant species, including potato (Mauk and Langille, 1978), sweet potato (Hashizume et al., 1982), *Dolichos lablab* (Yokota et al., 1981), hops (Watanabe et al., 1978, 1981), *Mercurialis* (Dauphin et al., 1979), rice (Izumi et al., 1988), wheat (Parker et al., 1989), and oat (Parker et al., 1989). The *cis*-derivatives were prominent in underground

parts, roots of rice and tubers of potato and sweet potato, and in xylem sap of wheat and oat collected just above ground level. It is tempting to speculate that the absence of light may have precluded isomerization to the *trans* forms. Significant quantities of *cis*-zeatin riboside were also found in cones of hops (Watanabe et al., 1982), in immature seeds of Dolichos lablab (Yokota et al., 1981) and in shoot apices of *Mercurialis* (Durand et al., 1992). In the latter, formation of male fertile and male sterile flowers was correlated with the occurrence of *trans*- and *cis*-zeatin derivatives respectively (Durand et al., 1992). Thus, interconversion between *cis*- and *trans*-zeatin may play a role in developmental events such as shoot differentiation and male gamete production.

The dependence of zeatin *cis-trans* isomerization on the presence of light brings to mind many processes that are equally influenced by cytokinins and light. Cytokinins mimic the effect of light on various aspects of plant growth and development. For example, light and cytokinin can singly induce the accumulation of the light-harvesting chlorophyll a/b binding protein (Cab), the small subunit of ribulose-1,5-bisphosphate carboxylase (SSU) and the corresponding mRNAs (Fierabend and De Boer 1978; Lerbs et al., 1984; Flores and Tobin, 1986; Flores and Tobin 1988), thus enhancing chloroplast development and the photosynthetic capability of the plant. In addition, nitrate reductase activity was induced by light or, in the dark by cytokinin (Lips and Roth-Bejerano, 1969; Kende et al., 1971). Furthemore, the decrease in phytochrome mRNA levels observed upon treatment of etiolated cucumber cotyledons with light or cytokinin (Cotton et al., 1990), led Crowell and Amassino (1994) to propose a process of light- and cytokinin-induced desensitization of plant cells to light. The ability of light

to increase the level of active cytokinins through the potential action of the *cis-trans* isomerase also suggests another process by which light and cytokinin interaction could affect plant growth and development. It would be interesting to elucidate at the molecular level any relationship that might exist between light, the possible blue light photoreceptor and cytokinins.

In conclusion, our discovery of an isomerase system capable of converting the biologically inactive *cis*-zeatin derivatives to their *trans*-zeatin counterparts indicates that the indirect pathway of cytokinin biosynthesis could contribute to the pool of active cytokinin in plants. It is difficult to estimate the extent of this contribution. Purification of the isomerase to homogeneity and isolation of the corresponding gene from plant tissues and identification of their respective properties might prove useful in evaluating the importance of the zeatin *cis-trans* isomerase to cytokinin biosynthesis and to plant growth and development.

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