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Doctoral Thesis

Biosynthesis of Indole-3-Acetic Acid in Higher Plants

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Dedicated to my respectable supervisor, the late Dr. Susumu Kuraishi, who introduced me into the field of plant physiology and giving me the very interesting and exciting theme, study of indole-3-acetic acid biosynthesis.

CONTENTS

Abbreviations

ABP, auxin binding protein

AP, apoplast

AS, ammonium sulfate

ATP, adenosine 5'-triphosphate

CWF, cell-wall fraction

D-CS, D-cycloserine

GA, gibberellin

GC-(SIM-)MS, gas chromatography-(selected ion monitoring-) mass spectrometry

HPLC, high-performance liquid chromatography

IAA, indole-3-acetic acid

IAAld, indole-3-acetaldehyde

IAM, indole-3-acetamide

IAN, indole-3-acetonitrile

IAox, indole-3-acetaldoxime

IEt, indole-3-ethanol

IGP, indole-3-glycerol phosphate

IPA, indole-3-propionic acid

IPyA, indole-3-pyruvic acid

NAA, 1-naphthaleneacetic acid

N-OH-Trp, *N*-hydroxy-tryptophan

SEF, soluble enzyme fraction

SY, symplast

TLC, thin layer chromatography

TMS, trymetylsyllil

TNH_2, tryptamine

Trp, tryptophan

Me, methyl

Chapter 1

General Introduction

1.1 Discovery of First Plant Hormone, Indole-3-Acetic Acid

Plant growth and differentiation are regulated by plant hormones, such as auxin, gibberellins, cytokinins, abscisic acid and ethylene. Auxin was found as a plant growth substance for the first time. The term auxin is derived from the Greek *auxein*, which means "to grow". The discovery of auxin can be traced back to an original observation by Darwin who investigated the phototropic curvature of coleoptiles of grass seedlings (Darwin 1880). From partial illumination experiments he deduced that illumination of the organ tip (from the side) has a "causal influence on curvature growth" of the lower part of the organ. The Dutch plant physiologist, Went, found evidence in his classical experiments of 1926 that led to the physiological proof of auxin as a plant "growth substance". In his experiments he used the coleoptile of the oat seedling (*Avena sativa*), a juvenile organ able to elongate rapidly, and which is still used today as the standard material for auxin research. The experiment of a growth-triggering substance but is also a paradigm for the separation, of *incompetent site of formation* and *competent site of action*. The "growth substance" of oat coleoptiles may be demonstrated specifically and determined quantitatively with a sensitive *biotest* developed by Went (1928). This agent was soon found to be also produced in the plants in "diffusible" form. However, the amounts obtained in this way are so extremely small that initially it was not possible to identify the effective component chemically. Kögl and Haggen-Smit (1931), starting with 33 gallons of human urine, performed a series of purification steps, and after each step they tested biological activity using the *Avena* curvature test. Their final purification step yielded 40 mg of purified compound which they called *Auxin A* (*auxentriolic acid*). Later Kögl et al. (1934a, 1934b) once again analyzed human urine and found a compound similar in structure and activity to auxin A and give it the name *auxin* (*auxenolenic acid*). Also contained in this extract was *heteroauxin* (*other auxin*), now known as indole-3-acetic acid (IAA), which had been initially discovered and characterized by Salkowski (1885). Kögl and Kostermans (1934) isolated IAA from yeast and Timann (1935) isolated IAA from cultures of *Rhizopus suinus*. It was not until 1946 that Haggen-Smit et al. isolated pure IAA from the endosperm of immature corn grains showing that IAA was found in a higher plant. Vliegenthart and Viegenthart (1966) presented

evidence that auxin A and B are not natural plant products; however, IAA has since been isolated from numerous plant species and has been shown to be ubiquitous in the plant kingdom. Went's auxin was probably mostly IAA, however, other growth promoters may have been in his initial diffusion studies on the phototropic response, possibly IAA derivatives.

Members of almost every group of living organisms known to be capable of producing IAA; it is formed by numerous species of bacteria (Robarts and Robarts 1939, Stowe 1955, Wichner and Libbert 1968) and fungi (Gruen 1959); it has been found in a variety of animals (Went and Thimann 1973, Gordon and Buess 1967) and is produced in developing chick embryos (Robinson and Woodside 1973). Considerable quantities are excreted in human urine, which is one of the sources from which IAA was first isolated. The IAA in human urine is not simply derived from plant material in the diet, nor can more than a third of it be attributed to auxin production by the microflora of the gut: the majority is actually formed in the human body (Weissbach et al. 1959). The rate of human auxin production, expressed in terms that permit comparison with auxin production in plants, is about $5–50\times10^{-12}$ g/mg/h. Coleoptile tips of *Avena* yield 50×10^{-12} g/mg/h (Went and Thimann 1937). This comparison emphasizes that IAA production in plants should not be regarded as an isolated biochemical phenomenon.

1.2 Research of Indole-3-Acetic Acid Biosynthesis Pathway in Higher Plants

Although IAA is most probably the universal auxin of higher plants and the biosynthesis pathway has been extensively studied, the pathway and the site has not yet been demonstrated unequivocally. Following the discovery of IAA, many indole compounds have been reported in plant; however, auxin activity of each of these can probably be attributed to their conversion to IAA. The site of IAA synthesis is not clear, although the site is thought generally in leaf primordia and young leaves, and in developing seeds.

IAA Synthesis from Tryptophan

Tryptophan (Trp) is now generally accepted as the primary precursor for the biosynthesis of IAA, since Trp has a close structure to IAA and also Trp is ubiquitous in plant tissues. Thimann (1935) showed that Trp could be converted to IAA in mold. Wildman et al. (1947) found an enzyme system capable of converting Trp to IAA in spinach leaves. Since this time many other

researchers have demonstrated that Trp is the primary precursor in higher plants. This pathway came under fire by researchers suggesting that bacterial contamination was responsible for the production of IAA (Libbert et al. 1966). However, it was then shown that Trp could be converted to IAA under sterile conditions by Muir and Lantican (1968). In the study using [^{14}C]Trp as a tracer, Koshiba et al. (1995) suggested that in maize (*Zea mays* L.) coleoptiles a major portion of IAA was synthesized from Trp.

An enormous body of knowledge demonstrates that IAA can be synthesized from Trp; a summary of some of the known reactions is presented in Fig. 1.2.1. Two main pathways of IAA biosynthesis from L-Trp have been proposed.

One hypothesis is that L-Trp is converted to indole-3-pyruvic acid (IPyA) by an aminotransferase and then IPyA is converted to IAA via indole-3-acetaldehyde (IAAld). Accordingly, the only enzyme required for formation of IAA in the system is an L-Trp aminotransferase, for which very high K_m values for L-Trp have been reported, e.g. 0.33 mM in Phaseolus aureus (Truelsen 1972), 5.0 mM in tomato shoots (Gibson et al. 1972a). The IAA concentration in growing tissue is maintained in the nM range and it is hardly likely that such a low level is regulated by an enzyme with so high a K_m value (Sheldrake 1973). Moreover, although enzyme activities for each step in the IPyA pathway have been identified in plants, and the genes encoding IPyA decarboxylase have been isolated from *Enterobacter cloacae* (Koga et al. 1991) and *Azospirillum brasilense* (Costacurta et al. 1994), none of the plant genes encoding these enzymes has been cloned. Law (1987) suggested that IAA was synthesized by the conversion of L-Trp to IPyA via D-Trp, and that the Trp racemase (the activity of which was stimulated by GA), might regulate conversion of L-Trp to IAA in dwarf pea plants. McQueen-Mason and Hamilton (1989) proposed that a D-Trp aminotransferase is important in IAA biosynthesis in tall pea plants.

Another hypothesis is that L-Trp is converted to tryptamine by Trp decarboxylase, and tryptamine (TNH_2) is converted to IAAld by monoamine oxidase (Hill and Mann 1964, Sherwin 1970). This pathway is also doubtful because neither enzyme exist universally in plants (Gibson et al. 1972a,b). The gene encoding this enzyme has been isolated from *Catharanthus roseus* (De Luca et al. 1989) and TNH_2 has been identified as a native compound in tomato (Cooney and Nonhebel, 1991). Transgenic tobacco expressing the *C. roseus* Trp decarboxylase gene under control of the cauliflower mosaic virus 35S promoter accumulated TNH_2 but not IAA (Songstad et al. 1990). This appears to negate a role for TNH_2 in IAA biosynthesis, although it could be argued that 35S promoter did not direct expression of Trp decarboxylase in a manner that was temporally

and spatially compatible with the other enzymes in this pathway.

A possible role for aldehyde oxidase in IAA biosynthesis was suggested in maize coleoptile (Koshiba et al. 1996). Aldehyde oxidase could catalyze the oxidation of IAAld to form IAA (Rajagopal 1971, Bower et al. 1978, Miyata et al. 1981). Koshiba and Matsuyama (1993) reported that an in vitro system of maize coleoptile extracts catalyzed the production of IAA from Trp and that the IAA-forming activity was co-purified with an IAAld oxidase.

For most of the last half century, research on biosynthesis of IAA has focused on different possible routes for the conversion of Trp into IAA. Other pathways excepting two main pathways also were discussed. The indole-3-acetaldoxime (IAox) — indole-3-acetonitrile (IAN) pathway for the conversion of Trp to IAA is characteristic of the Brassicaceae (Kutacek and Kefeli 1968). With the unequivocal demonstration of IAox, which appears to be synthesized from *N*-hydroxy-tryptophan (N-OH-Trp), in some higher plants (Ludwig-Müler and Hilgenberg 1988), other routes became apparent: IAox → IAAld → IAA; and IAox → IAN → IAA. Especially, IAox conversion to IAN has been demonstrated in plasma membrane of Chinese cabbage (Ludwig-Müler and Hilgenberg 1990). The nitrilase that converts IAN to IAA has been cloned in *Arabidopsis* (Bartel and Fink 1994, Bartling et al. 1994).

Pseudomonas savastanoi, by over-producing and secreting IAA into infected tissues, causes galls in olive and oleander (Nester and Kosuge 1981). Through a unique feat of genetic engineering, it transfers a piece of its plasmid DNA (tumor-inducing or Ti-DNA) to the host genome, where it gets integrated stably. This T-DNA contains genetic information for the production of two enzymes, a Trp monooxygenase and an amidohydrolase. While the monooxygenase converts Trp to indole-3-acetamide (IAM), the second enzyme, amidohydrolase, produces IAA from the amide (Prinsen et al. 1990). The functions and locations of the genes coding for these two enzymes have been determined on the T-DNA introduced into the host subsequent to infection.

The occurrence of IAM has been reported in mung bean seeds (Isogai et al. 1967), *unshiu* orange fruits (Takahashi et al. 1975), the club-root of chinese cabbage (Tamura et al. 1972), wounded potato tuber (Rausch et al. 1985) and aseptically raised cherry seedlings (Saotome et al. 1993). IAM hydrolysing activity leading to IAA formation was detected in sterile calli of wild and cultivated rice varieties (Kawaguchi et al. 1991); and in cell-free preparations obtained from either surface-sterilized or peeled tissues of young *trifoliata* orange fruits (Kawaguchi et al. 1993). The conversion of L-[^{14}C]Trp to IAA via IAM has been demonstrated (Kawaguchi et al. 1993).

Despite these observations, the natural occurrence of IAM in higher plants and its precursory role in IAA biogenesis are doubted (Schröder et al. 1984, Weiler and Schröder 1987).

Concerns about the low rate of labeling from Trp of the IAA pool (Greenberg et al. 1957, Wright et al. 1991) were largely overwhelmed by the preponderance of studies on Trp conversion in the literature. Recently it has been established that in some plants the quantitative importance of Trp conversion relative to other possible sources is minor (Baldi et al. 1991) and that plants that cannot make Trp at all are, nevertheless, able to make IAA *de novo* (Normanly et al. 1993, Wright et al. 1991). There are several potential pitfalls in this field, not the least of which includes the enormous disparity in the amounts of Trp and IAA in tissues. The pool size of Trp is three, or more, orders of magnitude larger than that of IAA (Epstein et al. 1980).

De novo synthesis of IAA not involving tryptophan

Although early studies equated IAA synthesis from Trp with *de novo* biosynthesis, recent more exacting and critical evaluations of when in a plant's life it beings or stops making IAA from early precursors have yielded surprising results. Experiments from several laboratories in which young plants of *Zea mays*, *Arabidopsis*, or pea, and cell cultures of carrot were grown on 30% deuterium oxide demonstrate that IAA is made by a route resulting in the incorporation of deuterium molecules into non-exchangeable positions of the indole ring of IAA to a greater extent than that found in Trp (Cooney and Nonhebel, 1991, Michalczuk et al. 1992, Normanly et al. 1993, Pengelly and Bandurski 1983, Wright et al. 1991). Other experiments (Bandurski et al. 1992) employing the deuterium incorporation technique, indicated that Trp synthesis begins before IAA biosynthesis in germinating *Zea mays* kernels. Thus, Trp and IAA synthesis appear to occur independently, and there is not *necessarily* an incorporation of deuterium from Trp into IAA

Perhaps the most striking of these isotopic labeling studies is the report on the *orange pericarp* (*orp*) mutant of maize. This plant carries a double recessive trait caused by mutation of both genes in maize that encode the protein for Trp synthase b. Despite this metabolic block in the terminal step for Trp biosynthesis, the *orange pericarp* mutant produces IAA *de novo* and, in fact, accumulates up to 50 times the level of IAA as do non-mutant seedlings. Labeling studies establishes that the *orp* mutants are able to convert [^{15}N]anthranilic acid to IAA, but do not convert it to Trp. Neither *orp* seedlings nor control seedlings convert Trp to IAA in significant amounts even when the *orp* seedlings are fed levels of stable isotope labeled Trp high enough to reverse the lethal effects of the mutation (Wright et al. 1991). These results established that non-Trp

biosynthesis of IAA does occur, and suggested that the non-Trp pathway actually predominates over the Trp pathway.

Despite the demonstration and now wide acceptance that IAA biosynthesis can occur without the amino acid Trp as an intermediate, the exact pathway for the production of IAA by such a route is not yet known. *In vivo* labeling techniques using *Arabidopsis* mutants (Normanly et al. 1993) have extended the findings from the *orange pericarp* maize study, and suggest that the branch point for IAA production is probably at the point of indole (following Trp synthase a) or its precursor, indole-3-glycerol phosphate (IGP, the conversion of indoleglycerol phosphate to indole is a reversible reaction). Most current evidence favors the condensation of indole with a two carbon unit with a nitrogen at the terminal carbon, followed by conversion to the carboxylic acid. Until the pathway can be established using both *in vivo* and *in vitro* techniques, the data is only useful as a guide for further investigations. Rekoslavskaya et al. (1992) reported obtaining an *in vitro* system from maize endosperm capable of converting radioactive indole into IAA by a reaction which is not inhibited by the addition of unlabeled Trp. The availability of this *in vitro* system should now make it possible to establish the biochemistry of the conversion. Moreover, although earlier studies suggest that IAN is derived from Trp, IAN accumulates in the *Arabidopsis trp2* mutant, indicating that it could be derived independently of Trp (Normanly et al. 1993).

Whether is Tryptophan or Non-Tryptophan ?

These recent developments have certainly changed my concepts of IAA biogenesis from what I know only a few years ago. It is important to keep in mind, however, that the establishment of the existence of a non-Trp pathway does not change the fact that many plant species have been shown to convert Trp to IAA, and in some cases this conversion is clearly at rates that make it important for the auxin economy of the plant. For example, in the bean seedling, *de novo* IAA biosynthesis begins even before the stored conjugates are fully used up (Bialek and Cohen 1992, Bialek et al. 1992), and this biosynthesis comes primarily from Trp conversion. Similarly, Michalczuk et al. (1992) showed that in embryogenic carrot suspension cultures, the conversion of Trp to IAA is the primary route. However, when 2,4-dichlorophenoxyacetic acid was removed from the medium, which induces these carrot cells to form embryos, the conversion of Trp to IAA was no larger the primary route, and the non-Trp pathway appeared to predominate. These metabolic interactions, and the regulation of these pathways in relation to particular developmental programs, are clearly exciting topics for further detailed studies at a molecular level.

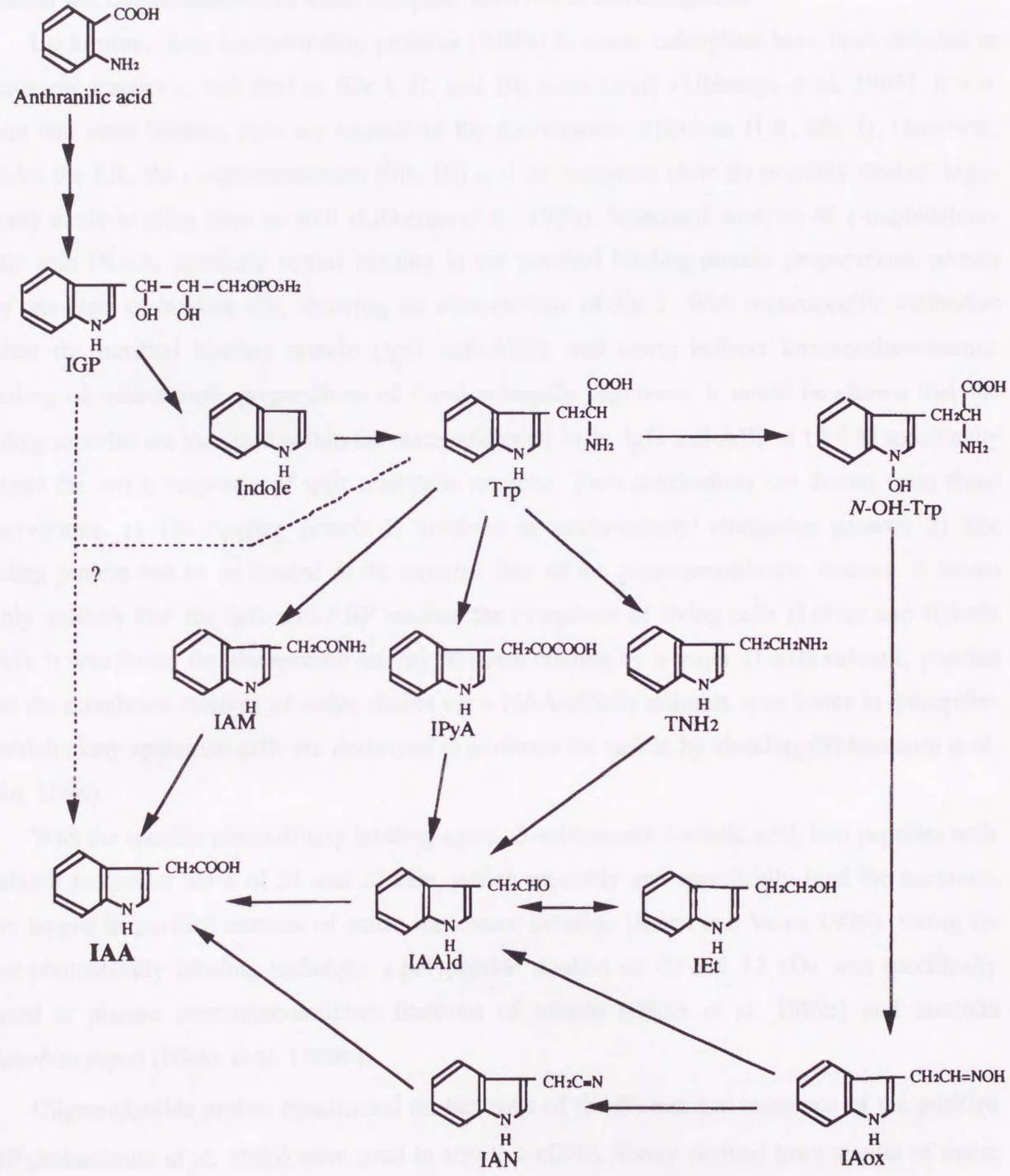

Fig. 1.2.1. Hypothetical pathways of IAA biosynthesis.

1.3 Auxin Receptor for Cell Elongation in Higher Plants

In auxin-receptor research coleoptiles from *Zea mays* have been studied most extensively. Therefore the results obtained with this system illustrate the progress that has been made in the isolation and characterization of auxin receptors involved in cell elongation.

Up to now, three auxin-binding proteins (ABPs) in maize coleoptiles have been detected in microsome fractions, indicated as Site I, II, and III, respectively (Libbenga et al. 1985). It was found that most binding sites are located on the endoplasmic reticulum (ER, Site I). However, besides the ER, the plasmamembrane (Site III) and the tonoplast (Site II) possibly contain high-affinity auxin-binding sites as well (Libbenga et al. 1985). Scatchard analysis of 1-naphthalene-acetic acid (NAA, synthetic auxin) binding in the purified binding-protein preparations reveals only one class of binding site, showing the characteristic of Site I. With monospecific antibodies against the purified binding protein (IgG anti-ABP), and using indirect immunofluorescence labelling of microscopic preparations of fixed coleoptile segments, it could be shown that the binding proteins are localized within the outer epidermal cells. IgG anti-ABP at 10^{-8} M specifically reduces the auxin response of split coleoptile sections. Two conclusions are drawn from these observations. 1) The binding protein is involved in auxin-induced elongation growth; 2) The binding protein has to be located at the external face of the plasmamembrane, because it seems highly unlikely that the IgG anti-ABP reaches the cytoplasm of living cells (Löbler and Klämbt 1985). It was found that the specific activity of auxin binding by a major 21-kDa subunit, purified from the membrane fraction of maize shoots via a NAA-affinity column, was lower in coleoptiles in which many epidermal cells are destroyed to perforate the cuticle by abrading (Shimomura et al. 1986, 1988).

With the specific photoaffinity labeling agent, 5-azidoindole-3-acetic acid, two peptides with a subunit molecular mass of 24 and 22 kDa, which saturably and specifically bind the hormone, were tagged in purified extracts of maize membrane proteins (Jones and Venis 1989). Using the same photoaffinity labeling technique a polypeptide doublet of 40 and 42 kDa was specifically labeled in plasma membrane-enriched fractions of tomato (Hicks et al. 1989a) and zucchini (*Cucurbita pepo*) (Hicks et al. 1989b).

Oligonucleotide probes constructed on the basis of the *N*- terminal sequence of the purified ABP (Shimomura et al. 1986) were used to screen a cDNA library derived from shoots of maize seedlings (Inohara et al. 1989). The predicted precursor for the binding protein contains 201

amino acid residues and has a molecular weight of 22 kDa. The sequence indicated a 38 amino acid signal peptide as had been calculated from *in vitro* translation experiments (Löbler et al. 1987). The hydrophobic *N*- terminal leader sequence could represent a signal for translocation of the ABP to the ER. The mature ABP contains a potential *N*- glycosylation site as predicted before (Löbler et al. 1987, Napier et al. 1988), and, at the C-terminus, a tetrapeptide sequence, KDEL (lys-asp-glu-leu), known as a common signal for protein that are retained within the lumen of the ER. The presence of a KDEL sequence is in accordance with the finding by two groups that the 22-kDa ABP subunit and Site I activity comigrate (Jones et al. 1989, Shimomura et al. 1988).

Functional evidence for an auxin receptor localized at the plasmamembrane was reported for tobacco mesohyll protoplasts (Barbier-Brygoo et al. 1989). Auxin-induced hyperpolarization of membrane potential can be measured by a microelectrode technique (Ephritikhine et al. 1987), and this functional test was used to show the effects of polyclonal antibodies raised against the ABP of maize (Löbler and Klämbt 1985). Of particular interest is the finding that addition of purified maize ABP increases the sensitivity of the protoplasts towards NAA (Barbier-Brygoo et al. 1990).

Impermeant auxin analogues (Venis et al. 1990) are capable of inducing membrane hyperpolarization and extension growth, though only when the cell walls were removed, or the epicotyls were abraded to perforate the cuticle, respectively. This shows that the functional auxin receptors are localized on the outside of the plasmamembrane. It also indicates that auxin can produce both, rapid and long-term responses, without entering into the cell.

1.4 Purpose of This Study

How is IAA synthesized? Where is IAA synthesized? What compound does convert to IAA? What regulates the synthesis of IAA? These riddles have not been solved yet, though it has took about 70 years to study the analysis of IAA synthesis pathway. Although IAA is very simple structure, it may be difficult to analyze the pathway that the compound is very low amounts in the plant tissue and is, moreover, fairly unstable.

Recently, the researches of Cohen's group (Michalczuk et al. 1992, Normanly et al. 1993, Wright et al. 1991) have suggested that IAA is not synthesized from Trp. But, they could not suggest the alternative pathway of IAA synthesis, though indole, which is a precursor of Trp, may be a precursor of IAA. Koshiba et al. (1995), however, suggested that a major portion of IAA was

synthesized from Trp. The pathway of IAA synthesis is still kept completely in darkness.

Therefore, to review the pathway and the regulation site of IAA synthesis, I investigated the activity of IAA synthesis to use various compounds, which may be the precursors of IAA, in monocotyledonous coleoptile of barley mutant, which is a mutant of lower endogenous IAA (Chapter 2.1, 2.2), and dicotyledonous squash roots (Chapter 3.1, 3.2).

The study of impermeant auxin analogues (Venis et al. 1990) shows that the functional auxin receptors are localized on the outside of the plasmamembrane. If IAA acts on cell elongation from outside of the cell, IAA should be present at high concentration in apoplastic space and may be synthesized even in the outside of the cell in the growing region in the plant. I also studied the role of the outside space of the cell to IAA synthesis (Chapter 2.2), and the distribution of IAA in apoplast and symplast (Chapter 2.3).

Chapter 2

Biosynthesis of Indole-3-Acetic Acid in Shoots in Higher Plants

The site of IAA biosynthesis is thought generally to exist in shoot apexes, coleoptile tips in young plant seedlings. In this chapter, at first, I studied the possibility of the synthesis of IAA via Trp. Next, I describe the possibility of IAA synthesized in cell wall (apoplastic space?) in monocotyledonous barley coleoptile and IAA existence in apoplast in growing region of dicotyledonous squash hypocotyls.

2.1 Conversion of D-Tryptophan to Indole-3-Acetic Acid in Coleoptiles of A Normal and A Semi-Dwarf Mutant Barley (*Hordeum vulgare*) strain

Abstract

Exogenously applied D-Trp was more effective than L-Trp in inducing elongation of coleoptile segments of a normal barley (*Hordeum vulgare* L. cv. Akashinriki) strain and a semi-dwarf strain with lower endogenous IAA level. D-cycloserine (D-CS), an inhibitor of D-Trp aminotransferase, completely inhibited both the D- and L-Trp-induced elongation of both strains. Addition of D-Trp increased IAA level in both strains 4-fold over endogenous levels. The increase in IAA level was completely inhibited by D-CS. The endogenous L-Trp level of semi-dwarf coleoptiles was similar to that of normal ones. These results suggested that IAA is synthesized by the conversion of L-Trp to IPyA via D-Trp in both strains, and that the lower IAA level of the semi-dwarf strain probably is a result of the impeded IAA biosynthesis involved in D-Trp.

Introduction

Most of physiological studies of the cause of dwarf plants have been focused on the role of gibberellins (Brian and Hemming 1955, Lockhart 1956, Cooper 1958, Loy and Liu 1974, Perez et al. 1974). However, dwarfism has not always been explained only by the deficiency of

gibberellins (Kuraishi 1974, Wylie and Ryugo 1971). Van Overbeek (1938) first showed that less auxin content in dwarf corn coleoptile is one of the causes of dwarfism. Inouhe et al (1982) demonstrated that the dwarfism of coleoptiles of eleven barley strains (Hordeum vulgare L. cv. Akashinriki) is primarily caused by low levels of endogenous IAA in the dwarf strains.

The pathway of IAA biosynthesis has been extensively studied, but has not yet been demonstrated unequivocally. Trp has been suggested as a primary precursor of IAA (Gordon 1954, 1958, Gibson et al. 1972b, Monteiro et al 1988), since Trp has a close structure to IAA and also Trp is ubiquitous in plant tissues.

Two pathways of IAA biosynthesis from L-Trp have been proposed. One hypothesis is that L-Trp is converted to IPyA by an aminotransferase and then IPyA is converted to IAA. Accordingly, the only enzyme required for formation of IAA in the system is an L-Trp aminotransferase, for which very high K_m values for L-Trp have been reported, e.g. 0.33 mM in Phaseolus aureus (Truelsen 1972), 5.0 mM in tomato shoots (Gibson et al. 1972a). The IAA concentration in growing tissue is maintained in the nM range and it is hardly likely that such a low level is regulated by an enzyme with so high a K_m value (Sheldrake 1973). Another hypothesis is that L-Trp is converted to tryptamine by Trp decarboxylase, and tryptamine is converted to IAAld by monoamine oxidase (Hill and Mann 1964, sherwin 1970). This pathway is also doubtful because neither enzyme exist universally in plants (Gibson et al. 1972a,b). Consequently, the possibility of a new pathway of IAA biosynthesis remains open. Law (1987) suggested that IAA was synthesized by the conversion of L-Trp to IPyA via D-Trp, and that the Trp racemase (the activity of which was stimulated by gibberellin), might regulate conversion of L-Trp to IAA in dwarf pea plants. Recently McQueen-Mason and Hamilton (1989) proposed that a D-Trp aminotransferase is important in IAA biosynthesis in tall pea plants.

To analyze the cause of lower IAA levels of coleoptiles of dwarf barley, the present experiments were undertaken to investigate the importance of D-Trp in IAA biosynthesis pathway and also to try to show the regulation site of IAA biosynthesis in semi-dwarf barley coleoptiles.

Materials and Methods

Plant materials

Two isogenic strains (uzu or semi-brachytic and its corresponding normal line) of the barley

(*Hordeum vulgare* L.) cultivar, Akashinriki, were supplied by The Research Inst. for Resource, Okayama Univ. in 1991. About 700-800 seeds were soaked for 8 h in water, then germinated in the dark in plastic boxes (34 cm × 23 cm × 4 cm) filled with vermiculite at 25.5 ± 0.5°C for 3 days. Seedlings were selected for uniformity of coleoptile length (15-30 mm for the semi-dwarf and 30-50 mm for the normal strain), and 5 mm long coleoptile segments were excised form the region 2 mm below the apex using a double-bladed cutter.All manipulations were conducted under dim green light (0.02-0.04 μmol $m^{-2}s^{-1}$).

Growth experiments

Fifteen coleoptile segments were transferred into a 4-cm Petri dish containing 4 ml of solution consisting of 10 mM K-citrate-phosphate (pH 6.5) with test substances (D-Trp, L-Trp, IAA, D-CS) and 0.1 mM ampicillin (Law 1987). The dishes were incubated at 25.5 ± 0.5°C in the dark on a reciprocating shaker (EYELA, SS-80, Tokyo, Japan) at 60 rpm. The segment length was measured to the nearest 0.05 mm using a binocular microscope (Olympus, Tokyo, Japan).

Extraction and determination of IAA

IAA was extracted and analyzed according to the method of Kuraishi et al (1989). Tow hundred coleoptile segments were incubated at 25.5 ± 0.5°C in the dark for 10 h in a 9-cm Petri dish containing 10 ml of the treatment solution. After incubation, coleoptile segments were blotted dry and blended for 1 min using an ultrasonic homogenizer (Ika Werk, Breisgau, FRG, Ultratullax Tp 18/10 S2) in 70 ml of 80% ethanol containing 50 mg of butylated hydroxy toluene and 0.5 g of polyvinylpyrrolidone (Katayama, Osaka, Japan) and 1 nmole indole-3-propionic acid (IPA) as internal standard.

The homogenate was filtered and the residue was again extracted with ethanol. The filtrates were combined and reduced to 20 ml in vacuo. The pH of the concentrate was adjusted to 3.5 with solid tartaric acid followed by three extractions with petroleum ether. IAA was extracted from the water layer three times with diethyl ether. The combined diethyl ether fractions were evaporated, and the residue was dissolved in 15 ml of 5 mM sodium acetate in 60 % ethanol. The extract was loaded on a DEAE Sephadex A-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) column (1 cm × 6 cm) which was washed with 40 ml of 5 mM sodium acetate in 60% ethanol before elution of IAA with 70 ml of 60% ethanol containing 0.5 M NaCl.

The elute from the DEAE A-25 column was evaporated and the residue was dissolved in 10

ml of 5 mM sodium acetate (pH 3.5). The sample was loaded on a SEP-PAK C-18 cartridge (Waters Associates Co., Milford, MA, USA). The cartridge was washed with 10 ml of deionized water and IAA was eluted with 100% methanol.

The eluate was evaporated and the residue was dissolved in 1 ml of 20 mM sodium acetate in 25% CH_3CN. IAA was assayed using an HPLC system (Japan Spectoscopic Co. Ltd., Tokyo, Japan , TRI ROTOR) equipped with a fluorometric detector (Japan Specrtoscopic Co. Ltd., FP-100). The HPLC column was a Radial Pak μ-Bondapak C-18 (Waters Associates) column. The sample was eluted with 25% CH_3CN solution (pH 3.5, 20 mM sodium acetate) at a flow rate of 2 ml min^{-1}. IAA was determined fluorometrically with an excitation wavelength of 280 ± 5 nm and an emission wavelength of 350 ± 5 nm. The IAA content was calculated from the ratio of the peak area of IPA to that of IAA as reported previously (Kuraishi et al. 1989).

Extraction and purification of the endogenous free Trp

Amino acid in the barley coleoptile segments were extracted and analyzed according to the methods of Hatanaka (1996) and Kuraishi (1973). One hundred coleoptile segments (5 mm in length) were incubated at 25.5 ± 0.5°C in the dark in a 6-cm Petri dish containing 8 ml of the test solution used for growth tests with or without 1 mM L-Trp. After 6-24 h incubation, coleoptile segments were homogenized using a mortar and pestle in 6 ml of 80% ethanol containing 185 Bq of L-[3'-^{14}C]Trp (Amersham International, Amersham Laboratories, Buckinghamshire, UK, 20 GBq mol-1) as internal standard. The homogenate was centrifuged for 5 min at 1600 × g and the pellet was washes twice with 80% ethanol. The combined supernatants were evaporated and then dissolved in 5 ml of deionized water. The sample was loaded on a Dowex 50W-X4 (Muromachi Chemical, Tokyo, Japan) column (1.5 cm × 1 cm) which was washed with 10 ml of deionized water before elution of amino acids with 10 ml of 2 M NH_4OH. The eluate was evaporated and then dissolved in 1 ml of 0.2 M sodium citrate in 5 % ethanol (pH 3.5). Trp was assayed using an HPLC (Gilson Medical Electronics Inc., model 302, Middleton, WI, USA) equipped with a UV detector (Shimadzu Corporation). For the HPLC separation, the starting eluant was 0.2 M sodium citrate in 5% ethanol (pH3.5 adjusted by perchloric acid) and increased to 0.6 M sodium citrate in 5% ethanol (pH 10.0 adjusted by sodium hydroxide) using a linear gradient for 15 min at a flow rate of 0.5 ml min^{-1}, and then 0.6 M sodium citrate in 5% ethanol was run isocratically until Trp was eluted. Trp was eluted from 25 to 34 min. Trp fractions were pooled, evaporated and

dissolved in 0.5 ml of 0.1% perchloric acid in 10% methanol. The sample was eluted from a chiral column (CROWNPAC CR, Daiseru Chemical Industries, Ltd., Osaka, Japan) with 0.1% perchloric acid in 10% methanol [Flow rate 1 ml min^{-1}, UV detection (254 nm)]. D-Trp was completely separated from L-Trp. The retention times of D- and L-Trp were 26 and 34 min, respectively. For the detection of D-Trp, a fluorometric detector (FP 110, Japan Spectroscopic Co. Ltd., Tokyo) was also used. The excitation and emission wavelengths were 280 ± 5 and 350 ± 5 nm, respectively. A portion of the L-Trp fraction was mixed with 12 ml of scintillation cocktail (ACS-II, Amersham), and radioactivity of L-[3'-^{14}C]Trp as internal standard was measured using a scintillation counter (Aloka, Tokyo, Japan, LSC-701). The endogenous free Trp content was calculated from the recovery of the internal standard.

Incorporation of L-[3'-^{14}C]Trp into D-Trp

Fifty barley coleoptile segments were incubated for 2-8 h at 25.5 ± 0 .5°C in the dark in a 6-cm Petri dish containing 8 ml of the test solution used for growth studies with 37 kBq of L-[3'-^{14}C]Trp. Trp isomers were purified according to the methods described above. Radioactivity of the separated L-Trp and D-Trp fractions were determined with a scintillation counter.

Results and Discussion

Effect of Trp on the growth of barley coleoptile segments

Barley seedlings were grown in the dark for 3 days and their coleoptiles were cut and floated on L- or D-Trp (Fig.2.1.1). Without exogenous Trp, the growth of coleoptile segments of the normal strain was much more than that of the uzu strain, probably because the normal coleoptiles had more IAA (Inouhe et al. 1982) and more extensible cell walls than the uzu coleoptiles (Sakurai and Kuraishi 1984). After the addition of L- or D-Trp growth stimulation of both normal and uzu coleoptiles was observed. The growth stimulation of uzu coleoptile by L-Trp was also reported by Kuraishi (1974). Thus, the result implies that both L- and D-Trp may be precursors of IAA. Furthermore, the growth of both types of coleoptile segments was stimulated more by D-Trp than by L-Trp. The results suggest the presence of a pathway of conversion of L-Trp to D-Trp by tryptophan racemase, as proposed by Law (1987), and the greater growth obtained with D-Trp might implicate D-Trp as a more direct precursor for IAA.

Effect of D-CS on growth of barley coleoptile segments

Since D-Trp stimulated the growth of coleoptile segments, I investigated the amount of inhibition of D-Trp-induced coleoptile growth after the treatment with D-CS, an inhibitor of D-Trp aminotransferase (Law 1987). If the growth of the coleoptile segments is promoted by an increased level of IAA which is formed directly from D-Trp, their extension should be blocked by D-CS. The coleoptile segments were incubated for 24 h in the presence of 1 mM D-Trp, L-Trp or 10 μM IAA with or without 1 mM D-CS (Table 2.1.1). Although D-CS did not inhibit the IAA-induced elongation, it inhibited control elongation. D-CS possibly inhibited the endogenous conversion of D-Trp to IAA. D-Trp-stimulated elongation of both strains was inhibited by D-CS by about 50%. The results suggest the possibility that D-CS inhibits D-Trp aminotransferase-catalyzed formation of IPyA. Furthermore, L-Trp-stimulated elongation of both strains was also inhibited by D-CS. Although D-CS at high concentrations might act as a racemase inhibitor (Law 1987), the inhibition of L-Trp-induced elongation suggests that the direct formation if IPyA from L-Trp is relatively small in the barley coleoptiles. Unspecific inhibitory effect of D-CS on the other sites can not be excluded.

Effect of D-Trp on IAA biosynthesis

We examined if the addition of D-Trp increased the endogenous IAA levels in the barley coleoptile segments of both normal and uzu strains (Table 2.1.2). In the absence of exogenous D-Trp, the IAA level was lower in the uzu strain than in the normal strain. Addition of D-Trp increased IAA levels in both strains 4-fold. D-CS completely inhibited IAA biosynthesis in both normal and uzu strains. The results suggest that D-Trp is converted to IAA by D-Trp aminotransferase in both strains. The results agree with a recent finding of D-Trp aminotransferase involvement in IAA biosynthesis in Alaska pea by McQueen-Mason and Hamilton (1989).

Endogenous Trp level

If the endogenous L-Trp level in uzu coleoptiles is lower than that in normal coleoptiles, the low production of IAA results from the low substrate level. Thus endogenous L-Trp levels in the presence or absence of exogenous L-Trp were determined in both strains (Table 2.1.3). In the absence of exogenous L-Trp, endogenous L-Trp levels of normal coleoptile segments and those of the uzu strain were similar during the incubation period up to 24 h. Thus, the endogenous L-Trp level did not directly relate to the lower IAA levels in the uzu strain. When L-Trp was added to

coleoptile segments, L-Trp contents in segments of both strains increased during the experimental period, but the level in the uzu strain was lower than that of the normal strain at 24 h. Furthermore, the L-Trp level of freshly harvested uzu or normal coleoptile segments, 2.9 nmol per segment^{-1} [380 nmole (g fresh weight)$^{-1}$], was roughly comparable with the levels of L-Trp demonstrated by other studies; 98-225 nmols (g fresh weight)$^{-1}$ in wheat leaves (Kim and Rohringer 1969), barley leaves and shoots (Schneider et al. 1972), and 10-83 nmole (g fresh weight)$^{-1}$ in Solanum nigrum leaves (Wakhloo 1965), and tomato shoots (Schneider et al. 1972).

We attempted to measure the endogenous D-Trp level by use of a fluorescence detector. Although the detection limit for D-Trp [5 pmole (g fresh weight)$^{-1}$, S/N=2] was comparable to the endogenous IAA level, D-Trp was not detectable in either strains. The data indicate that the endogenous D-Trp level is lower than the IAA levels in both strains.

Conversion of L-Trp to D-Trp using L-[3'-^{14}C]Trp

Since direct measurement failed to detect endogenous D-Trp, conversion of L-Trp to D-Trp was studied using L-[3'-^{14}C]Trp (Table 2.1.4). L-[3'-^{14}C]Trp used in this experiment was found to contain 6.8 ± 0.4% (n=3) of D-[^{14}C]Trp by a chiral HPLC system. Incorporation of labeled L-Trp into coleoptile segments increased with time in both lines. Although incorporation of labeled D-Trp also increased for both strains, the relative amount of D-Trp decreased from 6.8% to ca 3% in both strains at the end of incubation. The decrease in the percentage of D-Trp suggests that the metabolism of D-Trp is more rapid than the conversion of D-Trp by a racemase. The metabolism of D-Trp may include the conversion of D-Trp to malonyl-D-Trp, as suggested by Law (1987).

From these experiments I conclude that in barley coleoptiles of both normal and uzu strains, D-Trp is a more direct precursor for IAA synthesis than L-Trp, since D-Trp was more effective in stimulating elongation of the coleoptile segments and D-CS inhibited not only D-Trp-induced elongation but also L-Trp-induced elongation. Law (1987) described the mechanism of gibberellin-induced stem growth in a dwarf pea cultivar in which gibberellin stimulated Trp racemase activity, leading to increased production of IAA. For uzu, a dwarf barley strain, exogenously applied gibberellin did not affect the coleoptile growth (Kuraishi 1974). Therefore it is unlikely that the endogenous L-Trp level results in the lower production of IAA, since the level was comparable to that of normal strain. Fluorometric detection of endogenous D-Trp indicated that the D-Trp level is similar to, or even lower than the IAA level. Accordingly aminotransferase may not be important

for the regulation of endogenous D-Trp level is more important. Therefore, the low endogenous level of IAA in uzu coleoptiles probably results from the impeded activity of racemase and/or of the enzyme that produces a malonyl-D-Trp conjugate.

Baldi et al. (1991) tested the hypothesis that D-Trp is the IAA precursor using the aquatic monocot *Lemna gibba* as a model system, but they could find no evidence for this pathway. With the *Lemna* system, the experiments could be done under sterile conditions and uptake of both D- and L-form of Trp from the medium occurred rapidly. It was found that even after several days, the D-[^{15}N]Trp taken up from the medium was not converted into [^{15}N]IAA, although they measured a several hundred fold enrichment of the D-Trp pool. They also reported finding only low levels of L-Trp conversion, and this L-[^{15}N]Trp to [^{15}N]IAA labeling occurred without detectable labeling of the D-Trp pool.

Conversion of *N*-malonyl-Trp, a compound thought to be found *in vivo* predominantly in the D-Trp form, to IAox and then to IAA has been proposed as another route to IAA (Rekoslavskaya 1986). However, Ludwig-Müller and Hilgenberg (1989) showed that while *N*-malonyl-Trp was converted to IAA, *N*-malonyl-L-Trp was substrate for this reaction, not the D-form. Recent publications from Marumo's laboratory (Sakagami et al. 1993) on the occurrence of 4-Cl-Trp and malonyl-Trp in pea, report that only about 2% of the 4-Cl-Trp is in the D-form and bulk of 4-Cl-Trp is the L-isomer. They also present evidence that, contrary to the initial reports, the malonyl-Trp in plants is predominantly in the L-form.

Unfortunately, I could not also find the more evidence of the role of D-Trp as the intermediate of IAA synthesis, because I could not detect natural D-Trp and not detect an appreciable amount of activity of D-Trp transaminase in the barley coleoptile. Therefore, I must concluded that the pathway of IAA synthesis via D-Trp would not play the role of endogenous IAA synthesis and regulation, although D-Trp appears the precursor of IAA as the results of my experiments applying D-Trp to coleoptile segments.

Next, I have focused on the intermediate, for example IAN, IAM, IAAld, directly before IAA synthesis in the barley seedlings.

Table 2.1.1. Effect of D-cycloserine on elongation of barley coleoptile segments induced by D-Trp. Coleoptile segments (5 mm long) were incubated for 24 h in 10 mM K-citrate-phosphate (pH 6.5) containing 1 mM D-Trp, 1 mM L-Trp or 10 μM IAA with or without 1 mM D-CS. Data are means ± SE (n=15).

Strain	Treatment	Elongation, mm	
		- D-CS	+ D-CS
normal	Control	2.48 ± 0.09	1.62 ± 0.12
	L-Trp	3.73 ± 0.14	2.61 ± 0.14
	D-Trp	4.18 ± 0.16	2.21 ± 0.11
	IAA	4.43 ± 0.12	4.31 ± 0.10
uzu	Control	0.90 ± 0.05	0.45 ± 0.05
	L-Trp	2.98 ± 0.16	1.11 ± 0.12
	D-Trp	3.89 ± 0.13	2.02 ± 0.11
	IAA	3.20 ± 0.09	3.89 ± 0.13

Table 2.1.2. IAA synthesis from D-Trp in barley coleoptile segments. Two hundred coleoptile segments were incubated for 10 h in 10 mM K-citrate-phosphate (pH 6.5) with or without D-Trp (1 mM) and with D-Trp and D-CS (1 mM). Data are means ± SE (3 injections).

Strain	IAA, pmol (g FW)$^{-1}$ (10 h)$^{-1}$		
	- D-Trp	+ D-Trp	+ D-Trp + D-CS
normal	18 ± 1	72 ± 2	21 ± 2
uzu	10 ± 1	39 ± 2	11 ± 1

Table 2.1.3. Variation of endogenous L-Trp in barley coleoptile segments after the addition of exogenous L-Trp. Coleoptile segments were incubated in 10 mM K-citrate-phosphate (pH 6.5) with or without 1 mM L-Trp.

Strain	Exogenous L-Trp	Endogenous L-Trp (nmol segments^{-1})		
		0 h	6 h	24 h
normal	-	2.9	5.4	4.2
	+	-	15.6	66.2
uzu	-	2.8	3.2	4.1
	+	-	20.5	35.4

Table 2.1.4. Incorporation of radioactivity from L-[^{14}C]Trp into D- and L-Trp in barley coleoptile segments. Coleoptile segments were incubated in 10 mM K-citrate-phosphate (pH 6.5) containing L-[^{14}C]Trp (37 kBq). Data are means of two injections. Figures in parentheses are % of radioactivity of D-Trp.

	Radioactivity, Bq (50 segments)$^{-1}$			
	normal		uzu	
Time, h	L-Trp	D-Trp	L-Trp	D-Trp
2	500	21 (4.0)	450	24 (5.0)
4	790	17 (2.1)	790	35 (4.2)
6	1860	30 (1.6)	1730	54 (3.0)
8	1880	50 (2.6)	2480	81 (3.2)

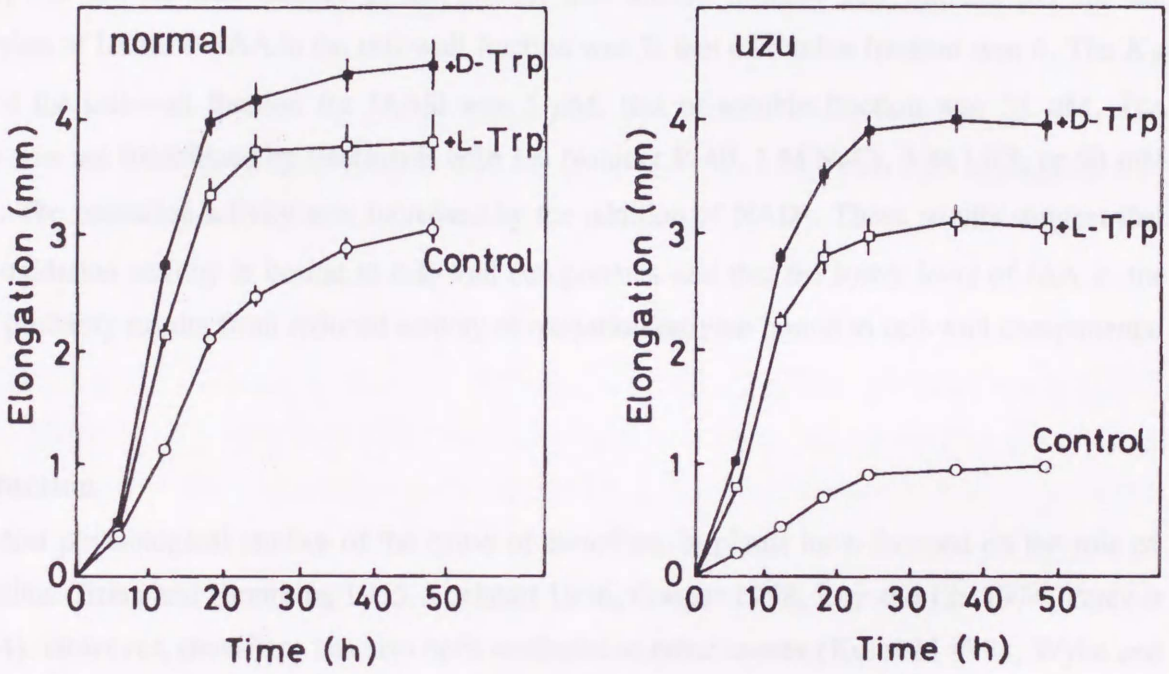

Fig. 2.1.1. Effect of D- or L-Trp on elongation of barley coleoptile segments. Coleoptile segments (5 mm long) were incubated in 10 mM K-citrate phosphate (pH 6.5) containing 1 mM D-Trp (+ D-Trp), 1 mM L-Trp (+ L-Trp) or no Trp (Control). Data are means ± SE (n = 15).

2.2 Conversion of Indole-3-acetaldehyde to Indole-3-acetic Acid in Cell-wall Fraction of Barley (*Hordeum vulgare*) Seedlings

Abstract

The cell-wall fraction of barley seedlings was able to oxidize IAAld to form IAA, whereas the fraction did not catalyze the conversion of IAN or IAM to IAA. The activity was lower in a semi-dwarf mutant that had an endogenous IAA level lower than that of the normal isogenic strain [Inouhe et al. (1982) *Plant Cell Physiol.* 23: 689]. The soluble fraction also contained some activity; the activity was similar in the normal and mutant strains. The optimal pH for the conversion of IAAld to IAA in the cell-wall fraction was 7; that of soluble fraction was 6. The K_m value of the cell-wall fraction for IAAld was 5 μM; that of soluble fraction was 31 μM. The activity was not solubilized by treatments with 1% Nonidet P-40, 1 M NaCl, 3 M LiCl, or 50 mM $MgCl_2$. The oxidation activity was increased by the addition of NAD^+. These results suggest that IAAld oxidation activity is bound to cell-wall components and that the lower level of IAA in the mutant probably results from reduced activity of oxidation enzyme bound to cell-wall components.

Introduction

Most physiological studies of the cause of dwarfism in plants have focused on the role of gibberellins (Brian and Hemming 1955, Lockhart 1956, Cooper 1958, Loy and Liu 1974, Perez et al. 1974). However, dwarfism has also been attributed to other causes (Kuraishi 1974, Wylie and Ryugo 1971). Van Overbeek (1938) first showed that lowered auxin content in dwarf corn seedlings is one of the causes of dwarfism. Inouhe et al. (1982) demonstrated that the dwarfism of etiolated seedlings of eleven barley isogenic strains (*Hordeum vulgare* L. cv. Akashinriki) is primarily caused by low levels of endogenous IAA in these strains.

The pathway of IAA biosynthesis has been extensively studied, but has not yet been demonstrated unequivocally (Bandurski et al. 1995, Normanly et al. 1995). Trp has been suggested as a primary precursor of IAA (Gordon 1954, Gibson et al. 1972, Monteiro et al. 1988, Cooney and Nonhebel 1991, Bialek et al. 1992, Koshiba and Matsuyama 1993, Koshiba et al. 1995), because Trp is similar in structure to IAA and is ubiquitous in plant tissues.

Two pathways of IAA biosynthesis from L-Trp have been proposed in higher plants: Trp →

IPyA → IAAld → IAA; or Trp → TNH_2 → IAAld → IAA. With the unequivocal demonstration of IAox, which appears to be synthesized from *N*- OH-Trp, in some higher plants (Ludwig-Müler and Hilgenberg 1988) and its onward metabolism to IAA in several higher plants (Rajagopal et al. 1993), two other routes became apparent: IAox → IAAld → IAA; and IAox → IAN → IAA. IAM has been reported as an important precursor of IAA in some higher plants (Kawaguchi et al. 1991, 1993, Saotome et al. 1993, Rajagopal et al. 1994). On the other hand, IAA may not be directly synthesized from Trp but from anthranilic acid or indole, which are precursors of Trp (Baldi et al. 1991, Wright et al. 1991, Michalczuk et al. 1992).

The question of IAA synthesis involves not only the primary precursor but also the direct precursor of IAA. IAAld (Rajagopal 1967a, b, 1968, 1971, Bower et al. 1978, Purves and Brown 1978, Miyata et al. 1981, Koshiba et al. 1996), IAN (Normanly et al. 1993, Bartel and Fink 1994, Bartling et al. 1994) and IAM (Kawaguchi et al. 1991, 1993, Saotome et al. 1993, Rajagopal et al. 1994) have been suggested as direct precursors, but the existence and amounts of these compounds and their substrate specificities are still controversial.

Recently, a possible role for aldehyde oxidase in IAA biosynthesis was suggested in maize coleoptile (Koshiba et al. 1996). Aldehyde oxidase could catalyze the oxidation of IAAld to form IAA (Rajagopal 1971, Bower et al. 1978, Miyata et al. 1981). Koshiba and Matsuyama (1993) reported that an in vitro system of maize coleoptile extracts catalyzed the production of IAA from Trp and that the IAA-forming activity was co-purified with an IAAld oxidase. However, the actual function of IAAld oxidase in IAA biosynthesis is not yet known.

To determine the cause of the lower level of endogenous IAA in dwarf barley (Inouhe et al. 1982), the present experiments were undertaken to investigate the contribution of IAAld to the IAA biosynthesis pathway in the normal and isogenic dwarf mutant of barley. I demonstrated that the insoluble cell-wall fraction exhibited substantial IAAld oxidation activity which converted IAAld to IAA and revealed that the activity of this cell-wall fraction was lower in the dwarf barley strain, which had been previously shown to be contain lower levels of endogenous IAA than the normal strain.

Materials and Methods

Plant materials

Two isogeneic strains (uzu and its corresponding normal line) of barley (*Hordeum vulgare* L. cv, Akashinriki) were harvested at an experimental farm of Research Institute for Bioresources, Okayama University, in 1993. About 700-800 seeds were soaked for 8 h in water and then germinated in the dark in plastic boxes (34 cm × 23 cm × 4 cm) filled with moistened vermiculite at 25.5 ± 0.5°C for 3 d. Coleoptiles, including first leaves, selected for uniformity of seedling length (15-30 mm for the uzu strain and 30-50 mm for the normal strain) were weighed. The material was immediately used for experiments or frozen at -80°C until use.

Enzyme fractionation

All manipulations were performed at 2-4°C. One hundred samples (normal, ca. 3.5 g in fresh weight; uzu, ca. 2.4 g in fresh weight) were homogenized by mortar and pestle in 4 to 8 ml of 50 mM Tris-HCl (pH 7.2) containing 5 mM Na-EDTA, 1 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM benzamidine, and 0.1 mM 4-(2-aminoethyl) benzensulfonyl fluoride. The homogenate was centrifuged at 1,000 × *g* for 20 min at 4°C. The supernatant was designated as soluble enzyme fraction (SEF). The pellet was suspended in homogenizing medium with 1% Nonidet P-40 and 1 M NaCl and homogenized again. The homogenate was centrifuged at 1,000 × *g* for 20 min, washed 3 times with the homogenizing medium, and suspended in the same medium (4 to 8 ml). This suspension was designated as cell-wall fraction (CWF). When ATPase activity was measured as a plasma membrane marker enzyme, the seedlings were homogenized in previous homogenizing buffer with 1% Nonidet P-40 and 1 M NaCl. Homogenate was centrifuged at 1,000 × *g* for 20 min at 4°C. The pellet was washed 3 times with the homogenizing medium and suspended in the same medium for ATPase assay.

Protein content in SEF was determined by the Bio-Rad Protein Assay Kit (Bradford 1976). Total sugar in CWF was determined by the phenol-sulfuric acid method (Dubois et al. 1956).

Enzyme assay

The incubation medium usually contained 400 μl of SEF or CWF and 100 μl of 1 mM free-IAAld (prepared from IAAld bisulfite as described by Bower et al. [1978]) and was kept for 60-90 min at 25°C. At the end of the incubation period, 500 pmol of IPA was added as the internal standard for recovery calculations. The pH of the medium was adjusted to pH 2.5 with 1 M H_3PO_4. The incubation mixture was then extracted by diethyl ether. The organic phase was

evaporated to dryness in N_2 gas and dissolved in 500 μl of 20% acetonitrile (pH 5.0 adjusted by 20 mM triethylamine-acetic acid) containing 50 mM $NaHSO_3$. An aliquot was loaded on an HPLC system.

Activity of ATPase as a plasma membrane marker enzyme was determined by measuring phosphate released from ATP (Fiske and Subbarow 1925). The 500 μl of incubation medium contained about 30 μg of protein of SEF or CWF equivalent to 200 μg of sugar, 3 mM ATP, and 3 mM $MgSO_4$ in homogenizing medium and was kept for 2 min at 30°C. The reaction was stopped by the addition of 300 μl of 20% SDS. The mixture was added to 4 ml of a mixture of 10 mM ammonium molybdate : 2.5 M sulfuric acid : acetone (1 : 1 : 2 v/v), then was mixed with 0.4 ml of 1 M citric acid. The color intensity was measured at 355 nm.

HPLC

Separation of the products was achieved on an Inertsil ODS-2 column (4.6 × 150 mm, GL Sciences Inc., Japan), eluted isocratically with 20% acetonitrile (pH 5.0 adjusted with 20 mM triethylamine-acetic acid) at a flow rate of 0.8 ml/min. The HPLC system consisted of a pump unit (model LC-6A, Shimadzu Inc., Kyoto) connected to a system controller (model SCL-6A, Shimadzu Inc.). IAA and IEt were detected with an excitation at 280 ± 5 nm and an emission at 350 ± 5 nm by spectrofluorometry with a fluorometric detector (FP-200, Japan Spectroscopic CO., Tokyo). The IAA and IEt contents were calculated from the ratio of the peak area of IPA to that of IAA or IEt, as described by Kuraishi et al. (1989).

GC-MS

Following HPLC, the fractions corresponding to IAA or IEt retention time were pooled and evaporated to dryness. The dried samples were trimethylsilylated with a mixture of *bis*-trimethylsilyl trifluoroacetamide (BSTFA, Tokyo Kasei Kogyo Co., Tokyo) and acetonitrile (1:1, v/v) at 70°C for 1 h. The trimethylsilylated sample was characterized by GC-MS or GC-SIM-MS of QP-1000 (Shimadzu Inc.). Ionization of the compound was carried out by electron impact at 70 eV. The gas chromatograph was equipped with a 30 m fused silica capillary column (CBJ 17, Shimadzu Inc.).

Results

Comparison of the IAA synthesis activity of SEF and CWF

Table 1 shows the IAA synthesis activity from different precursors by SEF or CWF of the normal and uzu strains. When the activity is expressed per seedling, SEF converted IAAld to IAA more than did CWF in both the normal and uzu strains. In SEF, uzu showed slightly lower activity than normal on a seedling basis; the activity in CWF of uzu was only 28% of that of the normal strain. When the specific activity was calculated for SEF, normal and uzu exhibited similar activities. The activity in CWF was lost by preincubation of CWF at 90°C for 5 min, and the time course of the reaction showed linearity for 2 h. These results demonstrated that the reaction was caused by an enzyme catalyzing the oxidation.

We attempted to release the enzyme activity of CWF from the cell walls by 1 M NaCl, 3 M LiCl, 50 mM $MgCl_2$, and 1% Nonidet P-40, but the activity could not be solubilized. Solubilizing cell wall-bound proteins by commercially available cellulase or pectinase was attempted, but I found that these enzymes contained substantial aldehyde oxidase activity. Since I failed to release or isolate the soluble protein fraction containing aldehyde oxidase activity, specific activities of the enzyme in CWF were expressed as unit per mg cell-wall sugar. The specific activity converting IAAld to IAA in CWF was about two times higher in normal than in uzu (Table 2.2.1).

To check whether the CWF was contaminated with plasma membrane or not, ATPase activity was measured. The ATPase activity in SEF (221×10^3 unit/seedling) in the normal strain was about 20 times higher than that in CWF (12×10^3 unit/seedling). The results showed that about 5% of membrane remained in CWF.

SEF could convert IAN to IAA. The normal strain showed higher activity than uzu on a seedling basis. The specific activity of SEF, however, was slightly lower in the normal strain than in uzu. CWF did not convert IAN to IAA. Neither SEF nor CWF converted IAM to IAA in either barley strain.

Demonstration of conversion of IAAld to IAA by CWF

The reaction mixture of CWF including IAAld as substrate was analyzed by HPLC. The chromatograph clearly demonstrated that the CWF of normal and uzu strains converted IAAld to IAA (Fig. 2.2.1). The peaks corresponding to IAA retention time was identified as IAA by GC-MS (Fig. 2.2.2). Trimethylsilylated (TMS)-IAA had a retention time of 10.8 min and had two

major mass fragments at m/z = 202 and 319 (M^+). The HPLC chromatograph also revealed that IAAld was converted to IEt in both strains. The peak corresponding to IEt was identified as IEt by GC-SIM-MS as follows: the main mass fragments of standard TMS-IEt were m/z 202 (100%) and 305 (19%), and the fragments of the peak corresponding to retention time of IEt on the HPLC were m/z 202 (100%) and 305 (18%, normal; 19%, uzu).

Rough estimation of the level of IEt product on HPLC revealed that the reducing activity that converted IAAld to IEt was lower in the normal strain (0.49 unit/ mg sugar) than in the uzu (0.81 unit/mg sugar).

Effect of NAD^+ and NADH on the enzyme activity of CWF

To investigate the effect of electron acceptor and donor on IAAld oxidation and reduction activities, NAD^+ (200 μM) or NADH (200 μM) was added to the reaction mixture containing CWF. The addition of NAD^+ increased the activity converting IAAld to IAA by 63% and that of NADH increased the activity converting IAAld to IEt by 69%, suggesting that CWF contains aldehyde dehydrogenase requiring NAD^+ and aldehyde reductase requiring NADH.

Comparison of the properties of IAAld oxidation activity of SEF and CWF in the normal strain

The optimal pH of the IAAld oxidation activity of CWF differed from that of SEF (Fig. 2.2.3). The optimal pH of CWF was about 7; that of SEF was about 6. The activity of SEF was more affected by pH than that of CWF.

K_m values of SEF and CWF were determined as 31 μM and 5 μM, respectively (Fig. 2.2.4), indicating that the affinity of CWF to the substrate was six times higher than that of SEF.

Discussion

Inouhe et al. (1982) demonstrated that dwarfism of etiolated seedlings of several isogenic barley strains is primarily caused by lower levels of endogenous IAA in the dwarf strains. The cause of this lower level of IAA in uzu was proposed to be an impeded IAA biosynthesis pathway. There are three indole compounds nominated as direct precursors of IAA, IAAld (Rajagopal 1967a, b, 1968, 1971, Bower et al. 1978, Purves and Brown 1978, Miyata et al. 1981, Koshiba et al. 1996), IAN (Normanly et al. 1993, Bartel and Fink 1994, Bartling et al. 1994) and IAM

(Kawaguchi et al. 1991, 1993, Saotome et al. 1993). The normal strain of barley contained two to four times higher endogenous IAA than did the uzu strain (Inouhe et al. 1982). To specify the specific step in the IAA synthesis pathway that is impeded in the uzu strain, I tested the three indole compounds listed above using SEF or CWF in normal and uzu strains. IAN was converted to IAA in SEF, but not in CWF. The nitrilase activity in SEF of uzu was not lower than that in the normal on the basis of specific activity. Therefore, it is unlikely that the lower level of endogenous IAA in the uzu strain was caused by the lower nitrilase activity. Since IAM was not converted to IAA in either fraction of either strain, it is not involved in the impeded IAA synthesis in the uzu strain.

IAAld was converted to IAA, not only by SEF but also by CWF. Specific activity of conversion of IAAld to IAA in SEF was similar in normal and uzu, although the activity per seedling was two times higher in normal than in uzu. CWF in the normal strain, however, has twice as much oxidation activity as that in uzu on the basis of specific activity (/mg sugar) and 3.6 times as high as that in uzu per seedling (Table 2.2.1). Moreover, the activities on the basis of fresh weight were 12.3 unit/g for normal and 5.0 unit/g for uzu strain. These results correspond to differences between endogenous IAA levels of the normal and uzu strains (Inouhe et al. 1982: normal, 235 pmol/g in fresh weight; uzu, 54 pmol/g in fresh weight). Therefore, the lower conversion of IAAld to IAA in CWF probably results in the lower level of endogenous IAA in the uzu strain.

Oxidation enzyme(s) converting IAAld to IAA, particularly aldehyde oxidase, have been extensibility studied for IAA biosynthesis (Bower et al. 1978, Purves and Brown 1978). The oxidation activity found in CWF was slightly increased by NAD^+, suggesting that the CWF contained an aldehyde dehydrogenase activity requiring NAD^+ as well. However, since CWF was exhaustively washed with the buffer containing salts and detergent, it is unlikely that CWF contained NAD^+ to promote the activity. Therefore, most of the enzyme activity to convert IAAld to IAA in CWF is accounted for by aldehyde oxidase.

The facts that CWF has a lower K_m value for IAAld than SEF and that the pH optimum of CWF differs from that of SEF suggest that the enzyme(s) responsible for IAAld oxidation in CWF is different from that in SEF. Interestingly, the K_m value for IAAld in CWF (5 μM) was very similar to that of the partially purified enzyme found in maize coleoptile (Koshiba et al. 1996: K_m =3-5 μM). It will be intriguing to see if the maize aldehyde oxidase is secreted to the apoplast.

CWF also contained a reducing activity which converted IAAld to IEt. The specific activity

was higher in uzu than in normal. Generally, conversion of IAAld to IEt is proposed as a side branch of the pathway from IAAld to IAA and as a regulatory step in IAA synthesis (Ludwig-Müller and Hilgenberg 1990). The occurrence of endogenous IEt has been demonstrated in several plants (Rayle and Purves 1967, Rajagopal 1967a), and IEt metabolism involved in IAA synthesis has also been reported in several plants (Ludwig-Müller and Hilgenberg 1989, 1990). I identified IEt in barley seedlings, but could not quantify it, because I had no internal standard for IEt. The presence of IEt implies an inherent reaction from IAAld to IEt in barley.

IAAld has been found in several plants (Rajagopal 1968, Purves and Brown 1978, Ludwig-Müller and Hilgenberg 1990). Identification and quantitation of endogenous IAAld, however, were very difficult because of the notorious unstability of this compound in aqueous solvents. I were unsuccessful in isolating or identifying endogenous IAAld in barley seedlings.

We failed to solubilize the oxidation and reduction activities from CWF or to reduce these activities in CWF by treatment with salts and detergent. Although CWF included about 5% membrane, the results suggest that the enzyme is tightly or covalently bound to cell-wall components. The investigation of the spatial distribution of and the changes in these enzyme activities bound to cell walls may serve as an interesting clue in searching for the key regulation sites of IAA synthesis in barley.

IAA must first attach to a receptor for the elongation growth of the plants. This receptor may be on the outside of the plasma membrane or at some internal site in the cell (see Chapter 1.3). If IAA is synthesized for cell growth in cell-wall (apoplastic space ?) , IAA should be distributed in apoplast rather than in symplast. In the next section, I determined the distribution of IAA in apoplast and symplast of growing region of squash hypocotyl, from which I obtained more easily the apoplastic solution from barley coleoptiles, and I discuss the possibility of the cell elongation effect of IAA from outside of the plasmamembrane.

Table 2.2.1 Activities of IAA synthesis from various direct precursors in normal and uzu extracts

Substrate		unit/seedling		unit/mg protein	unit/mg sugar
		SEF	CWF	SEF	CWF
IAAld	normal	2.61	0.43	12.98	0.72
	uzu	1.72	0.12	13.87	0.33
IAN	normal	1.43	n.d.	7.02	n.d.
	uzu	1.10	n.d.	8.91	n.d.
IAM	normal	n.d.	n.d.	n.d.	n.d.
	uzu	n.d.	n.d.	n.d.	n.d.

The incubation medium contained 400 μl SEF or CWF and 100 μl of a 1 mM solution of IAAld, IAN or IAM, and was kept for 60 min at 25°C. One unit of enzyme activity was defined as the activity required to produce 1 pmol IAA min^{-1}. n.d., not detected. Data are means of two independent experiments.

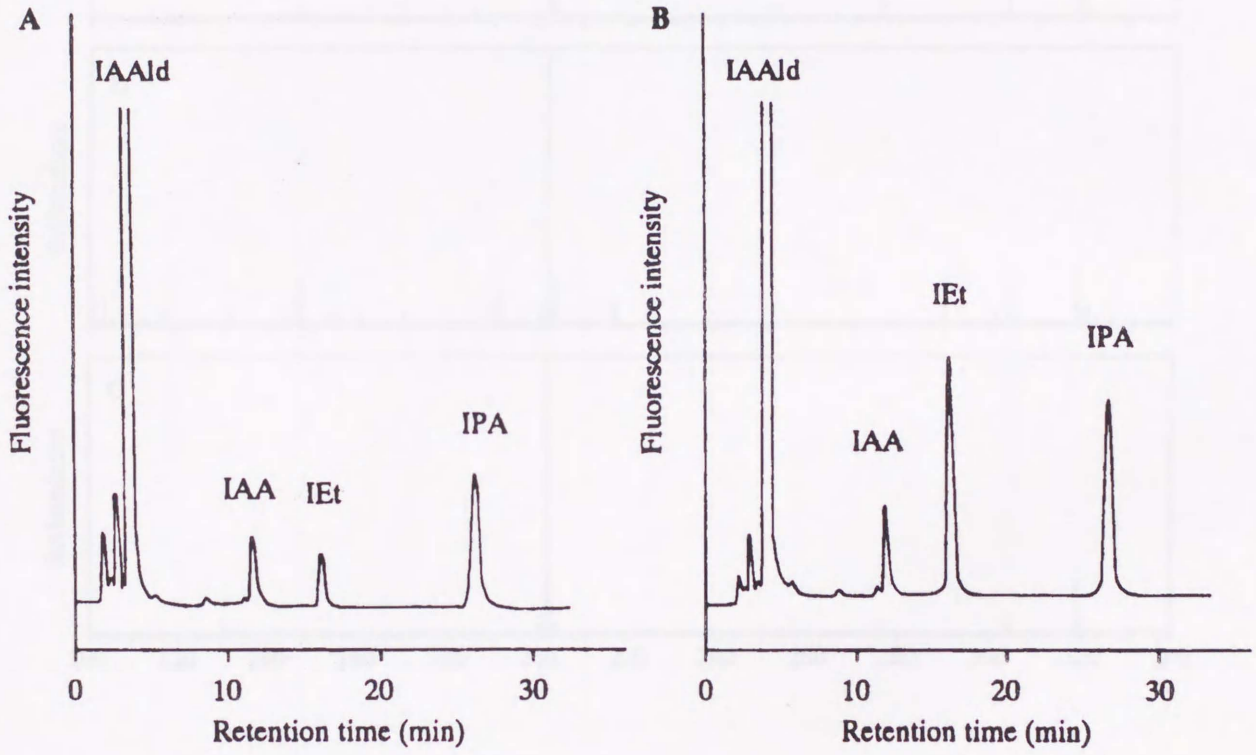

Fig. 2.2.1 HPLC profiles of the reaction mixture of IAAld metabolism in CWF of normal and uzu strains. A, profile of normal strain; B, profile of uzu strain. Indole compounds were detected by fluorometry.

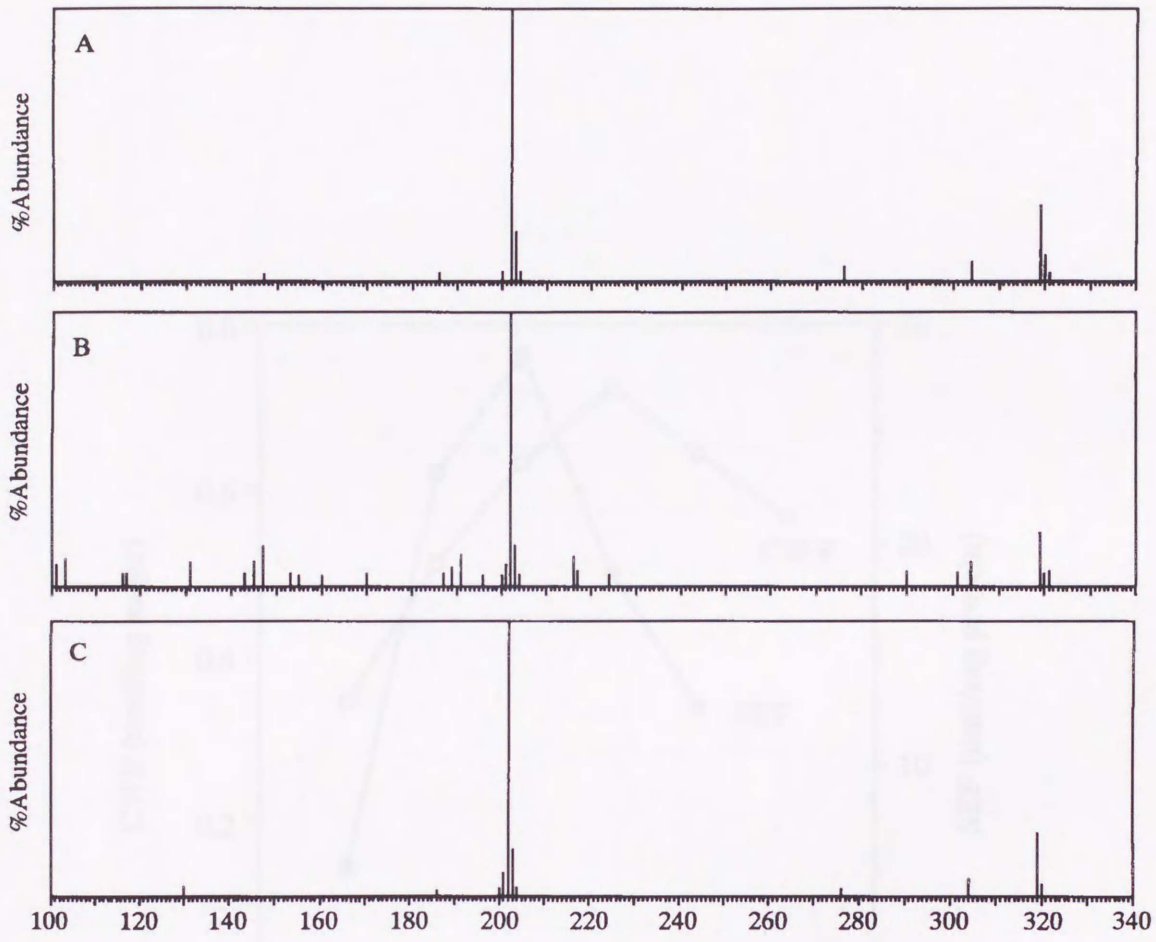

Fig. 2.2.2. GC-MS of IAA formed from IAAld by CWF of normal and uzu strains. A, standard TMS-IAA; B, TMS-IAA of normal strain; C, TMS-IAA of uzu strain.

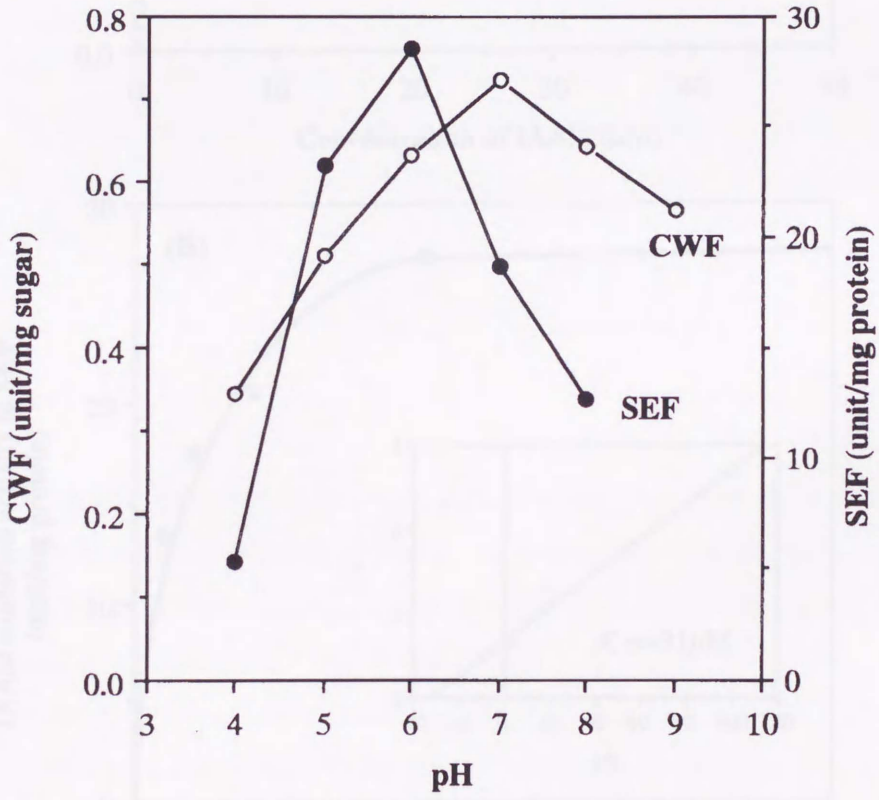

Fig. 2.2.3 Effect of pH on IAAld oxidation activity in CWF and SEF of the normal strain for 60 min at 25°C. ○, specific activity of CWF (unit/mg sugar); ●, specific activity of SEF (unit/mg protein).

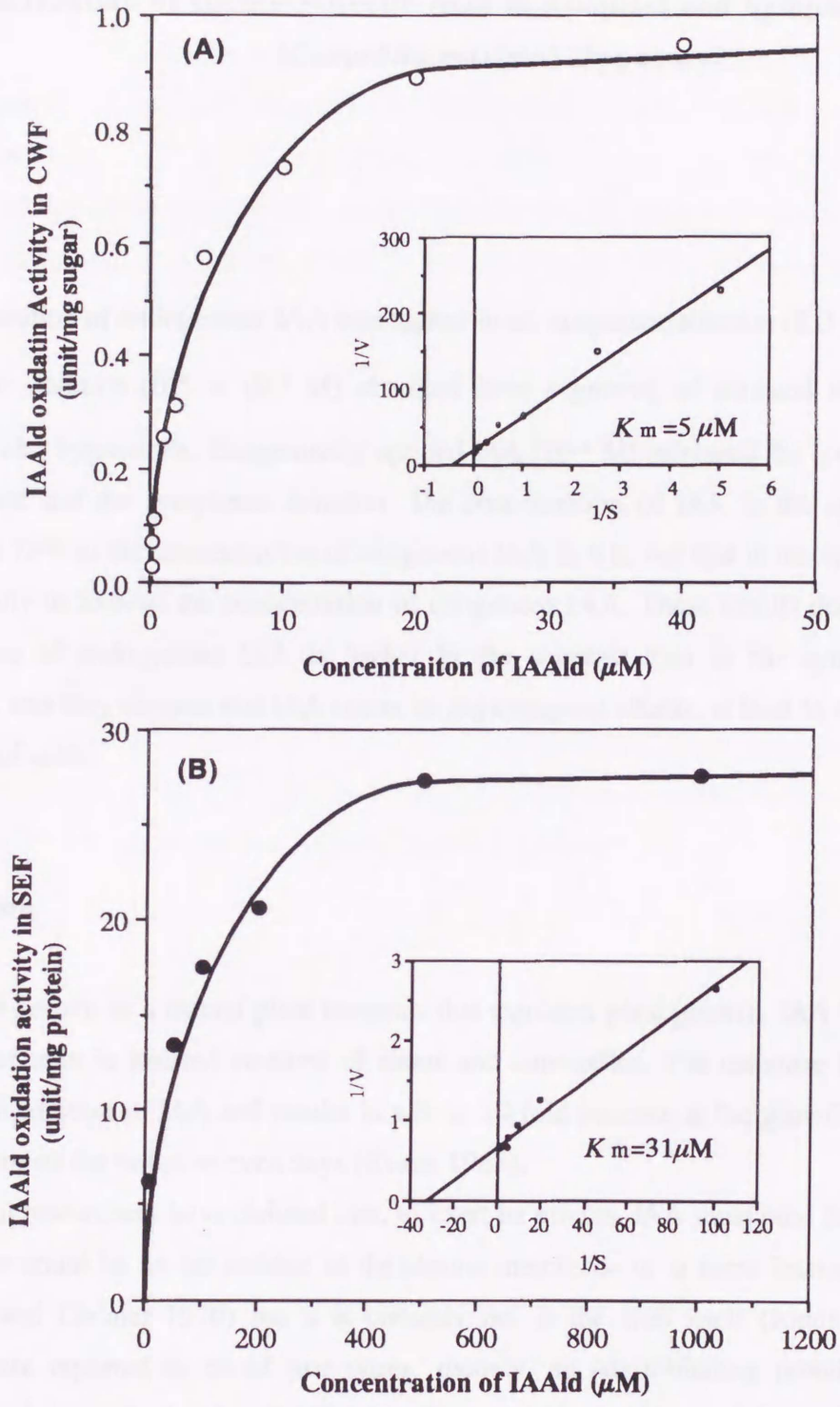

Fig. 2.2.4 Determination of K_m values for IAAld in CWF and SEF of the normal strain for 60 min at 25°C. (A), specific activity of CWF (unit/mg sugar); (B), specific activity of SEF (unit/mg protein). Inset: Lineweaver-Burk plots of CWF and SEF give K_m values of 5 and 31 μM, respectively.

2.3 Distribution of Indole-3-Acetic Acid in Apoplast and Symplast of Squash (*Cucurbita maxima*) Hypocotyl

Abstract

The concentration of endogenous IAA was higher in an apoplastic solution (2.3×10^{-7} M) than in a symplastic solution (0.5×10^{-7} M) obtained from segments of etiolated squash (*Cucurbita maxima* Duch.) hypocotyls. Exogenously applied IAA (10^{-5} M) increased the level of IAA in both the apoplastic and the symplastic solution. The concentration of IAA in the apoplastic solution increased to 75% of the concentration of exogenous IAA in 4 h, but that in the symplastic solution increased only to 23% of the concentration of exogenous IAA. These results demonstrate that the concentration of endogenous IAA is higher in the apoplast than in the symplast of squash hypocotyls, and they suggest that IAA exerts its physiological effects, at least to some extent, from the outside of cells.

Introduction

IAA is known as a natural plant hormone that regulates plant growth. IAA induces the rapid elongation of cells in isolated sections of stems and coleoptiles. The response begins within 10 min after the addition of IAA and results in a 5- to 10-fold increase in the growth rate. Moreover, the effect persists for hours or even days (Evans 1985).

Several researchers have claimed that, to exert its effects, IAA must bind first to a receptor. This receptor could be on the outside of the plasma membrane or at some internal site within the cell (Rayle and Cleland 1970) but it is certainly not in the wall itself (Jones 1990). Putative receptors were reported to be of two types, namely, an auxin-binding protein at the plasma membrane and a protein that is solubilized in the cytoplasm. Löbler and Klämbt (1985) reported that an auxin-binding protein, located at the plasma membrane, recognized auxin and was concentrated in the outer epidermis, as compared to the inner tissues of maize coleoptiles. Venis et al. (1990) demonstrated that exogenous application of impermeant analogs of auxin caused a rapid response (hyperpolarization of the plasma membrane) in tobacco mesophyll protoplasts, as well as

long-term elongation growth of segments of pea epicotyls. The results suggest that auxin causes a rapid growth, related response in plant cells by exerting its effect on cells from the outside of the cell and, moreover, that auxin does not necessarily need to enter the cell to exert its effect on elongation growth.

Optimal concentrations of exogenous IAA for the elongation of sections of stems and coleoptiles are usually in the range of 10^{-5} to 10^{-6} M (Nissen 1985), but measured concentrations of endogenous IAA extracted from sections of growing zones of squash hypocotyls were only about 4×10^{-8} M (Sakurai et al. 1985). The reason for the difference between the optimum concentration of exogenously applied IAA and the endogenous concentration has not yet been elucidated. There is a possibility that the low concentration of endogenous IAA found in the tissue is due to the low concentration in the symplast. The volume of the symplast is more than ten times that of the apoplast. Even if endogenous IAA were to be distributed preferentially in the apoplast, the concentration of IAA extracted from whole tissues could be estimated to be lower than the concentration in the apoplast.

To demonstrate directly that IAA is distributed preferentially in the apoplast and to determine whether it is likely that IAA acts from the outside of cells, I measured levels of IAA in the apoplast and symplast of growing tissues of etiolated squash hypocotyls.

Materials and Methods

Plant materials

Squash seeds (*Cucurbita maxima* Duch. cv. Houkou-Aokawaamaguri; Takayama Seed Co., Kyoto) were soaked for 16 h in tap water and then they were allowed to germinated for 2 d in darkness on two layers of moistened filter paper. The germinated seeds were placed on a stainless-steel mesh in plastic boxes (34 cm × 23.5 cm × 4.6 cm) that contained two liters of 1/5-strength Hoagland solution, as described previously (Sakurai et al. 1985), and they were cultured hydroponically at 25.5 ± 0.5°C for 2 or 3 d in darkness. After 2 or 3 d, seedlings that had hypocotyls of 4-6 and 8-10 cm in length, respectively, were selected for experiments.

Collection of apoplastic and symplastic solutions

Apoplastic solution was collected by the centrifugation method described previously by

Sakurai and Kuraishi (1988). Hypocotyl segments (1 cm in length) were excised from the upper part of hypocotyls, 5 mm below the cotyledonary node, and from the lower part of hypocotyls, 5 mm above the hypocotyl base. The segments were placed on a stainless-steel mesh in the barrel of a 6-ml plastic syringe (13 mm i.d.) which had been cut off at the 5-ml mark and was placed on an Eppendorf tube (1.5 ml) in a centrifuge tube (22.5 ml).

The tube was centrifuged for 20 min at 4,500 × g at 4°C for collection of apoplastic solution (AP solution) from the segments. The segments were frozen in liquid nitrogen immediately after centrifugation, thawed and recentrifuged for 20 min at 4,500 × g at 4°C for collection of cell sap (symplastic solution; SY solution). Weights of AP and SY solutions were calculated by subtracting the weight of the Eppendorf tube before collection of the AP or SY solution from the weight of the Eppendorf tube after collection of the AP or SY solution.

Extraction of IAA from the apoplastic and symplastic solutions

The AP and SY solutions in Eppendorf tubes were adjusted to pH 2.8 by two methods. In one method, solutions in five tubes were each adjusted to pH 2.8 with 200 μl of 2 M citric acid - NaOH buffer, and then the solutions were combined in a single test tube. In the other method, solutions of each of five tube were transferred to one test tube, then an aliquot of 2 M H_3PO_4, the volume of which was 10% the volume of the pooled solution, was added to the solution in the test tube. The two methods gave similar results.

[$^{13}C_6$]IAA (2 nmol; Merck, Sharp, and Dohme, Canada Ldt) was added to the sample solution in the test tube as an internal standard. The solution was then extracted twice with diethyl ether. The organic phase was evaporated to dryness under N_2 gas at room temperature or in a rotary evaporator (the recovery range from 90 to 100%). I also attempted to evaporate the organic phase to dryness with a stream of filtered air, but the recovery of IAA in such cases was fairly low (less than 10%). Therefore, this method was not used in these experiments. Each dried sample was dissolved in 0.9-1.5 ml of 25% acetonitrile that contained 20 mM acetic acid.

HPLC

IAA was purified by the method described by Akiyama et al. (1983) on an Inertsil ODS-2 column (4.6 mm i. d. × 150 mm; GL Sciences Inc., Tokyo) or on a Puresil C_{18} 120 Å column (4.6 mm i. d. × 150 mm; Nihon Millipore Ltd, Tokyo). Columns were eluted isocratically with

25% acetonitrile that contained 20 mM acetic acid at a flow rate of 0.8 ml/min. The HPLC system consisted of a pump unit (model LC-6A; Shimadzu Inc., Kyoto) connected to a system controller (model SCL-6A; Shimadzu Inc.). IAA was monitored with a fluorometric detector (model FP-200; Japan Spectroscopic Co., Tokyo) with excitation at 280 ± 5 nm and emission at 350 ± 5 nm. The IAA-containing fractions were collected for analysis by GC-SIM-MS.

GC-SIM-MS

After HPLC, appropriate IAA-containing fractions were pooled and evaporated to dryness on a rotary evaporator. Samples were dissolved in methanol and transferred to test tubes (the recovery range from 90 to 100%). I also attempted to dissolve dried samples in diethyl ether but the recovery of IAA was very low (less than 10%). Therefore, I did not use diethyl ether for dissolving the dried samples. Each sample in a test tube was evaporated to dryness under N_2 gas. The dried sample in the test tube was methylated with diazomethane, which was generated with a diazomethane generator (Wheaton, Millville, NJ, USA). IAA in the methylated sample was quantitated by GC-SIM-MS (model QP-1000; Shimadzu Inc.). Ionization of the compound was achieved by electron impact at 70 eV. The gas chromatograph was equipped with a 30-m fused silica capillary column (CBJ 17; Shimadzu Inc.). IAA content was calculated from the ratio of the peak area of [$^{13}C_6$]IAA (m/z=136) to that of the natural IAA (m/z=130).

Treatment of segments of squash hypocotyls with IAA

One-cm-long segments were incubated at 25.5 ± 0.5°C in darkness in a Petri dish that contained 10 ml of water with or without 10^{-5} M IAA. All manipulations were conducted under dim green light. After incubation, the segments were blotted with a Kim-wipe and placed vertically in a plastic syringe for collection of AP and SY solutions as described above. To estimate the amount of IAA that was transiently associated with the surface of segments but had not been absorbed by the segments, I dipped the segments in a solution of IAA for one second, then blotted and treated them as above. To calculate the exact amount of IAA in AP and SY solutions, I used the following equation:

$$\text{Amount of IAA} = A - (B - C)$$

where A is the amount of IAA in the AP or SY solution from IAA-treated segments after a given incubation period; B is the amount of IAA in the AP or SY solution from segments treated with IAA for one second; and C is the amount of IAA in the AP or SY solution from untreated

segments.

Results

Apoplastic and symplastic endogenous IAA in upper and lower segments of hypocotyls

Table 2.3.1 shows amounts and concentrations of endogenous IAA in AP and SY solutions obtained from upper and lower segments of hypocotyls of 2- or 3-d-old squash seedlings. The absolute amount of IAA in AP solutions was always lower than that in SY solutions for both upper and lower segments of hypocotyls of both 2- and 3-d-old seedlings. The upper segments yielded slightly larger amounts of IAA in the AP and SY solutions than the lower segments.

The concentrations of IAA were calculated from the weights of AP and SY solutions, on the assumption that the weight of each solution in grams was close to its volume in milliliters. Calculated concentrations of IAA in AP solutions from the upper segments were 8 times those in SY solutions on day 2. On day 3, the difference was smaller but was still 2.5-fold. Not surprisingly, the concentration of IAA in AP and SY solutions was always higher in the upper, growing region, than in the lower, non-growing region, on days 2 and 3. In the lower region, the difference between concentrations of IAA in AP and SY solutions was negligible on day 3.

Endogenous IAA in upper segments of hypocotyls from 2-d-old seedling

We confirmed the level of IAA in upper hypocotyl segments of 2-d-old seedlings (Table 2.3.2). The data in Table 2.3.2 are the averages of results from three independent experiments. The weight of the AP solution was approximately 3% of that of the SY solution. The amount of IAA in the SY solution was 7.2 times that in the AP solution, while the calculated concentration of IAA in the AP solution was 4.6 times that in the SY solution. The results confirmed that the concentration of IAA in the AP solution of the growing zone of the hypocotyl was higher than that in the SY solution.

Amounts of IAA in IAA-treated segments

When segments are treated with exogenous IAA, the concentration of IAA in the AP solution should be always higher than that in the SY solution. Furthermore, such results would validate my method for collecting AP solution. Fig. 2.3.1A shows changes in amounts of IAA in AP and SY

solutions in IAA-treated segments. The amount of IAA in both AP and SY solutions in the segments increased up to 4 h. Calculated concentrations of IAA in AP solutions were always higher than in SY solutions. The concentration of IAA in the AP solution reached 75% of the concentration of exogenous IAA (10^{-5} M) and that in the SY solution reached only 23% in 4 h (Fig. 2.3.1B). The difference in concentrations of IAA between AP and SY solutions decreased from 6.4-fold at 0.5 h to 3.3-fold after 4 h of treatment with IAA.

The concentration of IAA in the AP solution from segments that had been incubated in water for 2 h was below the limit of detection (below 6.20×10^{-8} M), but the concentration of IAA in the SY solution from segments that had been incubated in water for 2 h was still 4.46×10^{-8} M, being similar the concentration of endogenous IAA in the SY solution from intact segments, namely, 4.90×10^{-8} M (Table 2.3.2).

Discussion

In the present experiments, I used a centrifugation method to collect AP and SY solutions. The relative weight of AP solutions as a percentage of the total weight of solutions collected by centrifugation ranged from 2 to 17% (Table 2.3.1). Values were higher for the lower part than for the upper part of the hypocotyl. The values on day 2 for the upper and the lower part of the hypocotyl were lower than those on day 3. The volume of AP solution in growing stem tissues of various plants, as a percentage of tissue volume, was estimated to be about 4% in young growing epicotyls of pea by a totally different method (Cosgrove and Cleland 1983). Sakurai and Kuraishi (1988) reported that the volume of the AP solution in the upper part of the squash hypocotyl was always lower than in the lower part, and the volume of the AP solution in young hypocotyls was always lower than that in old hypocotyls. These results support the validity of my centrifugation method for collection of AP solution.

We identified and quantitated IAA in the AP solution by GC-SIM-MS and a centrifugation method. The presence of endogenous IAA in the AP solution was demonstrated for the first time in this study. The concentration of endogenous IAA was always higher in the AP than in the SY solution from squash hypocotyls, although the concentration in the AP solution (2.3×10^{-7} M) was lower than the optimal concentration (10^{-5} M) for induction of elongation growth by

exogenously applied IAA. The concentration of exogenous IAA that induces a directly related response in terms of the extent of elongation ranges from 1 to 6 × 10^{-7} M (Nissen 1985). Within this range, plants respond to the hormone very effectively: a small change in the concentration of IAA is sufficient to alter the growth rate. By contrast, around the optimum concentration of IAA, a small change in the concentration has only a small effect on the growth rate. The concentration of endogenous IAA in the AP solution (2.3 × 10^{-7} M) determined in this study falls within the range that is directly related to the extent of the response. These results support the hypothesis that IAA exerts its physiological effect on the cell from outside of the plasma membrane (Löbler & Klämbt 1985, Venis 1990).

The concentration of IAA in the AP solution from the upper part was higher on day 2 than on day 3. The upper part corresponds to the growing zone in the squash hypocotyl. It elongates at the maximum rate on day 2 and elongation slows down on day 3 (Sakurai et al. 1985, 1987). The decrease in the concentration of IAA in the AP solution from the upper part on day 3 corresponded to the decrease in the growth response of intact squash hypocotyls after the third day. The change in the concentration of IAA in the SY solution was, however, smaller than that in the AP solution. These results suggest that the hypocotyl growth is related to the concentration of IAA in the apoplast rather than in the symplast.

The concentration of IAA in the AP solution was immediately increased upon treatment of segments with exogenous IAA, and the concentration reached 75% of the exogenous concentration in 4 h. IAA was not detectable in the apoplast when segments had been incubated in water for 2 h. Therefore, IAA might have leaked from the apoplast to the ambient water or might have been absorbed by cells, probably moving freely in the apoplast. Cosgrove and Cleland (1983) reported that the AP solution was exchanged with the ambient solution for about 45 min in experiments to monitor diffusion of mannitol from segments of several plants. Yamamoto and Sakurai (1992) also reported that AP solution was exchanged with the exogenous solution for about 1 h in experiments designed to monitor diffusion of non-radioactive and radioactive mannitol from pea segments. These reports support my results for the rate of entry of IAA into the apoplast in my experiments and also demonstrate the validity of my centrifugation method for collection of AP solution.

We can not exclude the possibility that IAA diffuses from the symplast to the apoplast in squash hypocotyls, but the fact that the concentration of IAA in the symplast of intact segments was similar to that in the symplast of segments that had been incubated in water for 2 h might

exclude the possibility of the rapid outward transport of IAA across the plasma membrane, driven by an energy-consuming mechanism that involves an ATPase.

In this study, I revealed the unequal distribution of IAA in AP and SY solutions. The SY solution is thought to include vacuolar contents. IAA is unlikely to enter vacuoles, since IAA dissociates in the cytoplasm. Vacuoles in mature cells account for about 90% of the cell volume, but the vacuolar volume in younger, growing cells is not known. If increases in cell volume depend on increases in vacuolar volume, while the cytoplasmic volume is constant, in upper and lower parts of hypocotyls, I can calculate the vacuolar volume at a given stage by measuring cell volume. The epidermal cells in the upper, growing part were about 55 μm long and those in the lower, mature part were about 163 μm long. The vacuolar volume of cells in the upper part of the hypocotyl was estimated to be about 70% of the cell volume. If IAA is not present in vacuoles but is only found in the cytoplasm, the concentration of IAA in the SY solution in the upper part of 2-d-old hypocotyls can be calculated to be 16.3×10^{-8} M (4.90×10^{-8} M $\times$ 100% / 30%) from the data in Table 2.3.2, which is still lower than the concentration in the apoplast (22.6×10^{-8} M). If the assumption that IAA is present only in the cytoplasm is correct, the concentration of IAA in the symplast in the lower part of 2-d-old hypocotyls would be 27.1×10^{-8} M which is higher than that in the symplast in the upper part (17.9×10^{-8} M). In other words, mature cells would contain a higher concentration of IAA in their cytoplasm than younger, immature cells. This discrepancy should be clarified by separate measurements of levels of IAA in the cytoplasm and the vacuole.

Table 2.3.1. Levels of endogenous IAA in the apoplast and symplast of upper and lower segments of squash hypocotyls

Day	Part of hypocotyl	Amount of IAA (pmol/segment)		Weight (mg/segment)		Concentration of IAA (10^{-8} M)	
		AP (a)	SY (b)	AP (A)	SY (B)	AP (a/A)	SY (b/B)
2	Upper	0.44	2.86	1.08	53.16	40.54	5.38
	Lower	0.37	2.05	8.75	75.65	4.17	2.71
3	Upper	0.47	2.57	4.79	66.12	9.71	3.89
	Lower	0.29	1.39	16.55	79.87	1.76	1.74

One-cm-long segments were excised from upper (0 to 1 cm from the hook) and lower (0 to 1 cm from the base) parts of etiolated hypocotyls of squash seedlings after growth for 2 and 3 d. AP and SY solutions were collected by centrifugation. Levels of endogenous IAA in AP and SY solutions were determined by GC-SIM-MS. The concentration of IAA was calculated by dividing the amount of IAA by the weight (in grams) of AP or SY solution.

Table 2.3.2. Levels of endogenous IAA in the apoplast and symplast of the upper segments of hypocotyls from 2-d-old seedlings.

	Amount of IAA (pmol/segment)	Weight (mg/segment)	Concentration of IAA (10^{-8} M)
AP	0.37 ± 0.02*	1.64 ± 0.15	22.58 ± 2.79
SY	2.67 ± 0.16	54.52 ± 0.52	4.90 ± 0.33

One-cm-long segments were excised from the upper, growing zone (0 to 1 cm from the hook) of etiolated hypocotyls of squash seedlings that had been grown for 2 d in darkness. *: Data are means ± SE (n=3).

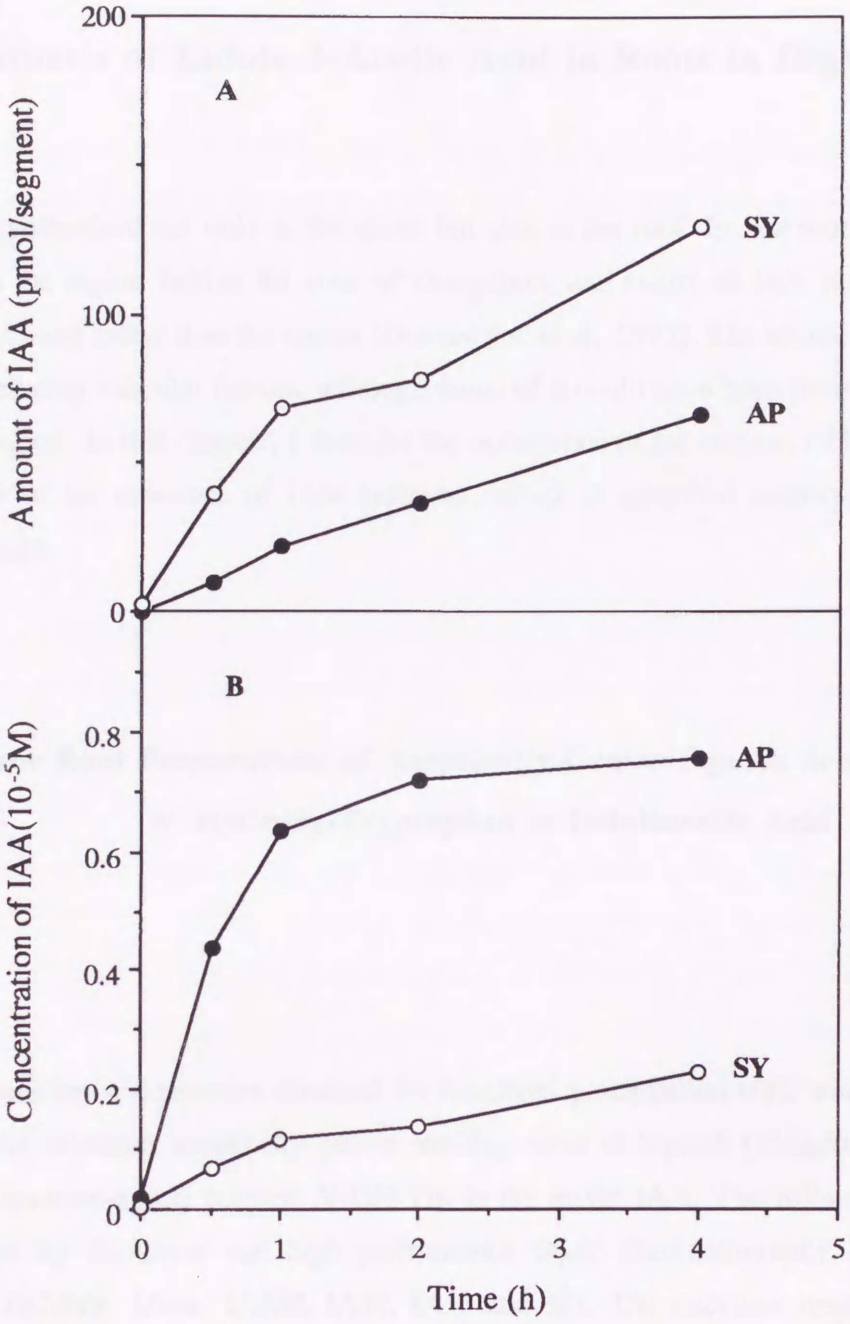

Fig. 2.3.1. Time course of changes in the amount (A) and in the concentration (B) of IAA in AP (●) and SY (○) solutions from hypocotyl segments that had been treated with 10^{-5} M IAA. Segments of squash hypocotyls (1 cm in length) from 2-d-old seedlings were incubated with 10^{-5} M IAA, then AP and SY solutions were separately collected by centrifugation. The amounts of IAA (A) in AP and SY solutions were determined by GC-SIM-MS. The amount of IAA was divided by the weight (in grams) of the AP or SY solution to calculate the concentration of IAA (B).

Chapter 3

Biosynthesis of Indole-3-Acetic Acid in Roots in Higher Plants

IAA may be synthesized not only in the shoot but also in the root. In *Zea* roots, the tip contains less IAA than the region behind the zone of elongation, and nearly all IAA is found in the stele (central part of root) rather than the cortex (Greenwood et al. 1973). The source of this IAA could be the differentiating vascular tissues, although some of it could have been transported acropetally in the stelar region. In this chapter, I describe the occurrence of the enzyme of IAA synthesis and the possibility of the existence of IAM pathway, which is microbial pathway, in the roots of squash seedlings.

3.1 Cell-Free Root Preparations of Aseptically Grown Squash Seedlings Convert *N*-Hydroxy-Tryptophan to Indoleacetic Acid

Abstract

Cell-free preparation and proteins obtained by fractional precipitation with ammonium sulphate, from 120-h old, etiolated, aseptically grown seedling roots of Squash (*Cucurbita maxima* Duch. cv. Houkouaokawaamaguri) convert *N*-OH-Trp to the auxin IAA. The following intermediates were separated by thin-layer and high performance liquid chromatography, and conclusively identified by GC-MS: IAox, IAAld, IAM, IAN and IEt. The enzymes responsible for these conversions could be separated into two fractions by fractional precipitation with ammonium sulphate viz., 0-35 and 35-70% fractions. Several pathways of IAA formation appear feasible in squash roots: 1. *N*-OH-Trp → IAox → IAAld → IAA. 2. *N*-OH-Trp → IAox → IAN → IAA; 3. IAN → IAM → IAA. Adding sodium bisulfite, an aldehyde trapping agent, eliminates most formation of IAA and IEt. Therefore, pathway 1 probably prevails in the metabolism of *N*-OH-Trp. Some of the enzymes involved in this conversion have been partially characterized. Chemical synthesis of *N*-OH-Trp is described.

Introduction

Among those naturally occurring, IAA is believed to be the principal auxin of higher plants. It occurs in every higher plant tested so far, and it has a profound influence on growth, development and differentiation (Davies 1987). While its physiological roles are well established, its biogenesis is still controversial. Notwithstanding evidence to the contrary (Baldi et al. 1991, Wright et al. 1991), several higher plants are capable of utilizing tryptophan (Trp) as a precursor of IAA (Helmlinger et al. 1987, Law 1987, Ludwig-Müller and Hilgenberg 1988, McQueen-Mason and Hamilton 1989, Tsurusaki et al. 1990, Cooney and Nonhebel 1991, Rajagopal et al. 1991, 1994, Bialek et al. 1992). Traditionally two routes of IAA biosynthesis are recognized in higher plants: Trp → IPyA → IAAld → IAA; or Trp → TNH_2 → IAAld → IAA. With the unequivocal demonstration of IAox in some higher plants (Ludwig-Müller and Hilgenberg 1988) and its onward metabolism to IAA in several higher plants (Rajagopal and Larsen 1972), two other routes became apparent: IAox → IAAld → IAA; or IAox → IAN → IAA. What is the origin of IAox? The in toto incorporation of the α-carbon and the amino-N atoms of Trp into indoleglucosinolates by Cruciferous plants suggested the intermediary formation of *N*-OH-Trp (see Mahadevan 1973 for a review). This compound was synthesized and found to be biologically active in several bioassays (Rajagopal et al. 1991). In the present study, I have investigated the metabolism of *N*-OH-Trp by cell-free preparations of the roots of squash seedlings, grown entirely under aseptic conditions and identified the various intermediates conclusively by mass spectrometry. The enzyme proteins of aseptically-grown squash seedlings were fractionally precipitated with ammonium sulphate, and the enzymes involved in converting *N*-OH-Trp to IAA were identified by intermediates (IAox, IAAld, IAN and IAM). The products of these reactions were analyzed by HPLC and GC-MS(GC-MS).

Materials and Methods

Plant materials

Etiolated squash (*Cucurbita maxima* duch. cv. Houkouaokawaamaguri) seedlings were grown in Hoagland's nutrient solution, diluted 5 times, and containing 10^{-4} M ampicillin (Sigma)

at 25°C, according to Sakurai et al. (1985). The seedlings were harvested after 120 h from the start of imbibition, when they were about 5-6 cm tall. for aseptic culture, the seeds were first disinfected by a 30 s dip in 80% ethanol, then transferred to 5% NaOCl (Kanto Chemicals, Japan) and kept stirred for 15 min. They were then washed agar, and incubated at 25°C in darkness until ready for use. All aseptic operations including harvesting were done in a laminar flow sterile air bench. The collected plant material, after weighing, was immediately frozen at -78°C.

Preparation of cell-free homogenate and enzymes

Frozen squash roots (30-60 g) were homogenized at 2°C in 100 mM acetate buffer, pH 5.2 filtered through two layers of nylon cloth (0.45 μm pore size, DIN 1304-140, Nytal, Switzerland), centrifuged at 35,000 × *g* (Kubota 6,000 refrigerated centrifuge with RA-300 rotor) for 1 h, and the clear supernatant was used as the cell-free homogenate.

For enzyme preparation, 30-60 g lots of frozen roots were homogenized at 2°C in 5 volumes of 100 mM phosphate buffer pH 7.2, containing 2 mM dithiothreitol, centrifuged for 30 min at 35,000 × *g*, and to the supernatant solid ammonium sulphate (AS) was added to give a saturation level of 35%, and left to stand overnight. After removing the precipitated proteins (0-35% fraction), more AS was added to the resultant supernatant to raise the level to 70% saturation. After standing for 3-4 h, the precipitated proteins (35-70% fraction) were collected by another centrifugation, dissolved in a small volume of buffer and homogenized in an all-glass Potter-Elvehjem homogenizer and finally dialyzed against 10 mM phosphate buffer pH 7.2 ; 2 mM dithiothreitol over 24 h with 3-4 changes of the buffer solution. After clearing the turbidity by another centrifugation at 35,000 × *g* for 30 min, the resultant solution was used as the source of aldehyde reductase and amide hydrolase. Protein was determined according to Bradford (1976). One gram fresh weight of roots from aseptically grown seedlings yielded 0.8-1 mg protein of the 0-35% fraction and 2.8-3.5 mg protein of the 35-70% fraction.

Enzyme assay

The incubation medium usually contained 0.5-2 mg protein + 1-2 μmols of the respective substrate in 2 ml of 100 mM acetate or phosphate buffer of appropriate pH, and kept for 1 h at 25°C. At the end of the incubation period, where needed, the internal standard was added (5-10 nmols of IPA or 12,000 dpm of [2-^{14}C]IAA (55 mCi/mmol, Amersham lot no. 910930) for

recovery calculation. The incubation mixture was then sequentially extracted at pH 8.5 (neutral indole fraction) and pH 2.9 (acidic indole) with dichloromethane (Cica-Merck, Japan). The organic extract was evaporated to dryness in vacuo at 35°C, dissolved in a small volume of pure methanol and an aliquot was loaded on the HPLC column.

HPLC

Separation of both the neutral and acidic indoles was achieved on an Inertsil ODS-2 column (4.5 x 250 mm, Gasukuro Kogyo Inc., Japan), eluted isocratically with 25% acetonitrile (Cica-Merck, Japan) + 20 mM acetic acid, pH 3.5, at flow rates ranging between 0.6 and 1 ml/min. For IAA, gradient elution (25-50% acetonitrile) was adopted in order to achieve a better separation. The HPLC system consisted of a Shimadzu LC-6A pump unit and a SCL 6-A Systems Controller. IAA was quantified by spectrofluorometry with a FP-210 fluorometric detector (Japan Spectroscopy Co., Tokyo) According to Akiyama et al. (1983) using IPA as the fluorescence ratio of IPA:IAA, the amount of IAA in the sample was calculated.

Gas chromatography-mass spectrometry

Pooled samples eluted from the HPLC column were further purified by thin-layer chromatography (TLC) on 0.25 mm silica gel plates (Merck, pre-washed in methanol and activated at 100°C for 10 min), and developed with chloroform + methanol (24:1, v/v) over a 15 cm front. The Rf values of some indoles under these conditions were: IAA = 0.12, IAox = 0.23, IAM = 0.28, IEt = 0.61, IAAld = 0.68 and IAN = 0.85. The spots corresponding to authentic markers were scraped off, eluted with methanol, evaporated to dryness, dried overnight in a desiccator and converted, where needed, to the TMS-derivative with a mixture of *bis*-trimethylsilyl trifluoroacetamide (BSTFA, Pierce Chemical Co., USA), acetonitrile and pyridine (2:2:1, v/v) at 60°C for 3 h. Aliquot of this derivatized indole was injected on to the GC-MS. For IAA, a slightly different procedure was adapted. The fraction corresponding to IAA from the first HPLC run was evaporated to dryness, methylated with diazomethane, filtered through an Ekicrodisc 13-HPLC prefilter (0.45 μm, Gelman Sciences Ltd., Japan) and re-run on HPLC, using gradient elution with 25-50% acetonitrile + 20 mM acetic acid at a flow rate of 0.6 ml/min. Fractions corresponding to authentic Me-IAA sample were collected, evaporated to dryness, taken up in pyridine and characterized by GC-MS in a Shimadzu Q-1000 quadrupole instrument. Ionization of the compound was carried out by electron impact at 70 eV. The gas chromatograph was equipped with a 30 m fused capillary column (Shimadzu CBJ 17; d_f = 0.25 μm). Two ions, at m/z 130 and

189 were monitored to identify Me-IAA.

Isolation of IAAld

To demonstrate the formation of IAAld, the cell free homogenate or the 0-35% As fraction was incubated with *N*-OH-Trp in 100 mM acetate buffer pH 5.2, without NADH or NADPH, to prevent the facile conversion of the aldehyde to IEt. But 5 mg of sodium bisulfite (Katayama chemicals, Japan) were added to trap the aldehyde formes as an addition product, and the period of incubation was prolonged to 4 h, after which the mixture was extracted thrice with dichloromethane to remove the other indoles except *N*-OH-Trp and IAAld-$NaHSO_3$. The aqueous fraction which contained the addition product was evaporated to dryness, taken up in a small volume of 25% acetonitrile + 20 mM acetic acid, an aliquot was injected directly on the HPLC and eluted isocratically with 25% acetonitrile. The IAAld addition product elutes in a sharp peak at 30.5 min and also co-chromatographs with authentic IAAld-$NaHSO_3$ (Fluka, Switzerland) solution. For GC-MS identification, the 0-35% AS fraction was incubated with *N*-OH-Trp in acetate buffer pH 5.5 + 5 mg $NaHSO_3$. At the end of the incubation, indoles other than IAAld-$NaHSO_3$ were removed with dichloromethane, after which the pH was raised to 8.5 with 0.5 M $NaHCO_3$. This liberated free IAAld, Which such fractions were pooled, subjected to TLC as described earlier, and the spot corresponding to authentic IAAld marker at R_f = 0.68 was eluted with methanol, and after transfer to pyridine was directly injected on to GC-MS without derivatization.

Recovery

The recovery of radioactive IAA from infiltration experiments ranged between 30 and 46% after extraction, HPLC and TLC purification steps. Omitting the TLC step led to recoveries of up to 70%. The extraction efficiency of dichloromethane was > 95% in enzyme experiments.

Synthesis of N-OH-Trp

Four mmoles (0.813 g) IPyA and 6 mmols (0.417 g) hydroxylamine-HCl were dissolved in a mixture of 7 ml water + 5 ml ethanol + 9 ml 1 M NaOH. This solution turns red due to the formation of indolepyruvic acid oxime. Under a continuous stream of nitrogen through this solution and constant stirring, 7 mmols (0.44 g) sodium cyanoborohydride were added and the pH was immediately adjusted to 4 with 1 M HCl with an automatic titrator (Shimadzu AT-117). *N*-

OH-Trp starts precipitating in the mixture. The reaction is allowed to proceed under N_2 for 24 h and during this time ca. 6 ml of 1 M HCl were consumed. Another portion of 0.44 g of $NaBH_3CN$ was added and the reaction allowed to continue for another 36 h, after which the pH was brought down to 2 with 1 M HCl and mixture was evaporated to dryness at 30°C. The white residue containing *N*-OH-Trp was extracted repeatedly with 50 ml portions of warm (50°C) ethanol. One ml of warm ethanol dissolves about 2 mg *N*-OH-Trp.

The pooled ethanol extract was concentrated at 30°C and the precipitated *N*-OH-Trp was redissolved by raising the temperature to 50°C. This warm solution was first cooled in an ice-bath and then in a freezer overnight at -18°C. The precipitated crystals of *N*-OH-Trp were filtered of and dried over P_2O_5.

Results

Separation of indoles by HPLC

Most neutral indoles separate clearly on an Inertsil ODS-2 column eluted isocratically with 25% acetonitrile + 20 mM acetic acid (Fig. 3.1.1). Only free IAAld is difficult to separate in this system as it runs close to IEt and also leaves a long tail. But IAAld-$NaHSO_3$ gives a sharp and clean peak at 30.5 min. IAA is best separated as its methyl derivative with gradient elution with 25-50% acetonitrile + 20 mM acetic acid at a flow rate of 0.8 ml/min.

N-OH-Trp metabolism by squash root cell-free homogenate

At pH 5.2 and in the presence of the cofactors NAD, NADH and pyridoxal phosphate, the cell-free homogenate of the roots of aseptically grown squash seedlings, converts *N*-OH-Trp to at least 6 indole metabolites, namely, IAox, IAAld, IAM, IAN, IEt and IAA. Quantitative data obtained by on-line spectrofuluorometry HPLC for 4 of these are presented in Table 3.1.1. In the presence of $NaHSO_3$, the amount of IEt and IAA formed are reduced to 11 and 19% respectively of the amounts formed without bisulfite; but ca. 17% more of IAox is formed. In the presence of bisulfite, no IAN could be detected.

Stepwise identification of intermediates

Fractional precipitation with AS yielded three fractions (0-35%, 35-70% and 70-100%) of the root proteins of aseptically grown squash seedlings. Each of these fractions was incubated

with *N*- OH-Trp as well as the intermediates IAox, IAN, IAM and IAAld to identify the relevant enzymes and the products of thier reaction by HPLC and GC-MS. No enzyme associated with *N*-OH-Trp metabolism was found in the 70-100% fraction. The activities associated with the other two fractions are shown in Table 3.1.2. All the enzymes needed to convert *N*- OH-Trp to IAA occur in the 0-35% fraction. The 35-70% fraction, which contains relatively more protein, contains two major activities - an amidohydrolase converting IAM to IAA. This is the subject of another paper (Rajagopal et al. 1994) and so will not be discussed here any further; and an aldehyde reductase transforming IAAld to IEt.

The major product of *N*-OH-Trp metabolism, eluting at 25.9 min and co-chromatographing with the authentic standard, is IAox (Fig. 3.1.2). It was identified in the free form by GC-MS (Table 3.1.3).

IAox to IAAld

The enzyme responsible for this reaction occurs in the 0-35% fraction and has a broad pH optimum. This enzyme does not attack indolepyruvic acid oxime. The reaction is enhanced by 10-20% by the addition of NAD or NADP, but these do not seem to be cofactors. The IAAld formed was trapped with sodium bisulfite and the addition product was identified by HPLC (R_t = 30.5 min). The free IAAld liberated from this adduct was characterized by GC-MS (Fig. 3.1.3). The M^+ ion at 159 and the quinolinium fragment at m/z 130 are typical of this compound.

IAAld to IAA

The enzyme effecting this conversion also occurs in the 0-35% fraction (Fig.3.1.4). It has a pH optimum around 5.2 and no IAA is formed at pH 7.2. The specific activity of this fraction is rather low compared to the activity of the cell-free homogenate (Table 3.1.1) suggesting the need for cofactors for maximal activity. Added NAD or NADP did not enhance IAA yielded (334 ± 18 pmols without cofactors against 383 ± 25 pmols/mg protein in 1 h in the presence of NAD or NADP). It does not appear to be a NAD-dependent dehydrogenase. The IAA formed was identified by GC-MS-SIM, following methylation. The M^+-ion (189) and the fragment 130 ion, characteristic for Me-IAA were identified. The ratio of the M^+-ion at 189 to the fragment at m/z 130 was 28%. The average ratio for authentic IAA was 19 ± 4%.

IAAld to IEt

The reduction of IAAld to the corresponding alcohol, IEt is carried out by a powerful reductase activity present in the 35-70% fraction (Fig. 3.1.5). The specific activity of this protein (30-42 nmols/mg in 1 h) is the highest noted in this work. Though optimally active at pH 7, the experiment of Fig.3.1.5 was done at pH 6 in 100 mM phosphate buffer, in order to remove the unreacted IAAld with $NaHSO_3$. This enzyme works equally well with both NADH and NADPH. IEt was conclusively characterized by the fragment at m/z 202 and the molecular ion at 305 (Table 3.1.3).

IAN to IAA

This reaction is due to the presence of nitrilase, a unique enzyme transforming the C≡N group directly to a -COOH, in the 0-35% fraction (Fig. 3.1.6). It has a broad pH optimum, needs no cofactors and being hydrolytic, proceeds equally well with or without oxygen. The product IAA was identified by HPLC, TLC and GC-MS-SIM. The quinolinium fragment ion at m/z 130 (100%) and the M^+-ion at 189 (12%) positively identify this compound (Table 3.1.3).

Discussion

Being unstable, *N*-OH-Trp breaks down spontaneously above pH 6 and one of the products is IAox. With increasing pH, more of the oxime is formed and in the presence of ammonia, it is converted to IAox almost quantitatively. This appears to be a general character of *N*-hydroxy-amino acids (Mϕller 1981). But it is quite stable below pH 5.5, in strongly buffered solutions; hence the pH of 5.2 was chosen for routine experimentation. This pH was also mandatory for another reason. The aldehyde oxidizing enzyme which converts IAAld to IAA works optimally below pH 5.5.

Roots of aseptically grown squash seedlings contain the enzymes needed to metabolize *N*-OH-Trp to IAA by more than one pathway. Based on the enzymic activities identified (Table 3.1.2), the following pathways are feasible:

N-OH-Trp → IAox → IAAld → IAA
↓
IEt

N-OH-Trp → IAox → IAN → IAA

IAN → IAM → IAA

Following the infiltration of *N*- OH-Trp into intact roots or hypocotyls (data not presented), the major products seen were IAox > IEt > IAM > IAA. The formation of large amounts of IEt and its abolition in the presence of $NaHSO_3$, suggests the intermediary formation of IAAld. This seems logical since the most active protein encountered in squash roots in this work is the aldehyde reductase (Table 3.1.2). The production of both IAA and IEt is drastically reduced by adding sodium bisulfite, an efficient aldehyde trapping agent, further lending credence to the assumption that IAAld is a normal intermediate between *N*-OH-Trp and IAA. Protein preparations from chinese cabbage (Helmlinger et al. 1987) and oat coleoptiles (Rajagopal et al. 1992) are also capable of converting IAox to IAA via IAAld. The small amounts of IAA formed in the presence of sodium bisulfite are probably the result of nitrilase/amidohydrolase action on IAN, since this nitrile could not be detected in the presence of $NaHSO_3$ (Table 3.1.1).

The amidohydrolase activity present in the 35-70% fraction has a broad substrate specificity (Kemper et al. 1985, Kawaguchi et al. 1991, Rajagopal et al. 1994). It acts upon IAN converting it to IAM and then hydrolyses this amide to IAA. Since neither the nitrilase nor the amidohydrolase require cofactors, the conversion of IAN and IAM to IAA are seemingly facile reactions. But the formation of IAN from IAox, by the removal of a molecule of water, seemingly requires pyridoxal phosphate, as in its absence only traces of IAN are seen. So this is probably a side reaction operating in the presence of high concentrations of pyridoxal phosphate. The significance of the IAM pathway is discussed elsewhere (Rajagopal et al. 1994).

At present, it is unknown whether the IPyA or the tryptamine pathways, mentioned in the Introduction, also operate in squash roots. Nor is the Quantitative importance of the various pathways in indole economy of squash clear. To elucidate the normal, in vivo pathway of IAA biosynthesis, it is necessary to label all the intermediates with a pervasive label like deuterium by growing the seedlings in D_2O and then isolate and identify the intermediates. Such experiments indicate the operation of the IPyA pathway in tomato shoots (Cooney and Nonhebel 1991) and a pathway not involving tryptophan in the *orange pericarp* mutant of maize (Wright et al. 1991). Squash seems to be exceptional in possessing a metabolic machinery capable of producing IAA by several pathways. This does not necessarily mean that all the pathways operate simultaneously. Which pathway operates when is probably developmentally determined, and also by the availability of cofactors, the equilibrium conditions prevailing at the subcellular site of IAA synthesis and the prevailing substrate concentrations. Enzymic compartmentation could also be involved in this regulation. The possibility of non-specific enzymes catalyzing one or more of the

steps can not be disregarded either. It is necessary to purify the various enzymes and study thier biochemical characteristics to assess their relative importance in IAA biosynthesis in squash.

The above results suggest the possible existence of many routes of IAA synthesis in the squash roots. Particularly, the pathway of IAA synthesis via IAAld is strongly suggested. But interestingly, the activity of conversion of IAM to IAA maybe also exist and act in squash roots. IAM pathway is known as the pathway of IAA synthesis of plant pathogens. The pathway from Trp via IAM is known to be present in crown gall tumors induced by infection with *Agrobacterium tumefaciens*, and the excessive production of IAA by the enzymes in this pathway, together with the excessive production of cytokinin, is responsible for formation of tumor (Morris 1985). Although the occurrence of IAM pathway has been reported in some plants (Isogai et al. 1967, Kawaguchi et al. 1991, 1993, Rausch et al. 1985, Saotome et al. 1993, Takahashi et al. 1975, Tamura et al. 1972), the role of IAM in IAA synthesis is doubted (Schröder et al. 1984, Weiler and Schröder 1987).

Next, I describe the possibility of the IAM pathway known as the pathway of pathogenic microbial, *Agrobacterium tumefaciens* and so on, in the aseptically-grown squash roots.

Table 3.1.1. Metabolism of *N*-OH-Trp in acetate buffer pH 5.2, by the cell-free homogenate of roots of aseptically grown squash seedlings + 1 mg each of NAD, NADH and pyridoxal phosphate, in the presence and absence of $NaHSO_3$. Data refer to nmols/mg protein at 25°C in 1 h.

Compound	$NaHSO_3$	Amount
IAox	-	7.90 ± 1.06
	+	9.20 ±0.94
IEt	-	1.30 ± 0.35
	+	0.14 ± 0.04
IAN	-	0.12 ± 0.03
	+	not detected
IAA	-	2.68 ± 0.44
	+	0.51 ± 0.10

Table 3.1.2. Ammonium sulphate fractionation of squash root proteins, probably involved in the conversion of *N*- OH-Trp to IAA. Data refer to nmols/mg protein in 1 h at 25°C.

Reaction	pH optimum	Sp. activity observed
<u>0-35% fraction</u>		
N-OH-Trp → IAox	?	8-15
IAox → IAAld	4.5-6.0	2-4
IAAld → IAA	5.2	0.34-0.45
IAN → IAA	6.5-7.5	3.5-5.9
<u>35-70% fraction</u>		
IAAld → IEt	6.0-7.5	30-42
IAM → IAA	7.0-7.5	1.8-5.2

Table 3.1.3. Relative abundances of the principal ions from electron impact mass spectra of indole compounds resulting from the metabolism of *N*-OH-Trp by enzyme preparations from the roots of aseptically grown squash seedlings.

Compound	m/z	% abundance	
		Standard	Sample
IAox	130	100	100
	174	78	68
	157	31	30
	156	26	33
	155	22	33
IAN	130	43.5	37
	155	98	85
	156	63	40
TMS-IEt	130	18	24
	202	100	100
	203	14	20
	305	36	27
Me-IAA	130	100	100
	189	17	12

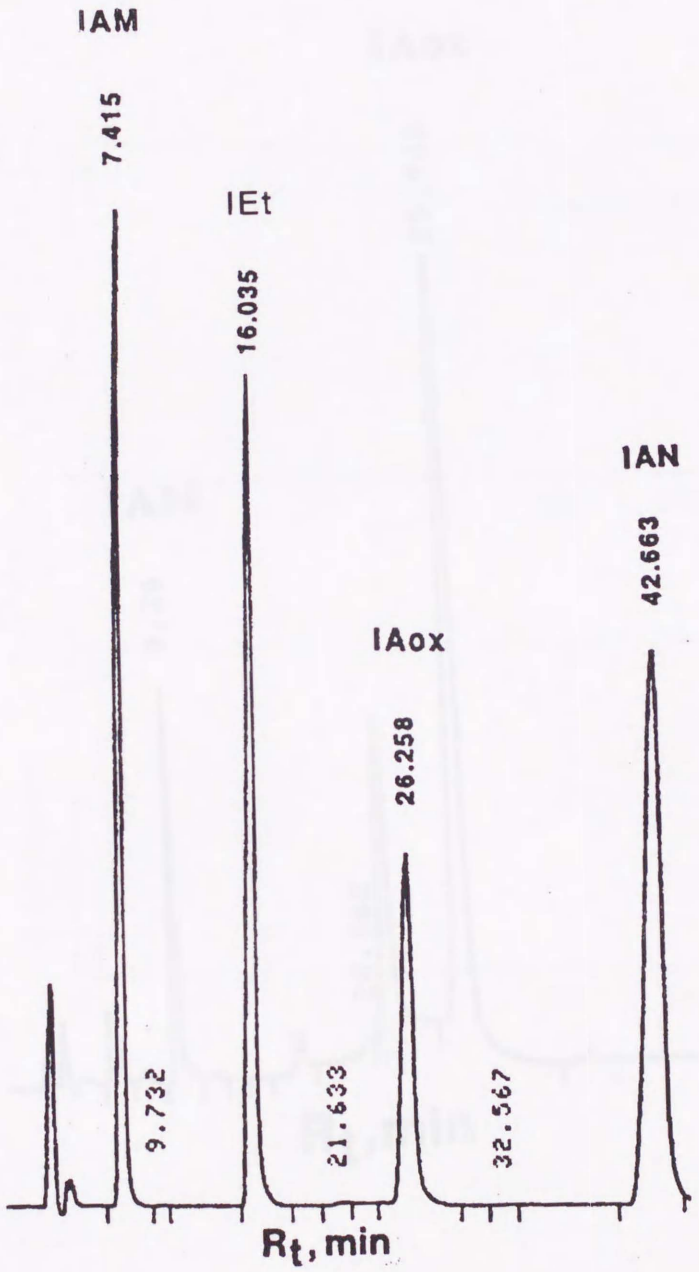

Fig. 3.1.1. HPLC separation of neutral indole standards, 100 pmole each, on Inertsil ODS-2 column-eluted with 25% acetonitrile @ 1 ml/min.

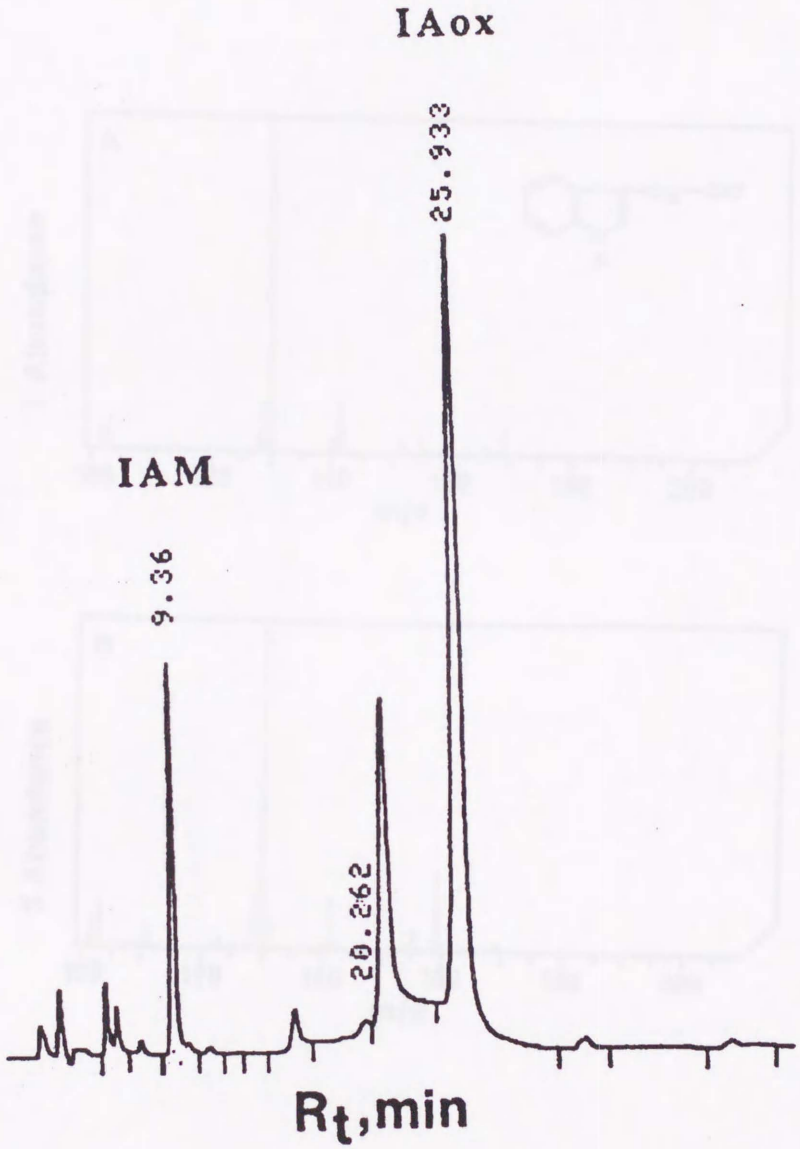

Fig. 3.1.2. Formation of IAox and IAM from *N*- OH-Trp by the cell-free homogenate of squash roots in acetate buffer pH 5.2. IAAld was removed by trapping with $NaHSO_3$.

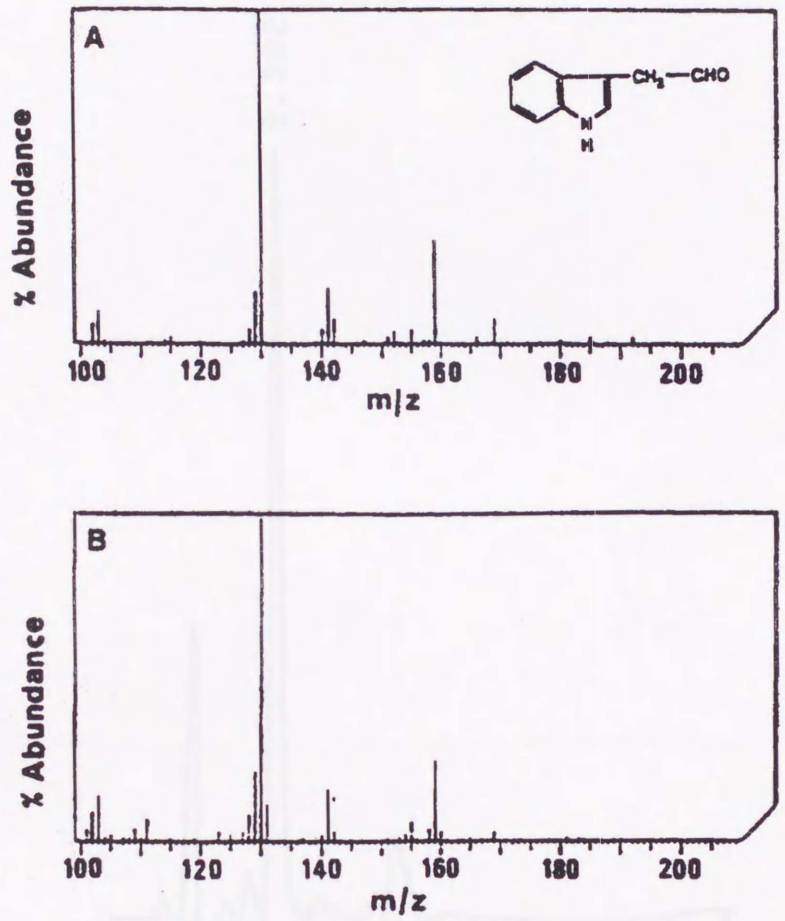

Fig. 3.1.3. Electron impact mass spectrum of IAAld. **A**. Authentic standard. **B**. IAAld produced from *N*- OH-Trp by the 0-35% fraction of squash roots at pH 5.5, trapped with $NaHSO_3$ and purified by TLC and HPLC.

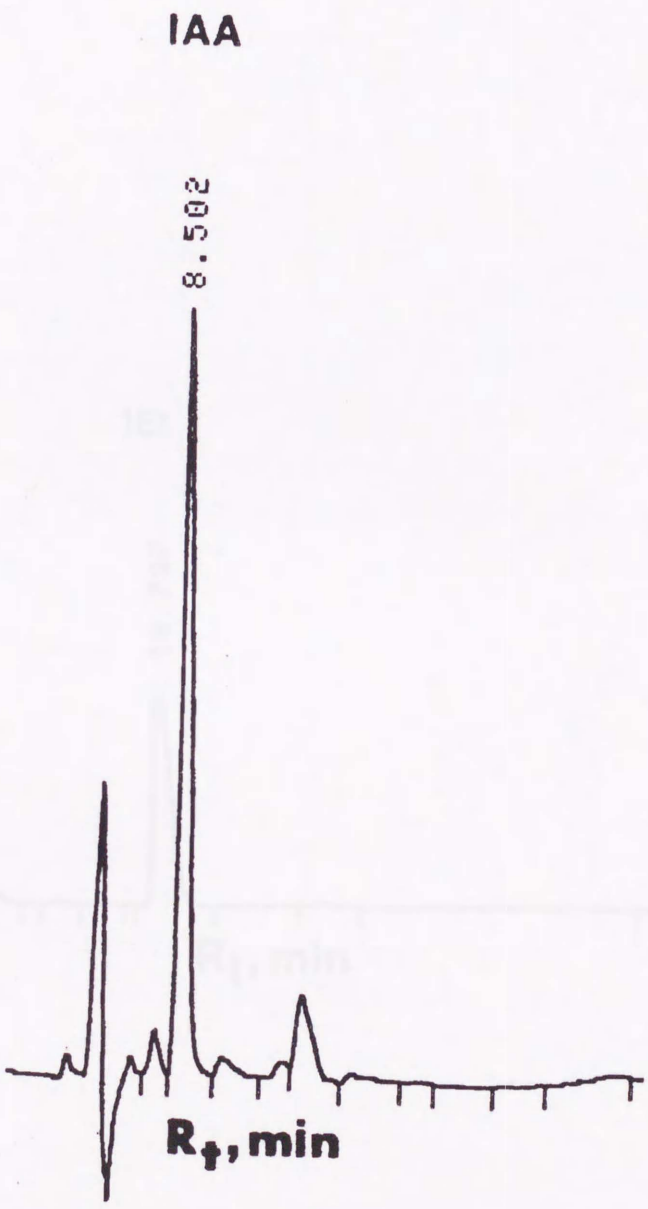

Fig. 3.1.4. HPLC profile of IAA formed from IAAld by the aldehyde oxidizing activity of the 0-35% fraction of squash roots at pH 5.2. 3.3% of the product was injected and eluted with 50% acetonitrile @ 0.5 ml/min.

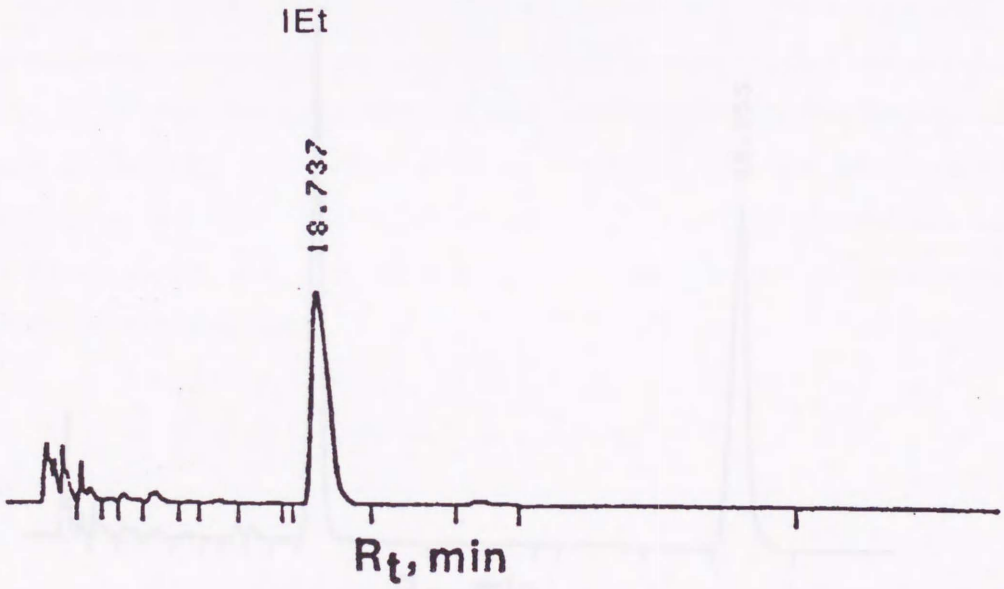

Fig. 3.1.5. HPLC profile of IEt formed by the reduction of IAAld by the 35-70% fraction of squash roots at pH 6. 0.05% of the product was injected.

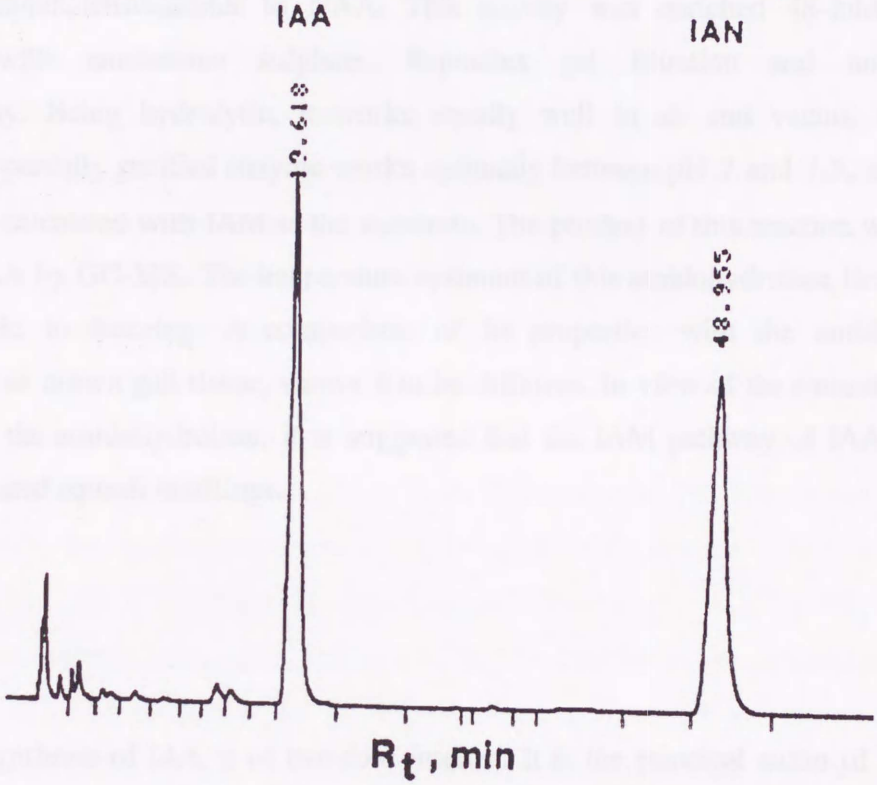

Fig. 3.1.6 HPLC profile of IAA formation from IAN due to the nitrilase activity present in the 0-35% of the product was injected.

3.2 Natural Occurrence of Indoleacetamide and Amidohydrolase Activity in Etiolated Aseptically-Grown Squash Seedlings

Abstract

Etiolated seedling tissues of aseptically grown squash (*Cucurbita maxima* Duch) contain IAM as a natural endogenous compound, conclusively identified by GC-MS. Roots of aseptically raised seedlings also contain amide hydrolysing activity, which converts IAM to IAA, IAN to IAM and IAA, and 1-naphthalenacetamide to NAA. This activity was enriched 48-fold by fractional precipitation with ammonium sulphate, Sephadex gel filtration and anion exchange chromatography. Being hydrolytic, it works equally well in air and vacuo, without added cofactors. The partially purified enzyme works optimally between pH 7 and 7.5, and a K_m value of 80 μM was calculated with IAM as the substrate. The product of this reaction was definitively identified as IAA by GC-MS. The temperature optimum of this amidohydrolase lies around 45°C, and it is stable to freezing. A comparison of its properties with the amidohydrolase of *Agrobacterium* or crown gall tissue, shows it to be different. In view of the natural occurrence of both IAM and the amidohydrolase, it is suggested that the IAM pathway of IAA biogenesis is feasible in etiolated squash seedlings.

Introduction

The biosynthesis of IAA is of two-fold interest. It is the principal auxin of higher plants, involved in many aspects of growth and development. It is also involved in tumorigenesis (Schell 1982). Both bacteria, which induce hyperplasia in infected tissues, and higher plants are capable of synthesizing IAA from Trp, though employing different pathways. *Pseudomonas savastanoi*, by over-producing and secreting IAA into infected tissues, causes galls in olive and oleander (Nester and Kosuge 1981). The soil-borne pathogen *Agrobacterium tumefaciens*, the causal organism of crown gall in several dicots employs a different strategy. Through a unique feat of genetic engineering, it transfers a piece of its plasmid DNA (tumor-inducing or Ti-DNA) to the host genome, where it gets integrated stably. This T-DNA contains genetic information for the

production of two enzymes, a Trp monooxygenase and an amidohydrolase. While the monooxygenase converts Trp to IAM, The second enzyme, amidohydrolase, produces IAA from the amide (Prinsen et al. 1990). The functions and locations of the genes coding for these two enzymes have been determined on the T-DNA introducted into the host subsequent to infection. Through deletion mutation and selective expression in transformed tissues, the enzymes as well as the products of thier reactions have been conclusively identified as IAM and IAA respectively (Inzé et al. 1984, Klee et al. 1984, Schröder et al. 1984, Follin et al. 1985, Kemper et al. 1985, von Onckelen et al. 1985, 1986, Thomashaw et al. 1986). A similar pathway of IAA production had earlier been established in *Pseudomonas savastanoi* (Comai and Kosuge 1980, 1982, Nester and Kosuge 1981).

Higher plants, on the other hand, are believed to synthesize IAA either via IpyA and IAAld, or through TNH_2 and IAAld (Sembdner et al. 1980). In some species, characterized by the production of glucosinolates or cyanogenic glucosides, IAA can also arise from IAox (Sembdner et al. 1980). The occurrence of IAM has been reported in mung bean seeds (Isogai et al. 1967), *unshiu* orange fruits (Takahashi et al. 1975), the club-root of chinese cabbage (Tamura et al. 1972), wounded potato tuber (Rausch et al. 1985) and aseptically raised cherry seedlings (Saotome et al. 1993). In these instances, amide formation as an isolation artefact was avoided. More recently, IAM hydrolysing activity leading to IAA formation was detected in sterile calli of wild and cultivated rice varieties (Kawaguchi et al. 1991); and in cell-free preparations obtained from either surface-sterilized or peeled tissues of young *trifoliata* orange fruits, The conversion of L-[^{14}C]Trp to IAA via IAM has been demonstrated (Kawaguchi et al. 1993). Despite these observations, the natural occurrence of IAM in higher plants and its precursory role in IAA biogenesis are doubted (Schröder et al. 1984, Weiler and Schröder 1987). The prevailing consensus seemingly favours the view that in tumorigenesis, caused by *A. Tumefaciens* the T-DNA operates by introducing new enzyme reactions into host cells which can not be controlled by the regulatory mechanisms of the host tissue, and that the uncontrolled over-production of IAA is in some way responsible for neoplastic growth (Schröder et al. 1984, Morris 1986), though this view seemingly does not apply to asparagus crown gall tissues (Prinsen et al. 1990). Implicit in this view is the assumption that IAA production via the IAM-pathway is the exclusive preserve of some pathogenic bacteria. I present herein evidence for the natural occurrence of IAM and of amidohydrolase activity which converts IAM to IAA, even in aseptically grown etiolated squash seedlings, opening the possibility that some higher plants too can operate this 'forbidden' pathway

of auxin production. Some of the biochemical parameters of this squash amidohydrolase have been characterized and by comparison with the properties of the enzyme from crown gall tissue, studied by Kemper et al. (1985), it is shown that the plant enzyme is probably different from its bacterial counterpart. This activity has also been partially purified (48-fold) from aseptic 120 h-old squash roots.

Materials and Methods

Plant material

Etiolated squash (*Cucurbita maxima* Duch. cv. Houkouaokawaamaguri) seedlings were raised at 25°C in 1/5th strength Hoagland's solution with 10^{-4} M ampicillin (Sigma), according to Sakurai et al. (1985), and used when they were 120 h old from the start of imbibition. For growing plants aseptically, the seeds were first disinfected by a 30 s dip in 80% ethanol following by transfer to 5% sodium hypochlorite (Kanto Chemicals, Japan) solution for 15 min, then washed free of chlorine with sterile distilled water and planted in 0.75% agar in 500 ml plastic tissue culture jars and incubated in darkness at 25°C until needed. All aseptic operations including harvesting, were performed in a laminar-flow clean-air bench. Collected material, after weighing, was immediately frozen at -78°C.

Isolation and identification of IAM

Frozen plant material, 30-100 g fresh weight, was homogenized in 5 volumes of cold 80% ethanol, containing 100 mg $liter^{-1}$ butylated hydroxy toluene, and filtered through a glassfiber filter (Kiriyama Roto S95, GF3) under suction. Ethanol was removed by rotary film evaporation at 35°C and the aqueous phase was clarified by centrifugation at 18,000 × *g* for 15 min. The pH of the clear supernatant was adjusted to 3.5 with phosphoric acid, and then extracted thrice with an equal volume of petroleum ether. The pH of the aqueous phase was then raised to 8.5 with 1 M KOH, and neutral indoles were removed by shaking thrice with dichloromethane. This fraction contained part of the IAM, which partitions in both the acidic and non-acidic fractions (more in the acidic fraction). The pH of the aqueous phase was then adjusted to 2.9 with 1 M HCl under constant stirring, and filtered successively through Millipore filters with pore sizes of 3, 1.2 and 0.45 μm and finally through a Sartolon 0.2 μm nylon filter (Sartorius GmbH, Göttingen,

Germany). Acidic indoles + IAM were extracted from this solution by shaking thrice with an equal volume of dichloromethane.

Following HPLC (described later on), the fraction corresponding to IAM was collected, evaporated to dryness, taken up in dichloromethane and subjected to TLC on 0.25 μm silica gel plate (prewashed in methanol and activated at 100°C for 10 min), developed with chloroform + methanol (24:1, v/v) over a 15 cm front. The spot corresponding to the marker IAM at R_f 0.28 was scraped off, extracted with methanol and again injected on HPLC, and eluted isocratically with 25% acetonitrile in 20 mM acetic acid, pH 3.5. The fraction peaking at 8.5-9.0 min which corresponded to the R_f of synthetic IAM was collected, evaporated to dryness, dried overnight in a desiccator and then silylated with a mixture of BSTFA, acetonitrile and pyridine (2:2:1, v/v) at 60°C for 3 h. After volume reduction under a stream of nitrogen, 1 μl of this TMS-IAM derivative was injected into the GC-MS.

Enzyme preparation

All operations in connection with the enzyme preparation were performed at 2-4°C. Thirty to 100 g lots of frozen squash roots were homogenized in 5 volumes of 100 mM phosphate buffer, pH 7.2, containing 5 mM Na-EDTA + 10 mM dithiothreitol (Sigma) + 10 mM $MgCl_2$ + 0.25 mM PMSF (Sigma), filtered through two layers of nylon cloth (DIN 130-140, Nytal, Switzerland) and centrifuged at 35,000 × *g* for 30 min in a Kubota 6000 or a Sorvall RC-2 refrigerated centrifuge at 4°C. The supernatant was saturated with solid ammonium sulphate (Katayama Chemicals, Japan) to give a saturation level of 35% and left to stand overnight. After removing the precipitated proteins by spinning at 35,000 × *g* for 1 h, the resultant supernatant was again saturated with ammonium sulphate to a level of 70%. After standing for 3-4 h, the precipitated proteins were collected by centrifugation, homogenized in an all glass Potter-Elvehjem homogenized, dissolved in 3-4 ml of homogenization buffer and dialyzed against 10 mM phosphate buffer containing 10 mM $MgCl_2$ + 5 mM EDTA + 2 mM dithiothreitol for 24 h, with 4 changes of the buffer solution. After clearing the turbidity by another centrifugation, the resultant clear solution was further purified by Sephadex G-75 (40 × 2.5 cm column, V_0=75 ml) gelfiltration, with 100 mM phosphate buffer, pH 7.2 + 2 mM dithiothreitol as the eluant. Active fractions were pooled, concentrated, desalted with Sephadex G-25 and finally cleared by centrifugation at 15,000 × *g* for 30 min. Protein was determined using BioRad Protein Assay Kit according to Bradford (1976).

Anion exchange chromatography

Anion exchange chromatography was performed at 4°C on a LKB Pharmacia SMART™ μ separation system, coupled to a SMART™ software manager (Pharmacia, Uppsala, Sweden), fitted with a MiniQ™ PC 3.2/3 column (3.2 × 30 mm) containing a strong anion exchanger (non-porous, 3 μm polymer beads with quaternary amino groups). Protein samples (100-500 μl) were loaded on the column and washed first with 100 mM phosphate buffer, pH 7.2 + 10 mM $MgCl_2$ + 5 mM dithiothreitol. The bound proteins were then eluted with a linear NaCl (1 M) gradient in the same buffer. Conductivity and absorbance at λ 260, 280 and 403 nm were monitored simultaneously and continuously, and 50 or 100 μl samples were collected. The protein content of these fractions was calculated by the formula: protein mg $ml^{-1}=1.55A_{280}-0.76A_{260}$.

Enzyme assay

Amidohydrolase activity was assayed in 100 mM phosphate buffer, pH 7.2, with 10 mM $MgCl_2$, 40 mM KCl, 5 mM DTT, 1-2 μmols of IAM (Sigma) and varying amounts of the enzyme (50 μg-2 mg depending on the fraction). After the incubation (usually 1 h), the medium was sequentially extracted with dichloromethane at pH 8.5 and pH 2.9, obtained with 0.5 M sodium bicarbonate and 1 M HCl respectively. The organic extract was taken to dryness in vacuo, dissolved in a small volume of methanol and an aliquot was loaded on the HPLC column.

HPLC

Separation of both neutral and acidic indoles was achieved on an Inertsil ODS-2 column (4.5 × 250 mm, Gasukuro Kogyo Inc., Japan), eluted isocratically with 25% acetonitrile + 20 mM acetic acid pH 3.5, at flow rates ranging between 0.6 and 1 ml min^{-1}. In a few cases gradient elution (25 to 50% acetonitrile) was adopted in order to achieve a better separation. The HPLC system consisted of a Shimadzu LC-6A pump unit coupled to Shimadzu SCL-6A systems controller. IAA was quantified by spectroscopic Co., Tokyo), according to Akiyama et al. (1983), using IPA (5-10 nmols) as the internal standard.

GC-MS

Following HPLC, the fractions representing IAA were pooled and methylated with

diazomethane. The methylated sample was filtered through an Ekicrodisc 13 HPLC pre-filter (0.45 μm, Gelman Sciences Ltd., Japan) and then put on the HPLC column and eluted with a gradient of 25-50% acetonitrile with 20 mM acetic acid. The peak corresponding to authentic Me-IAA was collected and characterized by GC-MS in a Shimadzu QP-1000 quadropole instrument. Ionization of the compound was carried out by electron impact at 70 eV. The gas chromatograph was equipped with a 30 m fused capillary column (Shimadzu CBJ 17; d_f=0.25 μm).

Results

Endogenous IAM

All parts of the 120 h-old, aseptically grown, etiolated squash seedling, contain naturally occurring IAM, which was identified by HPLC, TLC, and GC-MS. As measured by HPLC, the cotyledons contain the most and the hypocotyl the least amount of this compound (data not presented). Yet, for GC-MS identification, the hypocotyl was chosen as it provides relatively clean extracts with few interfering compounds. For want of a suitable internal standard, endogenous IAM could not be quantified by GC-MS. But there is no doubt as to its identity (Fig. 3.2.1) as the fragmentation pattern of the plant sample is in good agreement with the spectrum of the authentic standard.

Identity of the product of amidohydrolase action on IAM

The HPLC profile of the products of the reaction of the 35-70% ammonium sulphate saturated fraction with synthetic IAM is shown in Fig. 3.2.2. The peak at 18.6 min co-chromatogr aphs with authentic IAA. Other than IAA, there is no other product. Though a neutral compound, considerable amounts of IAM partition into the acidic fraction, due perhaps to its polar character. The mass spectrum of the methylated product of the enzyme reaction (Fig. 3.2.3) leaves no doubt as to its identity as IAA due to the fragments at m/z 130 (quinolinium ion) and 189, the molecular ion of Me-IAA.

Partial enzyme purification

Through the four-step protocol shown in Table 1, the amidohydrolase activity was enriched ca. 48-fold as compared to the activity of the cell-free homogenate. Sephadex gel filtration

removes high molecular weight compounds (Fig. 3.2.4) but does not give much purification. Following anion exchange chromatography, the activity elutes between 0.5 and 0.6 M NaCl (Fig.3.2.5). Through this fraction is heavily contaminated with nucleic acids, as is evident from the strong absorption at 260 nm, its specific amide hydrolysing activity is high compared to the starting material (Table 3.2.1). Protein recovery, however, is very poor, 102 g fresh weight roots yielding only about 1.3 mg of this fraction. Due to the limited availability of this fraction, only two parameters (effects of pH and substrate concentration) could be studied with this partially purified enzyme. Fractions 22 + 23 also show some IAM hydrolysing activity, but due to thier low protein content, not many experiments could be performed.

Properties of the enzyme

The amidohydrolase enzyme is present in both the aseptically grown as well as the non-aseptically grown, etiolated squash seedling roots, in about equal amounts on a fresh weight basis. Though its activity is promoted by the presence of $MgCl_2$ and KCl, it needs no cofactors as considerable activity is seen without any additions (data not presented). It works equally well in the presence (3.4 nmols IAA mg protein^{-1} h^{-1}) or absence of oxygen (2.9 nmols IAA mg protein^{-1} h^{-1}). Figs. 6-8 record some of the biochemical properties of this enzyme. The pH for optimal activity lies between 7 and 7.5, and for routine experimentation a pH of 7.2 was selected (Fig. 3.2.6). The increase in activity at pH 7 compared to that at pH 4.5 in more than 4-fold. The optimal temperature lies at around 45°C and activity falls off sharply on either side of this temperature (Fig. 3.2.7). The effect of substrate concentration (IAM) is shown in Fig. 3.2.8. Characteristically, there is no substrate inhibition even at high concentrations. A K_m value of 80 μmols was calculated from the double reciprocal plot.

Substrate specificity

Besides IAM, this enzyme is capable of converting 1-naphthaleneacetamide to its corresponding acid, but at a reduced velocity (Table 3.2.2). IAN is as good a substrate as IAM for this amidohydrolase, and the amount of IAA formed from the nitrile is ca. 90% of the amount formed from the amide. The HPLC profile of the reaction products (Fig. 3.2.9) shows considerable amounts of IAM besides IAA and the substrate IAN. The ethyl ester of IAA and the amino acid conjugate indoleacetylaspartate are not acted upon by this enzyme.

Comparison with the bacterial and crown gall tissue enzymes

A comparison of the properties of the amidohydrolase of squash roots with similar activities prepared from *A. tumefaciens* (cloned and expressed in *Eschericia coli*) and tobacco crown gall (Kemper et al. 1985) is revealing (Table 3.2.3). The squash root enzyme differs in all respects from its bacterial or crown gall counterpart. The bacterial enzyme has a relatively greater affinity for IAM than the squash root enzyme.

Discussion

Trp is generally believed to be the primary precursor of IAA in many but not all higher plants. The *orange pericarp* mutant of maize (Wright et al. 1991) and the IAA overproducing giant *Lemna gibba jsr. 1* (Baldi et al. 1991) appear to possess alternate pathways not involving Trp. Even in protoplasts prepared from the wild type of tobacco cv. SR1, an IAA biosynthetic pathway without the intervention of Trp is suspected to operate (Sitbon et al. 1993). IAA biosynthesis, in general, is confined mostly to meristematic tissues (shoot- root- and coleoptile-tips) and immature seeds. Due to compartmentation of Trp inside the cell (Widholm 1974), several sites of IAA biosynthesis are possible i. e., plastids (Fregau and Wightman 1983, McQueen-Mason and Hamilton 1989, Sitbon et al. 1993), mitochondria (Fregau and Wihgtman 1983) and in the cytoplasmic sub-microsomal system (Gordon 1958). Formation and hydrolysis of IAA conjugates (esters, amides, acyl anhydrides etc.) which are confined exclusively to the cytosol may also regulate endogenous levels of IAA (Sitbon et al. 1993). But IAM is the major intermediate in IAA biogenesis in some phytopathogens (Comai and Kosuge 1980, 1982), and in plant tissues transformed with bacterial plasmids containing oncogenes (van Onckelen et al. 1985, 1986, Prinsen et al. 1990). At present, only one plant tissue is known, namely the young fruit of *trifoliata* orange, which is capable of converting Trp to IAA via IAM (Kawaguchi et al. 1993).

In the present study, IAM has been unambiguously identified as a naturally occurring endogenous indole in etiolated squash seedlings (Fig. 3.2.1). Since these plants were raised under aseptic conditions, the IAM found should be an endogenous constituent and not a product of bacterial contamination. Based on GC-MS evidence, the normal occurrence of this amide has also been demonstrated recently in uncontaminated cherry seedlings (Saotome et al. 1993). In 120 h-old aseptically grown squash seedlings, The relative amounts of IAM based on fluorescence detection in HPLC are as follows: cotyledons > roots > hypocotyl. Being storage organs,

cotyledons contain the most and it is likely that this amide is transported to the roots from the cotyledons. Due to thier high solubility, amides are the preferred transport forms of nitrogenous compounds in plants (viz., asparagine, glutamine and the ureides in legumes). The advantages of transporting a precursor rather than IAA itself are obvious.

The demonstration as well as the partial purification of amide hydrolysing activity in aseptic squash seedlings also argues for its natural occurrence in this species. While the specific activity of the partially purified enzyme was ca. 47-fold higher than that of the starting material, the activity recovered is only about 15%. One single step, namely anion exchange chromatography, accounted for this great loss. Alternatively, the enzyme possibly occurs in very low amounts. It is necessary to devise less destructive methods of purification before this enzyme can be enriches to homogeneity. A comparison of the biochemical characteristics of the squash root enzyme with similar activities, prepared either by cloning and expressing the T-DNA encoded enzyme hydrolysing IAM in *E. coli*, or extracting it from transformed tobacco crown gall cells (Kemper et al. 1985), shows it to be different in all respects, i. e., optimal pH and temperature, substrate affinity and stability (Table 3.2.3). (The preparation of Kemper et al. (1985) was a crude, ammonium sulphate precipitated protein.) Their only common feature is a preference for an alkaline medium, indicating their possible occurrence and operation in the cytoplasm or one of its compartments. In tobacco cv. SR1 plants, transformed with the *Agrobacterium* oncogenes, the metabolism of IAM is said to be exclusively cytosolic (Sitbon et al. 1993). The K_m value of the squash root amidohydrolase for IAM is about 80 μM while the enzyme from tobacco crown gall tissue is reported to have a K_m value of 1 μM, indicating a relatively higher affinity. As determined by on-line HPLC fluorescence detection, squash roots contain ca. 8.8 + 0.4 pmols IAM (g fresh weight)$^{-1}$, but it is unlikely to be concentrated in the sub-cellular compartment wherein the amidohydrolase is also located, substrate saturation is conceivable. Due to its broad substrate specificity, it also acts on IAN which also occurs naturally in squash seedlings (unpublished observation), converting it to IAM (Table 3.2.1). This should further raise the IAM concentration at the site of IAA biogenesis.

From a partial literature survey, the K_m values of some higher plant enzymes probably involved in IAA biosynthesis, were collected and are presented in Table 4. Except for one, namely the IAox hydrolyase from chinese cabbage, all the other enzymes have K_m values ranging between 10^{-5} and 10^{-3} M. As their endogenous substrate levels rarely exceed μmolar amounts in plant tissues, it is obvious that plants have mechanisms for concentrating their substrates around the

enzymes at the site(s) of IAA biosynthesis. Or, enzyme affinity for its substrate could be altered by activators, particularly cofactors. Though it requires no added cofactors in vitro, the in vivo requirements of the squash root amidohydrolase are at present unknown. For the present therefore, the in vivo operation of this enzyme is unproven.

The specific activity of even the partially purified squash root enzyme is much greater than the IAM hydrolysing activity of rice callus (46 nmols IAA by the squash root enzyme as against the 63 pmols IAA formed (mg protein^{-1} h^{-1})) by the enzyme from sterile rice callus (Kawaguchi et al. 1991). The IAM hydrolase from young *trifoliata* orange fruit produces ca. 120 pmols IAA from IAM (mg protein^{-1} h^{-1}) at 30°C (Kawaguchi et al. 1993, Fig. 3.2.3). However, one line of Asparagus crown gall tissue transformed with the oncogenes of *A. tumefaciens* T-DNA showed considerable activity, i. e., 30 nmols IAA (mg protein^{-1} h^{-1}) (Prinsen et al. 1990).

Despite several earlier reports of the natural occurrence of IAM in higher plants (see Introduction), and of the IAM hydrolysing activity in rice calli (Kawaguchi et al. 1991), and in the young fruit of *trifoliata* orange (Kawaguchi et al. 1993), a role for this amide in IAA biosynthesis has not found support (Sembdner et al. 1980, Schröder et al. 1984, Weiler and Schröder 1987). The occurrence and onward metabolism of IAM are believed to be reactions of bacterial origin (Schröder 1984). But the present investigation wherein this enzymic activity was extracted from aseptically grown seedlings, and where the product of its reaction with IAM was conclusively identified as IAA, leaves little doubt of the feasibility of the operation of the amide pathway in the roots of etiolated, young squash seedlings. This belief is further strengthened by my demonstration of the natural occurrence of IAM in young squash seedlings, also beyond reasonable doubt (Fig. 3.2.1). It is true that squash seedlings also contain a powerful nitrilase enzyme, but this enzyme does not utilize IAM as a substrate. Moreover, it could be physically separated from the amidohydrolase activity by fractional precipitation with ammonium sulphate. The nitrilase is precipitated by 35% saturation while the amide hydrolysing activity is thrown down only when the salt concentration reaches 70%.

The question currently unresolved is the origin of IAM in squash seedlings. From structural similarity, Trp is the logical precursor. While phytopathogens like *Agrobacterium* and *Pseudomonas savastanoi* convert Trp directly to IAM with the help of a Trp monooxygenase, it is at present unknown whether a similar activity operates in squash seedlings. Preliminary experiments suggest such an activity in squash roots but definitive evidence is lacking (our unpublished observations). However, in young uncontaminated trifoliata orange fruit tissue, both

tryptophan monooxygenase and IAM hydrolase activities have been demonstrated, using [^{14}C]labeled Trp and IAM respectively (Kawaguchi et al. 1993). Alternatively, IAN can be transformed to the amide enzymically by the amidohydrolase itself, as shown in this paper (Table 3.2.2).

Naphthaleneacetamide is converted to its corresponding acid by the squash amidohydrolase, although at a much reduced velocity; but IAN is attacked almost as vigorously as IAM, since the enzyme seemingly recognizes only the C-N bond (Table 3.2.2). Though the squash root enzyme is inactive against the ethyl ester of IAA, its bacterial counterpart is reported to be capable of utilizing the glucose- and *myo*-inositol esters of IAA (Kemper et al. 1985). This has given rise to the suggestion that hydrolysis of stored forms of IAA may be a natural function of this enzyme (Kawaguchi et al. 1991). It is likely, as IAA conjugates are said to occur mostly in the cytoplasm (Sitbon et al. 1993) and since amidohydrolases, in general, have a wide spectrum of substrates (Varner 1960).

It is currently believed that the IAM pathway, introduced by phytopathogens, is 'foreign' to higher plants, and hence the host is unable to control the over-production of IAA which causes tumorigenesis (Schröder et al. 1984, Weiler and Schröder 1987). But *Cucurbita maxima* which possesses the metabolic machinery to produce IAA from IAM, is also known to be susceptible to infection by *Agrobacterium* (Lopatin 1936). It is not the pathway of IAA formation, per se, that is responsible for tumor induction but the over-production of IAA and perhaps also the sensitization of susceptible cells by the T-DNA genes, as is the case with the hairy root syndrome elicited by *Agrobacterium rhizogenes* (Cardarelli et al. 1987).

Table 3.2.1. Partial purification of the amidohydrolase activity from 102 g fr wt of etiolated squash seedling roots

Fraction	Protein (mg)	Specific activity (nmole)	Purification (-fold)
Cell-free supernatant	425	0.96	–
35-70% $(NH_4)_2SO_4$ fraction	119	3.30	3.4
Sephadex G-75	90.7	4.00	4.2
MiniQ™ PC	1.39	45.6	47.5

One unit is defined as the activity producing 1 nmole IAA from IAM under the assay conditions (2 μmole IAM + 50-2,000 μg protein + 100 mM phosphate buffer, pH 7.2 + 2 mM DTT + 10 mM $MgCl^2$ at 30°C, 1 h). Specific activity is nmole IAA formed at 30°C (mg protein)$^{-1}$ h^{-1}.

Table 3.2.2. Substrate specificity of the amidohydrolase activity of etiolated, aseptically grown squash seedling roots.

Substrate	Acid formed (nmole)
IAM	1.86
IAN	1.68
1-Naphthaleneacetamide	0.40
IAA-ethyl ester	not attacked
IAA-aspartate	not attacked

Data refer to the corresponding acid formed in nmole (mg protein)$^{-1}$ h^{-1} at 25°C

Table 3.2.3. Comparison of some biochemical parameters of the crown gall and squash root amidohydrolases

Parameter	Crown gall enzyme	Squash root enzyme
Optimal temperature	30-37°C	45°C
Optimal pH	8.5-9.5	7.0-7.5
K_m	1.2 μM	80 μM
Stability	unstable even at 4°C	stable to freezing

Parameters of the crown gall enzyme are those reported by Kemper et al. (1985).

Table 3.2.4. K_m values of some higher-plant enzymes probably involved in IAA biosynthesis

Enzyme	Reaction catalyzed	Plant source	K_m (M)	Reference
L-Trp decarboxylase	L-Trp → TNH_2	Tomato	3.0×10^{-3}	3
		Phalaris tuberosa	0.2×10^{-3}	1
L-Trp transaminase	L-Trp → IPyA	Tomato	5.0×10^{-3}	3
		Mung bean	3.3×10^{-4}	15
		Pea	0.4×10^{-3}	7
		Maize	6.8×10^{-4}	7
		Tomato	9.1×10^{-4}	7
IPyA decarboxylase	IPyA → IAAld	Yeast	1.2×10^{-3}	13
Amine oxidase	TNH_2 → IAAld	Pea	1.6×10^{-3}	11
L-Trp oxidase	L-Trp → IAox	Chinese cabbage	2.0×10^{-5}	8
Hydrolase	IAox → IAN	Chinese cabbage	3.3×10^{-6}	4
		Chinese cabbage	5.0×10^{-5}	5
Nitrilase	IAN → IAA	Barley	5.1×10^{-5}	14
Aldehyde oxidase	IAAld → IAA	Oat coleoptile	3.5×10^{-4}	12
		Maize coleoptile	5.0×10^{-6}	6
IAAld reductase	IAAld → IEt	Cucumber	1.2×10^{-4}	2
		Cucumber	2.5×10^{-5}	9
IAAld reductase	IAAld → IEt	Chinese cabbage	1.2×10^{-4}	10
		Tomato	5.0×10^{-5}	10
		Maize	1.6×10^{-5}	10
		Pea	2.0×10^{-5}	10
Amidohydrolase	IAM → IAA	Squash	8.0×10^{-5}	This study

References are 1, Baxter and Slaytor (1972); 2, Brown and Purves (1980); 3, Gibson et al. (1972); 4, Helmlinger et al. (1985); 5, (1987); 6, Koshiba et al. (1996); 7, Kutácek (1985); 8, Ludwig-Müller and Hilgenberg (1988); 9, (1989); 10, (1990); 11, McGowan and Muir (1971); 12, Rajagopal (1971); 13, Sukanya et al. (1971); 14, Thimann and Mahadevan (1964); 15, Turelsen (1972).

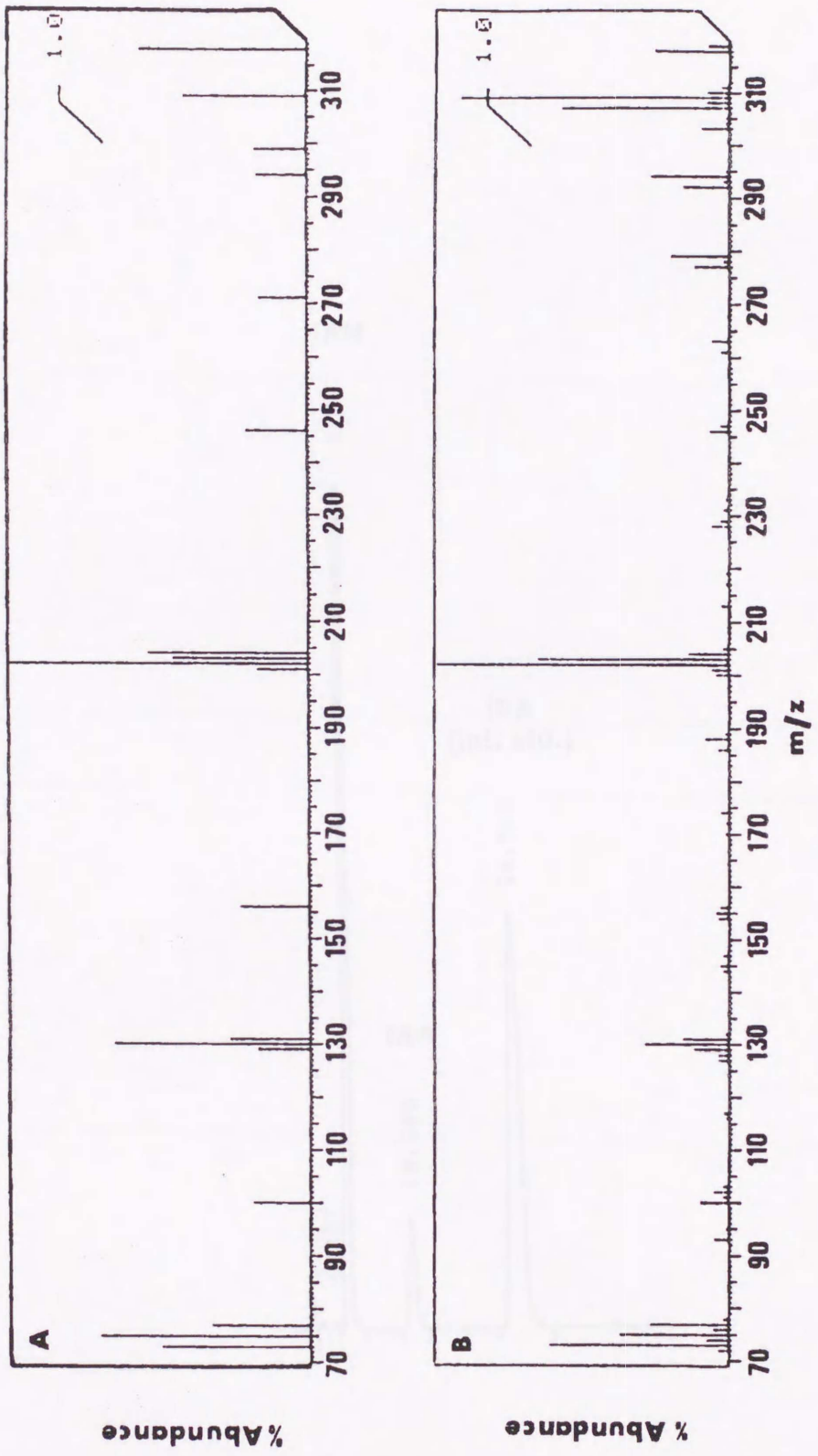

Fig. 3.2.1. Full-scan GC-MS of endogenous IAM from the hypocotyls of 120 h-old, etiolated, aseptically grown squash seedlings, silylated prior to analysis. **A**: authentic TMS-IAM; **B**: TMS-IAM from squash hypocotyl.

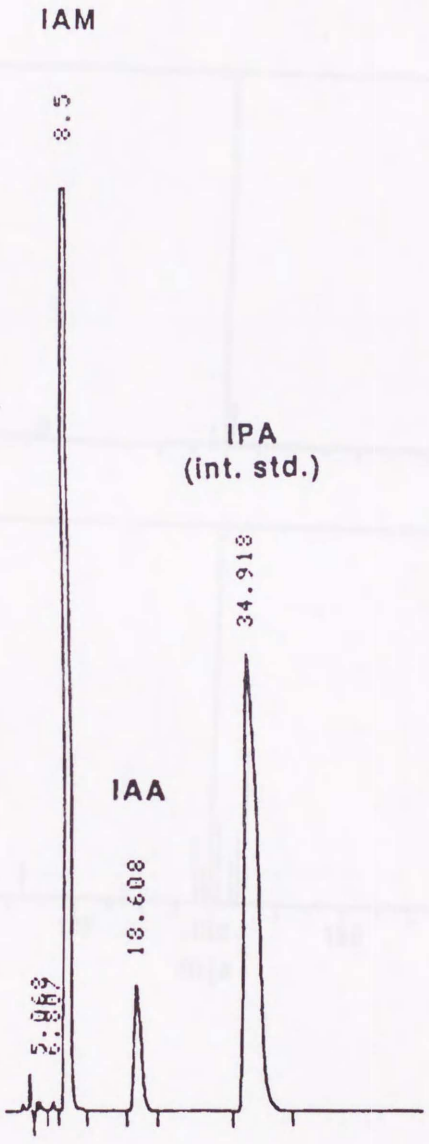

Fig. 3.2.2. HPLC profile of the reaction products of the IAM hydrolase activity at pH 7.2, 25°C and 1 h. 5% of the reaction product was injected.

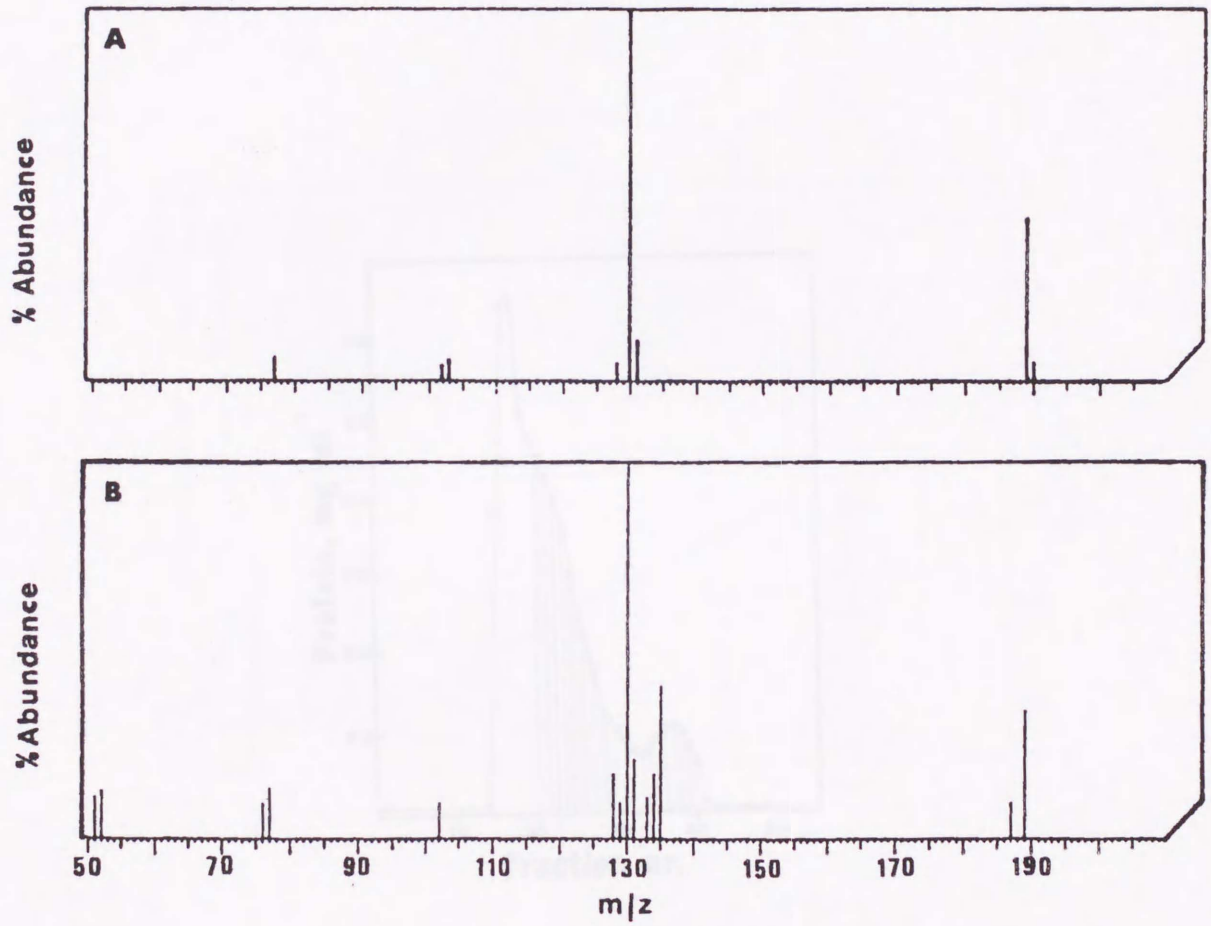

Fig. 3.2.3. GC-MS of IAA formed from IAM due to the squash root amidohydrolase activity. **A**: authentic IAA; **B**: IAA from the enzyme reaction. Both samples were methylated prior to analysis.

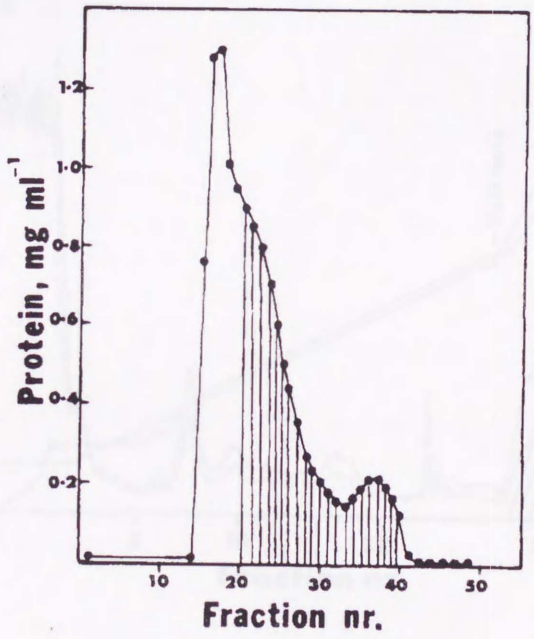

Fig. 3.2.4. Sephadex G-75 gel filtration pattern of the ammonium sulphate fractionated (35-70%) protein of squash roots. Fractions in the hatched area showed IAM hydrolysing activity.

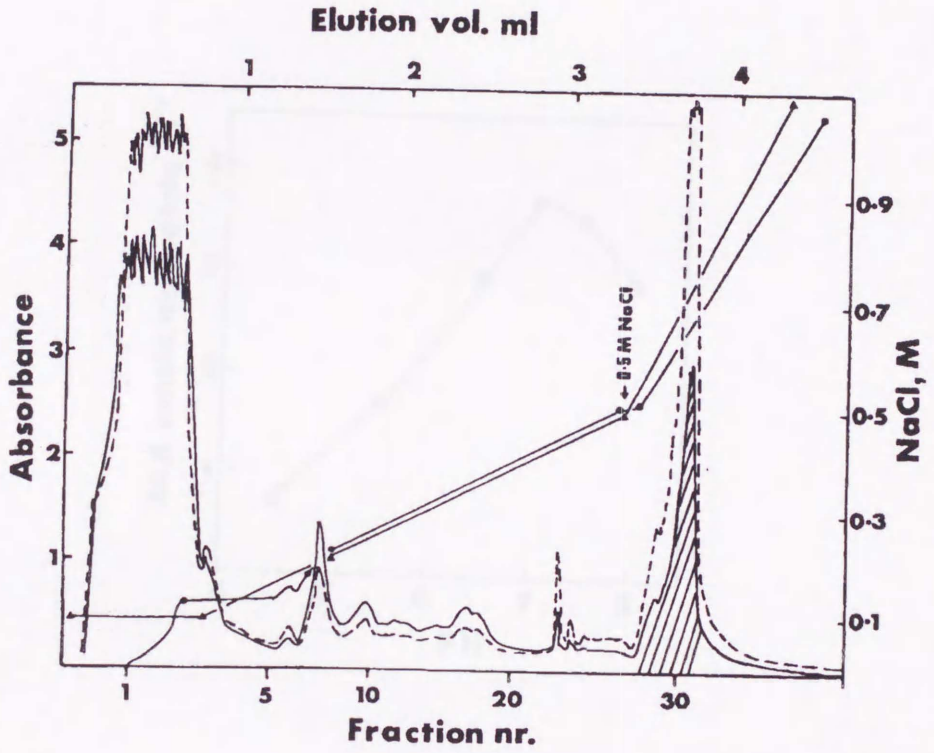

Fig. 3.2.5. Anion exchange chromatography of the active fractions from Sephadex gel filtration, on SMART-HPLC system on a MiniQ column, eluted with a linear NaCl gradient in 100 mM phosphate buffer, pH 7.2. --- A_{260} nm; — A_{280} nm; -●- conductivity; -▲- Nacl gradient. Hatched area (fractions 28-32) were pooled and dialyzed prior to assay.

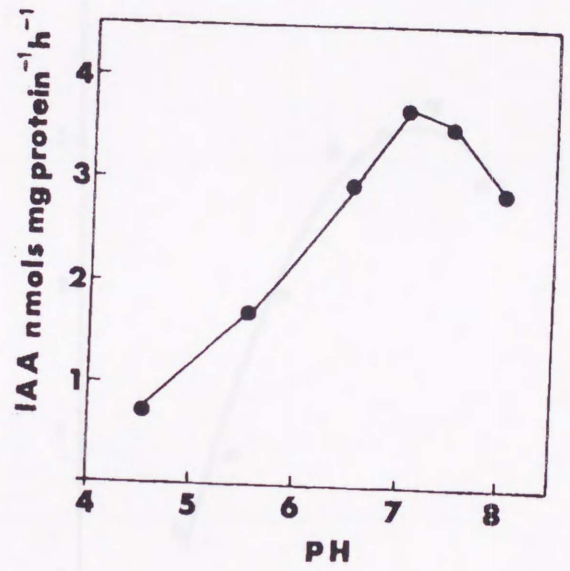

Fig. 3.2.6. Effect of pH on the partially purified IAM hydrolysing activity of squash roots at 30°C. pH 4.5-5.5—acetate buffer; pH 6.5-8.0—phosphate buffer, all at 0.1 M.

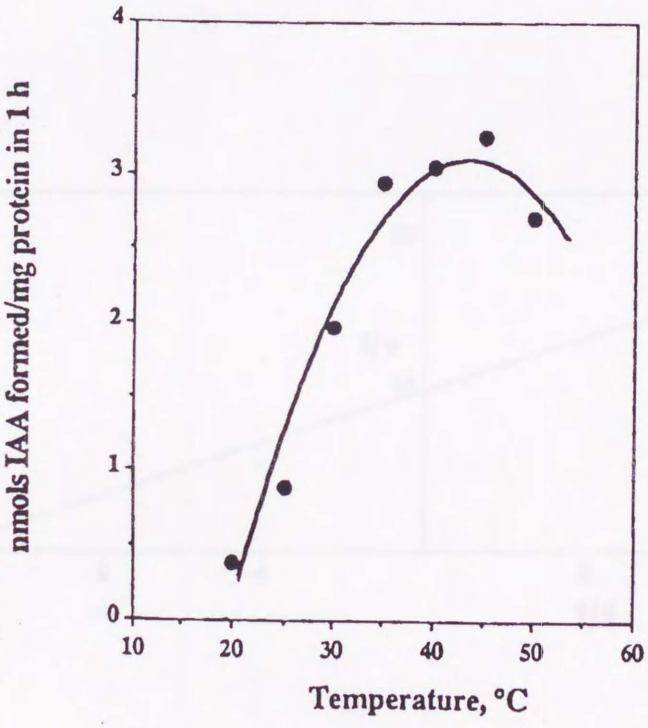

Fig. 3.2.7. Effect of temperature on the IAM hydrolysing activity of squash roots.

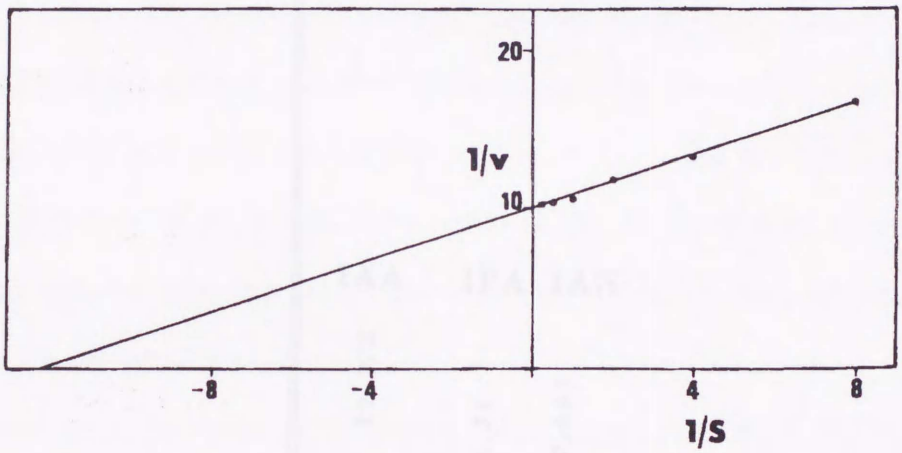

Fig. 3.2.8. Effect of IAM concentration on the partially purified IAM hydrolysing activity of squash roots, presented as a double reciprocal plot. $K_m = 8 \times 10^{-5}$ M.

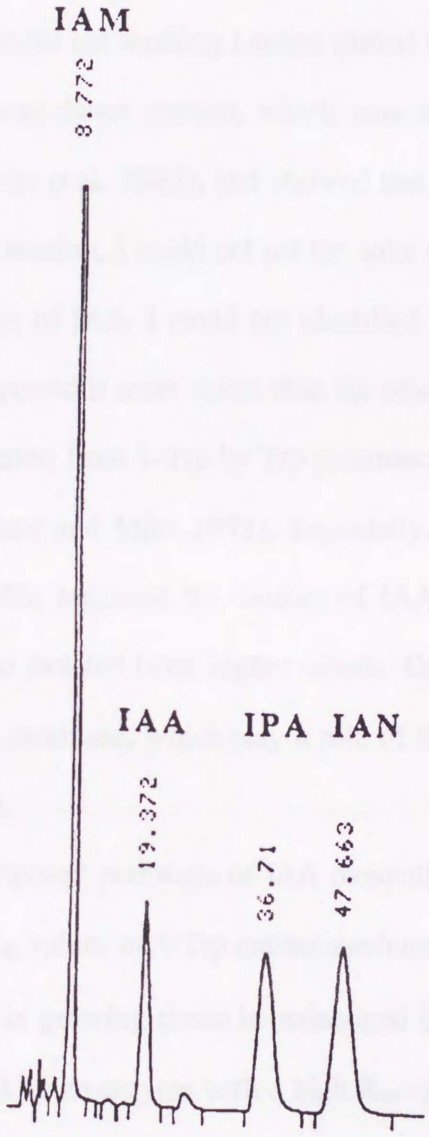

Fig. 3.2.9. Formation of IAM and IAA from IAN by the amidohydrolase of squash roots at 25°C and 1 h. IAA and IAM were also identified by GC-MS. 10% of the reaction product was injected on the HPLC column.

Chapter 4

General Discussion

Coleoptiles of monocotyledons have been used to analyze IAA synthesis and action, since the compound was discovered in the oat seedling (*Avena sativa*) in 1926 (see Chapter 1.1). I used the barley coleoptiles of the semi-dwarf mutant, which was known as a mutant of low IAA concentration in coleoptile (Inouhe et al. 1982), and showed that Trp might be a precursor of IAA (Chapter 2.1). But, in the same section, I could not get the solid evidence that D-Trp as the optical isomer of L-Trp was a precursor of IAA. I could not identified D-Trp as an intermediate of IAA synthesis, even though the compound is more stable than the other precursors of IAA. It was once suggested that D-Trp was converted from L-Trp by Trp racemase in dwarf pea (Law 1987) and in the cell culture of tobacco (Miura and Mills 1971). Especially, Law (1987) proposed that Trp racemase stimulated by gibberellin regulated the amount of IAA. The racemase itself, however, has not been reported yet to be isolated from higher plants. Only microbial racemase, such as alanine racemase and glutamate racemase, which play a role of the synthesis of peptide glycan in microbial cell walls are reported.

On the other hand, the proposed pathways of IAA biosynthesis from L-Trp have two weak points. One is the very high K_m values of L-Trp aminotransferase (Truelsen 1972, Gibson et al. 1972a). The IAA concentration in growing tissue is maintained in the nM range and it is unlikely that such a low level is regulated by an enzyme with a high K_m value (Sheldrake 1973). The other is that L-Trp decarboxylase is not ubiquitous in plants (Gibson et al. 1972a,b). D-Trp aminotransferase was reported to be partially purified in Alaska pea plastids (McQueen-Mason and Hamilton 1989) and in maize coleoptile (Koshiba et al. 1993). Both reports, however, did not show K_m value of D-Trp aminotransferase, and did not demonstrate that the substrate specificity

favored IAA synthesis.

Baldi et al. (1991) reported that D-Trp pathway did not function in an aquatic monocot *Lemna gibba*, using a stable isotope technique. They found the low conversion rate of L-Trp to D-Trp. Ludwig-Müller and Hilgenberg (1989) also showed that while *N*-malonyl-Trp was converted to IAA, *N*-malonyl-L-Trp was the substrate for this reaction, not the D-form.

Nevertheless the D-Trp pathway is the interesting hypothesis covering a weak point of L-Trp as a starting precursor. I also could not find the more evidence of the role of D-Trp as the intermediate in IAA synthesis. Therefore, I should conclude that the pathway of IAA synthesis via D-Trp would not play a major role in endogenous IAA synthesis and regulation, although D-Trp appears the precursor of IAA as the results of my experiments applying D-Trp to coleoptile segments.

At present, the hot topic of IAA synthesis is whether is Trp or non-Trp (see Chapter 1.2). Trp is not converted to IAA in a corn mutant by isotope experiments, but numerous plants have indeed the ability of the conversion of Trp to IAA. To decide whether is Trp or not, one needs to measure the intermediate compounds of IAA synthesis i.e., IAAld and IPyA. No one has yet been able to isolate and quantify the intermediates of IAA, because of their very unstable nature and the lack of suitable internal standards. Therefore, I focused on what compound is directly catalyzed to IAA, since I could not make clear whether IAA is synthesized from Trp or not. Several compounds are known as direct intermediates of IAA. Especially IAAld, IAN and IAM. IAAld was reported as the most common precursor of IAA in plants (Rajagopal 1971, Ludwig-Müller and Hilgenberg 1990). Since IAAld is very unstable compound, it is very difficult to be isolated and identified from plant tissues. Only one report showed the GC-MS data of natural IAAld isolated from chinese cabbage (Ludwig-Müller and Hilgenberg 1990). Nevertheless, since IEt converted from IAAld by reduction is known to exist in many plants (Rayle and Purves 1967, Lac

án et al. 1985), IAAld is the highest possible precursor of IAA. IAN is known as a precursor of IAA in Brassica plants (Helminger et al. 1985), but nitrilase that catalyzes IAN to IAA exists in only a few plants (Thimann and Mahadevan 1964). IAM is converted to IAA by amidohydrolase in crown gall tissues infected by *Agrobacterium* (Van Onckelen et al. 1985, Kemper et al. 1985) and *Pseudomonas* (Kosuge et al. 1966, Morris 1986). IAM and amidohydrolase occurs in some plants (Takahashi et al. 1975, Rausch et al. 1985, Saotome et al. 1993, Kawaguchi et al. 1991, 1993), but the role for IAA synthesis is doubted in higher plants (Schröder et al. 1984, Weiler and Schröder 1987).

I used three compounds described above in cell-free preparations of young seedling of barley including coleoptiles (the same barley sample in Chapter 2.1), and showed that IAAld was a most direct precursor of the known intermediates of IAA synthesis and that the activity of the conversion of IAAld to IAA was found in the cell-walls (Chapter 2.2). Cell-free soluble fraction of barley coleoptiles converted IAAld or IAN to IAA, but not IAM. The nitrilase and aldehyde oxidase activities in soluble fraction of semi-dwarf mutant, uzu, were similar to those in the normal on the basis of specific activity. Therefore, it is unlikely that the lower level of endogenous IAA in the uzu strain was caused by the lower nitrilase and aldehyde oxidase activities in the soluble fraction. Since IAM was not converted to IAA in either fraction of either strain, it is not involved in the impeded IAA synthesis in the uzu strain. Interestingly, the activity of IAAld to IAA existed in cell-wall fraction and the activity was higher in the normal strain than in uzu. IAAld was converted to IEt by the cell-wall fraction of which the activity is higher in uzu than in the normal (Fig. 2.2.1) and natural IEt was detected in the barley coleoptile (see Chapter 2.2). Therefore, the lower conversion of IAAld to IAA in cell-wall fraction in the uzu strain probably results in the lower level of endogenous IAA. After all, IAA may be synthesized via IAAld in outer space of cells including cell-walls, so-called apoplast, in barley coleoptiles.

If IAA is synthesized in apoplast, how does the compound influence plant growth and what

is the benefit for growth control in plants? In animal systems, the peptide hormone receptors are localized at the plasmamembrane, with the hormone-binding moiety facing outside the cell. These receptors function as sensory systems for external hormone levels and transduce the signal into intercellular signals. Moreover, in animal cells, the cells can send signals to other cells of the same type, and it follows from this that they can also send signals to themselves. In such autocrine signaling a cell secretes signaling molecules that can bind back to its own receptors facing outside the cells. During development, for example, once a cell has been directed into a particular path of differentiation, it may begin to secrete autocrine signals that reinforce this developmental decision from outside.

In plants, IAA receptor appears to exist on the outer surface of plasmamembrane (Chapter 1.3). In the segments of growing regions in plants, exogenously applied IAA causes rapid cell elongation response, which begins within 10 minutes. Moreover, I demonstrated that IAA could not freely move through plasmamembrane between apoplast and symplast (Chapter 2.3, Fig. 2.3.1). IAA, as well as animal hormone, must be synthesized in specific tissue or region, or group of cells, and it may be transported to target tissue. IAA, in turn, affect the target tissue which includes the cells that can synthesize IAA by the autocrine mechanism. It is not surprising that the receptive system of plant hormones may be similar to that of animal, although plants do not have the circulatory system like animal blood. I showed that IAA level was higher in apoplast than in symplast in growing region of squash hypocotyls (Chapter 2.3). The results suggest that plant cells, as well as animal cells, percepts the hormone concentration by the outside of the cell.

In general, IAA is thought to be transported by a one-directional polar transport system from axis to base through an auxin transporter. The occurrence of the ability of IAA synthesis, i. e. the conversion of IAAld to IAA, in apoplastic space (Chapter 2.2) and the high concentration of IAA in the apoplast of growing region (Chapter 2.3) leads a hypothesis that the cells in the growing region stimulated by IAA transported from the apex secret an IAA precursor, not IAA itself,

toward the apoplast by autocrine mechanism, and then IAA synthesized in outside stimulates the cell elongation from outside (Fig. 3.1). It was reported that IAA existed in the tip was not *de novo* synthesized in the tip, but was converted from IAA-conjugates (such as IAA-inositol, IAA-amino ester) carried by vessel stream from the seed to the tip in *Zea Mays* seedlings (Epstein et al. 1980), although IAA was reported to be *de novo* synthesized in the maize seedling tips (Iino and Carr 1982, Koshiba et al. 1995). It is possible that IAA from the tip acts as the first signal of autocrine of IAA in the cells of growing region to stimulate elongation growth.

In general, IAA is thought to be synthesized in shoot tips and transported from the shoots to roots (McDavid et al. 1972) where it may be conjugated or degraded, although IAA is produced in isolated roots cultured *in vitro* (van Overbeek 1939). In *Zea* roots, the tip contains less IAA than the region behind the zone of elongation, and nearly all IAA is found in the stele (central part of root) rather than the cortex (Greenwood et al. 1973). The source of this IAA could be the differentiating vascular tissues, although some of it could have been transported acropetally (toward root tip) in the stelar region. Therefore, I studied using squash roots whether or not IAA was synthesized in root, and whether or not root has the unique pathway of IAA synthesis.

At first, one derivative of Trp, *N*-OH-Trp, was shown to be a precursor, but not a direct precursor, of IAA in squash roots (Chapter 3.1). *N*-OH-Trp is proposed as an intermediate of the conversion of Trp to IAox (Mahadevan 1973). It was reported that IAox exists in some higher plants (Ludwig-Müller and Hilgenberg 1988) and is metabolized to IAA in several higher plants (Rajagopal and Larsen 1972). In Chapter 3.1, I showed that *N*-OH-Trp could be the intermediate of IAA synthesis via IAox in the squash roots. The results, furthermore, suggested that the possibility of the occurrence of many pathways of IAA synthesis in the roots. Based on the enzymic activities identified (Table 3.1.2), the following pathways are feasible (Fig. 4.2). *N*-OH-Trp is converted to IAox, and then IAox may be converted to two compound, IAAld and IAN.

The conversion of IAox to IAN is reported in some plants (Helminger et al. 1985, Mahadevan 1963, Rajagopal and Larsen 1972). The conversion of IAox to IAAld is also reported (Helmlinger et al. 1987,Rajagopal and Larsen 1972, Rajagopal et al. 1992). The formation of large amounts of IEt and IAA in the presence of *N*-OH-Trp, and its abolition in the presence of $NaHSO_3$ (Table 3.1.1), suggests the intermediary form of IAAld though IAAld itself was not quantified. The conversion to IAN, however, was lower than that to IAAld (or IEt) (Table 3.1.1), suggesting that IAN is supplied only in small amounts as an substrate for IAA synthesis in squash root, although the ability of the conversion of IAN to IAA by nitrilase and/or amidohydrolase exists in the cell-free preparation of squash roots (Table 3.1.2). These results support the idea that IAAld is a good and a direct precursor of IAA not only in the shoots but also in the roots. If IAN is abundant in squash roots, the pathway from IAN is important. Since IAA is an essential hormone of the growth of plants, it may be natural that the several bypasses or branched pathway of IAA synthesis should exist in the plant. Interestingly, in this study (Chapter 3.1), I also found the other pathway, which is similar to IAM pathway known as important pathway of plant pathogen, in the squash roots. In the next section, I investigated about the IAM pathway in detail.

I found the enzyme activity of the conversion of IAM to IAA and the occurrence of IAM in the aseptically grown squash roots (Chapter 3.2). It is currently believed that the IAM pathway, introduced by phytopathogens, is 'foreign' to higher plants, and hence the host is unable to control the over-production of IAA which causes tumorigenesis (Schröder et al. 1984, Weiler and Schröder 1987). *Cucurbita maxima* roots which possess the metabolic pathway to produce IAA from IAM, is also known to be susceptible to infection by *Agrobacterium* (Lopatin 1936). In plants, the occurrence of IAM has been reported in many plant species. Despite of these observations, the natural occurrence of IAM and the role as precursor in IAA biosynthesis were doubted in higher plants (Schröder et al. 1984, Weiler and Schröder 1987). The occurrence of IAM in the non-infected squash roots suggests the possibility of IAA synthesized from IAM in the

plant root tissues without microbial action. And the amidohydrolase that converted IAM to IAA was partially purified by ca. 48-fold (Table 3.2.1). The property of the enzyme in squash roots is different from that in crown gall (Table 3.2.3). Especially, the K_m value of the amidohydrolase activity for IAM was fairly lower than the other enzymes, which is thought to relate to IAA synthesis (Table 3.2.4). These results imply that IAM is also a direct precursor of IAA synthesis in squash roots. But, I could not detected IAM pathway in barley coleoptiles (Chapter 2.2). I think that the IAA synthesis pathway via IAM is not ubiquitous in plants, but the pathway may be one of the bypass working in specific tissues, for example root tissues.

IAA is known as a plant hormone having numerous important functions of plant growth and development. In this thesis, I indicate the various possible pathways of IAA synthesis. Particularly, I showed that IAAld should play an important role in IAA synthesis, while I could not demonstrate what compound (Trp or not?) is a precursor of IAAld. I also suggest that several bypasses of IAA synthesis including IAM pathway exists in root. It is no wonder that several bypasses function in response to various environmental stimuli. In the bean seedling, *de novo* IAA biosynthesis begins even before the stored conjugates are fully used up (Bialek and Cohen 1992, Bialek et al. 1992), and this biosynthesis comes primarily from Trp conversion. Michalczuk et al. (1992) showed that in embryogenic carrot suspension cultures, the conversion of Trp to IAA is the primary route. However, when 2,4-dichlorophenoxyacetic acid was removed from the medium, which induces these carrot cells to form embryos, the conversion of Trp to IAA was no longer the primary route, and the non-Trp pathway appeared to predominate. These reports would support my hypothesis that several bypasses of IAA synthesis exist and function in plants. The pathways of IAA synthesis may be different in the ages and the locations of the plants, and precisely selected by various environmental signals.

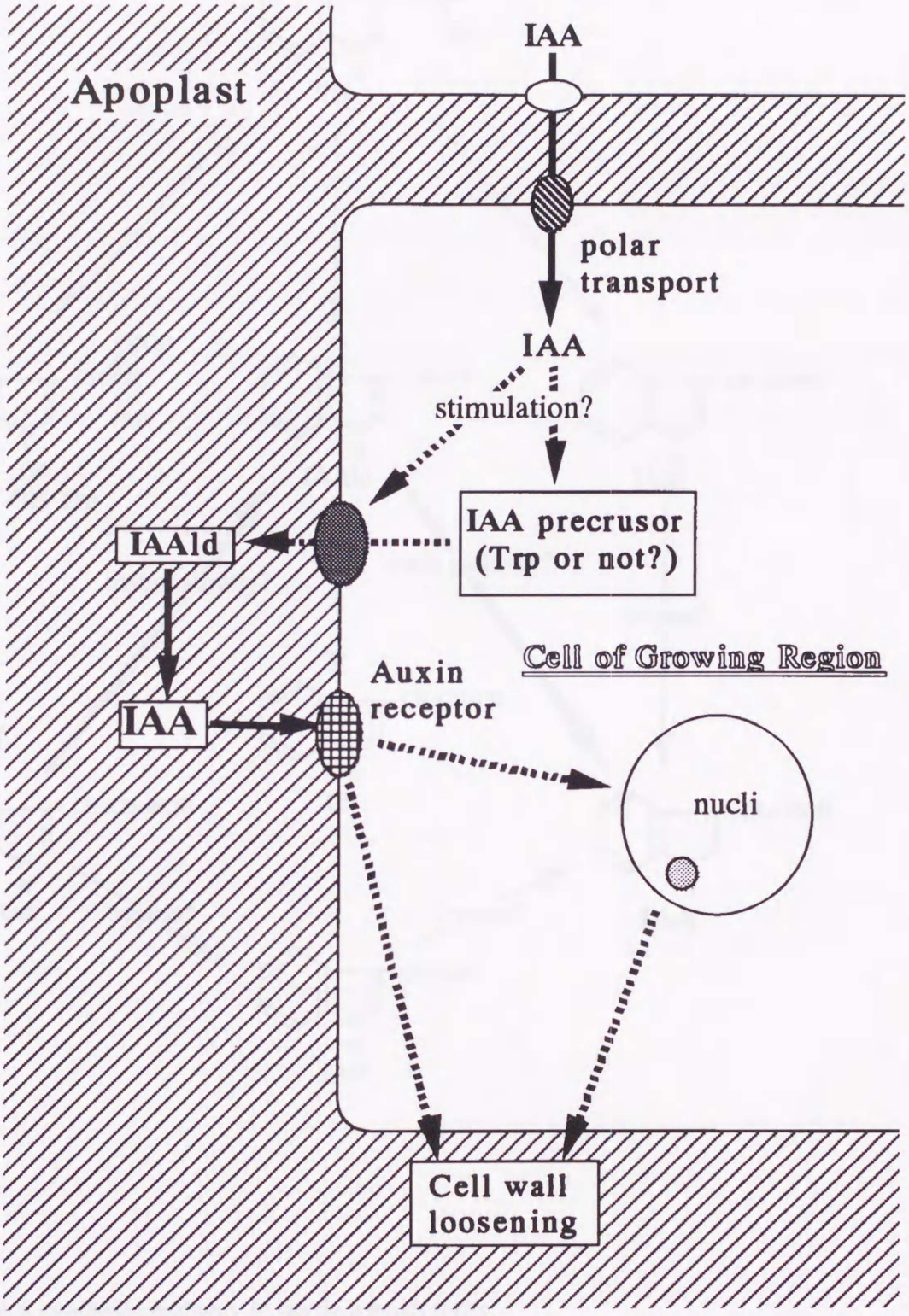

Fig. 4.1 Hypothesis of IAA synthesis in growing region and that action for elongation growth

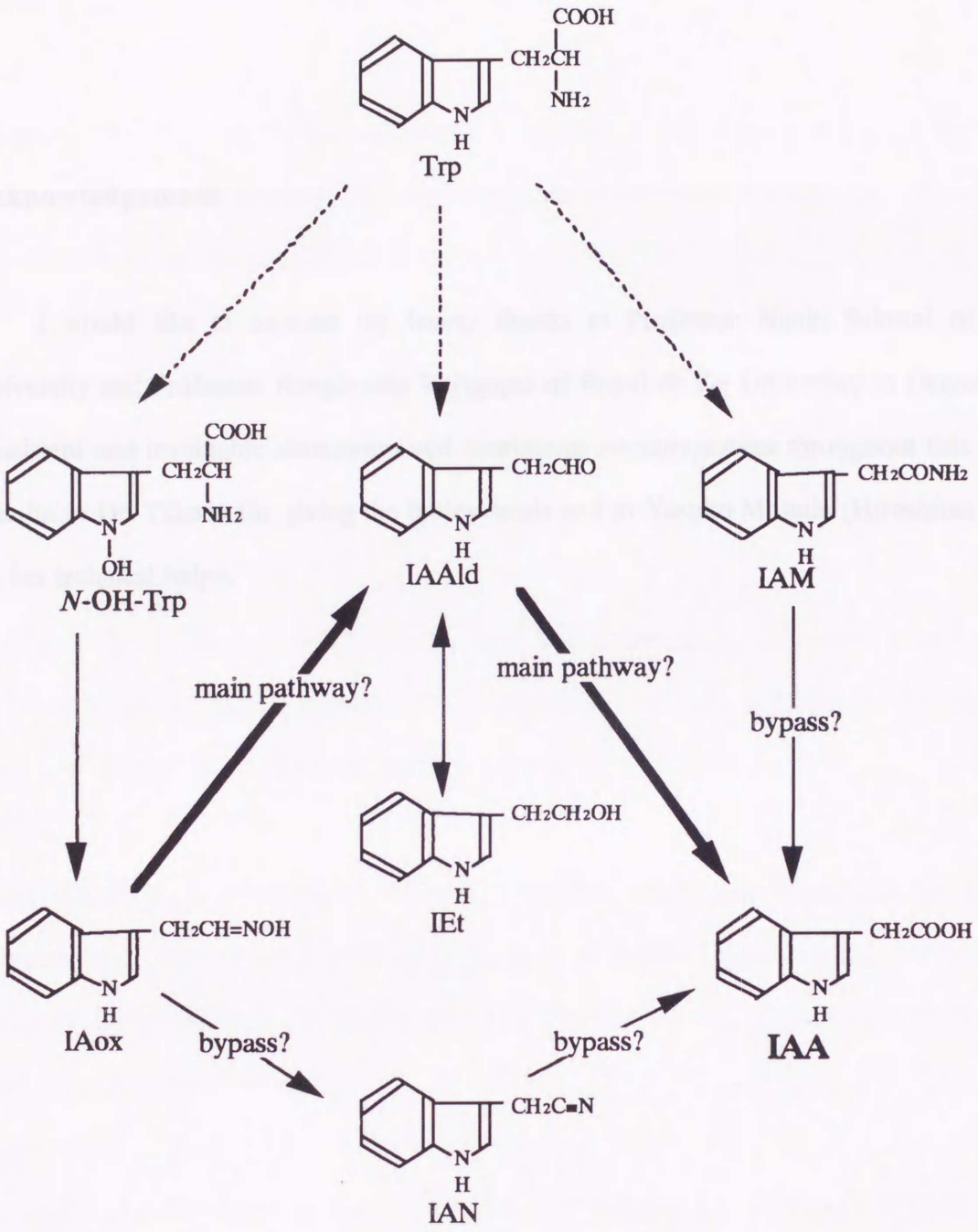

Fig. 4.2 IAA synthesis pathway in sterile squash roots.

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