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KATHERINE ALLISON KEITH

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AN IMMUNOLOGICAL APPROACH TO CYTOKININ BINDING AND A COMPARISON OF THE SUBMERGENCE RESPONSE IN DEEPWATER AND OTHER RICE

By

Katherine Allison Keith

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ABSTRACT

AN IMMUNOLOGICAL APPROACH TO CYTOKININ BINDING AND A COMPARISON OF THE SUBMERGENCE RESPONSE IN DEEPWATER AND OTHER RICE

By

Katherine Allison Keith

In order to search for a plant hormone (cytokinin) receptor, a rabbit antiserum with specificity for cytokinins was generated against a photoaffinity cytokinin. In order to covalently label the receptor, cytokinin-responsive tissue was treated _in vivo_ with this photoaffinity cytokinin, which is biologically active. The detection limits using western techniques were at the level expected for such a receptor. Tissue fractionation or use of ^{125}I-labelled antibodies may improve the sensitivity of this technique.

Deepwater, semidwarf, and short rice cultivars were analyzed for their response to partial submergence. All cultivars were capable of rapid internodal elongation when partially submerged. The deepwater cultivar expressed this capacity throughout its vegetative growth phase. The other cultivars only grew rapidly during the last weeks of vegetative growth. These cultivars differ not in the existence or absence of "elongation genes", but in the timing of expression of the ability to elongate.

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LIST OF ABBREVIATIONS

Ade	Adenine
Ag	Antigen
AMP	Adenosine monophosphate
AOV	Analysis of variance
AS	Antiserum
ATP	Adenosine triphosphate
BA	Benzyladenine
BA-BSA	A conjugate of benzyladenine and bovine serum albumin
BA-OVA	A conjugate of benzyladenine and ovalbumin
BAR	Benzyladenine riboside
BSA	Bovine serum albumin
CBP	Cytokinin binding protein, isolated from cereal embryos
c-Z	cis-Zeatin
ELISA	Enzyme-linked immunosorbent assay
GA	Gibberellic acid
Gua	Guanine
HA	Habiganj Aman
IgG	Immunoglobulin G
IPP	Isopentenyl pyrophosphate
KH	Kalar Harsall
LMN	Leb Mue Nahng
LSD	Least square difference

$8N_3BA$	8-azido-benzyladenine, a photoaffinity cytokinin
NBD	Norbornadiene
OVA	Ovalbumin
PAGE	Polyacrylamide gel electrophoresis
PIS	Preimmune serum
PG	Pin Gaew
t-RNA	Transfer ribonucleic acid
t-Z	trans-Zeatin

CHAPTER 1

AN IMMUNOLOGICAL APPROACH TO CYTOKININ BINDING USING A PHOTOAFFINITY DERIVATIVE OF BENZYLADENINE

LITERATURE REVIEW

Discovery and structure of cytokinins

The first pure substance known to induce cell division in callus culture was identified as 6-furfurylaminopurine, a degradation product of DNA, and was called kinetin (Miller *et al.*, 1955, 1956). Later, the first naturally occurring compound with kinetin-like properties was isolated from *Zea mays* kernels and given the name zeatin (Letham *et al.*, 1964). Following the discovery of a number of synthetic and naturally occurring compounds with similar effects on cell division, the name, cytokinin, was given to this class of compounds (Skoog *et al.*, 1965). The general structure of cytokinins is an N^6-substituted purine in either the free base or riboside form. The side chain attached at the N^6 position is generally composed of 5-6 carbons, usually an isoprenoid structure or a ring. The degree of unsaturation and substitution in the side chain is variable (Skoog *et al.*, 1967; Mok et al., 1978). Modifications of the purine ring may also occur, although most of these modifications greatly reduce cytokinin activity. Another class of compounds with weak cytokinin activity are the diphenylureas (Bruce and Zwar, 1966). However, there is speculation that the activity of these compounds may derive from their metabolism in plant tissue rather than through direct cytokinin activity (Letham and Palni, 1983).

Cytokinin biosynthesis

Based on indirect evidence, two pathways for the biosynthesis of cytokinins have been proposed: (1) de novo biosynthesis or (2) release of free intact cytokinin during tRNA degradation. Indirect evidence for de novo biosynthesis was obtained in several plant systems in which ^{14}C-adenine was incorporated into free cytokinins (Einset and Skoog, 1973; Chen and Petschow, 1978a). The possibility of obtaining free cytokinin via release of cytokinin nucleotides during degradation of tRNA was shown to exist when the incorporation of labelled Δ^2-isopentenyl pyrophosphate (IPP) into tRNA to produce cytokinin nucleotides was demonstrated (e.g. Walker et al., 1974; Murai et al., 1975). However, a number of lines of indirect evidence suggest that tRNA cannot be the sole source of free cytokinins. For example, the level of free cytokinin in pea root tips exceeds the amount of cytokinin present in tRNA by a factor of 27 (Short and Torrey, 1972). In addition to this, tissues shown to contain cytokinin in their tRNA still required exogenous supplies of cytokinin for growth (Burrows, 1976). Also, in some higher plants, cytokinins which occur in tRNA are not found as free cytokinins and vice versa (Burrows, 1978).

The first direct evidence for *de novo* biosynthesis was provided by Chen and Melitz (1979) when they demonstrated the existence of an enzyme which catalyzed the formation of free cytokinin in partially pure extracts of cytokinin autonomous tobacco callus. The enzyme, Δ^2-isopentenyl pyrophosphate: AMP-Δ^2-isopentenyl transferase catalyzes the formation of isopentenyl adenosine from AMP and Δ^2-IPP. Similar enzyme activities have been demonstrated in cultured tobacco cells (Nishinari and Syono, 1980) and in two bacterial plant pathogens, *Agrobacterium tumefaciens* (Morris, 1982) and *Corynebacterium facians* (Murai, 1981).

Cytokinin localization and biosynthetic sites

The distribution of cytokinin within the plant is very broad in that cytokinins have been found in many different tissues and organs. Included in these are endosperm, seeds, seedlings, roots, root exudate, stems, cambium, leaves, developing fruit, autonomous callus, and crown gall tumors (Kende, 1971). However, the sites of cytokinin biosynthesis within the plant have not been studied in great detail, and remain largely unknown. The observation by Chibnall in 1939 that senescence in detached leaves is reversed or prevented by adventitious root formation led him to propose that a substance (hormone) produced in roots prevented senescence in leaves and that deficiency of this factor lead to senescence. Following the discovery by Richmond and

Lang (1957) that the synthetic compound, kinetin, delayed senescence in detached leaves, Kulaeva (1962) provided proof for the first tenet of Chibnall's hypothesis. She showed that crude xylem exudate contained a compound which delayed senescence of detached tobacco leaves. For a number of years it was assumed that cytokinins were produced in the root and transported to the growing regions of the plant where it could exert its effects on cell division or any physiological process for which it was required. More recent evidence suggests that cytokinins are probably produced in meristems or tissues undergoing active cell division.

The first evidence for biosynthesis of cytokinins in tissues other than roots stems from a phenomenon observed in cultured plant tissues known as habituation. Cultured plant tissues, when established, usually require an exogenous supply of auxin and (or) cytokinin for growth. Habituated tissues are those which require these growth factors when established but at some point develop the capacity to grow without supplements of auxin and (or) cytokinin (Meins, 1982). Studies of cytokinin-habituated (or cytokinin-autonomous) tobacco cultures have shown that treatment of these tissues with cytokinin induces cytokinin habituation (Meins and Lutz, 1980) and that habituated tissues can "crossfeed" nonhabituated tissues (Meins and Binns, 1979). In other words, tobacco cultures requiring exogenous cytokinin for growth will grow in medium lacking cytokinin if cytokinin habituated tissues are growing on the same medium. This, of course, implies that cytokinin is being synthesized in the habituated cultures and that cytokinin either diffuses or is secreted into the culture medium.

Further evidence for cytokinin biosynthesis in cultured tissues comes from the work of Einset and Skoog (1973) who demonstrated ^{14}C-adenine incorporation into zeatin, ribosylzeatin, and isopentenyladenine in cytokinin autonomous tobacco cultures. No ^{14}C incorporation was found in the corresponding fractions isolated from cytokinin-dependent tobaccos cultures. Chen and Petschow (1978a) also provided evidence for cytokinin synthesis in tissues other than roots when they demonstrated ^{14}C-adenine incorporation into cytokinin isolated from cultured rootless tobacco shoots. Most recently, Chen et al. (1985) provided direct evidence on sites of cytokinin biosynthesis, when they demonstrated the presence of the cytokinin biosynthetic enzyme, Δ^2-IPP:AMP-Δ^2-isopentenyl transferase, in cultured leaves, stems and roots from pea. They also found this enzyme activity in carrot cambium (a meristematic tissue) but not in noncambial tissue. These and other similar data provide strong evidence for cytokinin biosynthesis in meristematic or dividing tissue.

Cytokinin metabolism

Inactive metabolites

Extensive studies of cytokinin metabolism have been caried out in a number of laboratories. These studies have provided information on cytokinin metabolites and some of the enzymes responsible for their synthesis. They have also provided information on the synthesis and interconversion of cytokinins of which at least 28 have been identified (Letham and Palni, 1983). Naturally occurring cytokinin metabolites include 7- and 9-glucoside derivatives of zeatin, dihydrozeatin and isopentenyl adenine and the corresponding ribosides. Zeatin and dihydrozeatin may also be glucosylated at the terminal oxygen on the isoprenoid side chain. Other less common modifications include addition of alanine at the 9 position of the purine ring (Entsch _et al_., 1982) or a methyl-thio group at the 2 position, the latter having been found only in cultures of pathogenic bacteria (Letham and Palni, 1983). The significance of these metabolites is not yet clear. However, it has been shown in detached bean leaves that the O-glucosides are more stable than the corresponding free bases and that dihydro derivatives are more stable than zeatin derivatives (Palmer _et al_., 1981). The O-glucosides of zeatin and dihydrozeatin have been shown to be rapidly cleaved by β-glucosidase in _Populus_ leaves to release free cytokinin (Duke _et al_., 1979). Thus, some of these metabolites may serve as storage forms of cytokinin which can be cleaved to release free cytokinin.

Interconversion

Another aspect of cytokinin metabolism, which is important when considering the pool size of active cytokinin in a tissue, is interconversion between the base, ribonucleoside and ribonucleotide forms of cytokinins. Chen and coworkers have partially purified and analyzed five of the enzymes which catalyze the interconversion of adenine, adenosine and AMP. The K_ms for these enzymes range from 1 μM to 74 μM when the appropriate form of adenine is used as a substrate. The K_ms for these enzymes are 1-4 times as large when the corresponding isopentenyl adenine derivative is used as a substrate (Chen, 1982; Chen and Eckert, 1977; Chen and Petschow, 1978b; Chen and Kristopeit, 1981a; Chen and Kristopeit, 1981b; Chen *et al*., 1982). The regulation of these enzymes with regard to cytokinin metabolism is unknown. Therefore, their relative contributions to cytokinin pool sizes is also unknown.

Degradation

The enzyme responsible for degradation of cytokinin bases has been studied in some detail. This enzyme, cytokinin oxidase, cleaves the sidechain from cytokinin bases to produce adenine. This activity has been partially purified by the same group (McGaw and Horgan, 1983a; McGaw and Horgan, 1983b) from two sources, *Zea mays* kernels and *Vinca rosea* crown gall tissue. The proteins responsible for these activities are very different. The maize protein is approximately 25 kda in size while the *Vinca* protein is approximately 94 kda. The structural requirements for substrates of the *Vinca* protein have been established. Since a double bond in the side chain is required for activity, dihydro derivatives may not serve as substrates. The addition of glucosyl, ribosyl or alanyl groups at the 7 or 9 position does not reduce the degradation of such derivatives by cytokinin oxidase; however, O-glucosyl derivatives are resistant to cytokinin oxidase activity. These data suggest that the substrate specificity of cytokinin oxidase is responsible, at least in part, for the relative stabilities of dihydro- and O-glucosyl cytokinin derivatives and provide further evidence for the function of O-glucosyl derivatives as storage forms of cytokinin.

Physiological effects of cytokinin

Delay of senescence

Since their discovery, cytokinins have been found to have many effects on physiology and metabolism in plants as well as in other organisms (Letham, 1978). Among the best known effects of cytokinins in plants is their ability to delay senescence. As previously mentioned, Richmond and Lang (1957) first demonstrated that kinetin treatment of detached leaves of Xanthium retarded the decline in protein levels and generally delayed senescence of this tissue. Later, Mothes and Engelbrecht (1961) showed that applied cytokinin directed transport of radiolabelled assimilates to the region of cytokinin application. This type of result has been obtained in many plant systems (Noodén and Leopold, 1978). Leopold and Kawase (1964) found that cytokinin application stimulated the growth of treated leaves while inhibiting the growth of untreated leaves in the same plant. These studies, in conjunction with other work on hormonal effects on transport into fruiting structures (e.g. Seth and Wareing, 1967), led to the notion that cytokinins might be responsible for coordination of senescence in the vegetative shoot with fruit development via assimilate partitioning (Kende, 1971).

Early biochemical investigations into the basis for the delay of senescence by cytokinin showed increased protein and RNA retention in treated tissues (Richmond and Lang, 1957; Osborne, 1962). Later work with radiolabelled precursors indicated that cytokinins delayed the degradation of these macromolecules (Kuraishi, 1968; Tavares and Kende, 1970).

In addition to studies of cytokinin effects on macromolecular activity, many other studies of cytokinin effects on metabolism in connection with delayed senescence have been made in an attempt to understand the mode of cytokinin action (Higgins and Jacobsen, 1978). This has resulted in a large literature documenting cytokinin effects on numerous aspects of plant metabolism in an equally large number of plant systems. The diversity of the enzymes and pathways affected and of response to cytokinin from tissue to tissue is so great that analysis of this body of literature, thus far, has not provided any general conclusions or direct evidence on the mechanism of cytokinin action.

Morphogenesis

In addition to their role in senescence and assimilate transport, cytokinins induce morphogenesis in a number of plant systems. Three of the best known systems are the development of gametophytic buds from specific caulonemal cells in the moss, *Funaria hygrometrica* (Brandes and Kende, 1968; Hahn and Bopp, 1968) and cytokinin induction of shoot formation in tobacco callus cultures (Skoog and Miller, 1957) and in crown gall tumors (Binns, 1983; Akiyoshi *et al*, 1984). The biochemical basis of these processes is not understood. Saunders and Hepler (1982) have shown that treatment of *Funaria* target cells with the Ca^{++} ionophore, A23187 and Ca^{++} can induce the initial cell division in the budding process in the absence of cytokinin. However, cytokinin must be present in order for the process to continue. Further work has shown that Ca^{++} antagonists and Ca^{++} channel inhibitors will block bud formation (Saunders and Hepler, 1983) and that a redistribution of Ca^{++}

begins in target cells within 5 minutes of cytokinin treatment (Saunders, 1986). This response is certainly very close to the primary biochemical events leading to cytokinin induced budding, however, the mechanism of this response is as yet unclear.

Cell division and growth

Cytokinins are also well known for their ability to promote cell division and growth in plants, the latter occurring when cell division is the predominant mode of growth (Wright, 1961; Holm and Key, 1969). Studies of cytokinin effects on soybean and tobacco cell cultures have shown that in the absence of cytokinin, DNA synthesis continues but cytokinesis will not proceed until cytokinin is returned to the medium (Fosket and Short, 1972; Jouanneau, 1971). In addition to this, Jouanneau (1975) demonstrated that protein synthesis must be functional while cytokinin is present in the medium in order for cytokinesis to proceed.

Cytokinins have also been shown to play a regulatory role in DNA synthesis in cultured mammalian cells. Blocking mevalonate synthesis in mammalian cells, by treating them with compactin, blocks progress through the cell cycle just prior to DNA synthesis. Supplying compactin-treated cells with mevalonate will permit DNA synthesis to occur and the cell cycle to continue. Isopentenyladenine or zeatin, both naturally occurring cytokinins, will also permit DNA synthesis and a continuation of the cell cycle. However, they do so at 100-fold lower concentrations than mevalonate (Huneeus et al., 1980). Mevalonate has been shown to be a precursor of isopentenyladenine synthesis in plant and bacterial systems. If a similar pathway is operative in mammalian cells, the effect of mevalonate on DNA synthesis may be mediated via synthesis of cytokinins or other related compounds.

Cytokinins in tRNA

Early work on cytokinin effects on protein and RNA synthesis in conjunction with reports of the presence of cytokinins in tRNA (Hall, 1967; Chen and Hall, 1969) led to the notion that cytokinins might act as effectors of transcription or translation. Thus studies of the relationship between cytokinins present in tRNA and the mode of cytokinin action began and the significance of this relationship became a subject of intense debate. Labelling experiments conducted to determine the source of cytokinins in tRNA showed that IPP was the precursor of the side chain in the modified bases in tRNA and that the addition of cytokinins to an _in vitro_ enzyme system did not reduce the incorporation of ^{14}C-IPP into tRNA (Kline _et al_., 1969). In addition, tissues which required an exogenous supply of cytokinin for growth were shown to be capable of modifying bases in tRNA to produce cytokinins (Chen and Hall, 1969; Burrows _et al_., 1971). It was also shown that pyrazolopyrimidine analogs of cytokinins had hormonal activity, but due to their structure were unlikely to be incorporated into tRNA (Skoog _et al_., 1973). These and other similar data led to the now broadly accepted conclusion that cytokinins do not act via modification of bases in tRNA (Kende, 1971; Kende and Gardner, 1976).

Cytokinin binding

The first studies of cytokinin binding were made based on the assumption that the primary effect of cytokinin was in the regulation of protein synthesis. Berridge _et_ _al_. (1970) reported reversible binding of radiolabelled cytokinins to 83S ribosomes from Chinese cabbage leaves using equilibrium dialysis, chromatography on Sephadex G-200 equilibrated with labelled cytokinins, and sucrose density gradient centrifugation. Although the observed binding correlated well with biological activity, binding was not saturable near the solubility limit of cytokinins and no binding kinetics were reported. The authors were also unable to demonstrate any effect of cytokinins on _in_ _vitro_ protein synthesis in this system.

Cereal embryo binding proteins

The best studied cytokinin-binding moiety is a protein which can be washed off cereal grain ribosomes in 0.5 M KCl (Fox and Erion, 1975). Using equilibrium dialysis and centrifugation assays, the K_d of binding to this protein was determined to be 0.5 μM for benzyladenine (Erion and Fox, 1981) and 0.2-7.5 μM for kinetin (Polya and Davis, 1978; Moore, 1979). The number of binding sites per molecule of CBP are reported to be between 1 and 1.7 (Erion and Fox, 1981; Polya and Davis, 1978; Moore, 1979). CBP has a molecular weight of 160,000 and is composed of 3 identical subunits of molecular weight 54,000 (Brinegar and Fox, 1985). This suggests that the cytokinin binding site is in a domain to which each of the three subunits contributes equally.

Immunological studies with CBP antiserum in other organs of wheat have shown that an antigenically related protein is present in the ovule and surrounding tissue during early floral development, but is not present in any other parts of the flower (Keim et al., 1981). Brinegar et al. (1985) used antibodies to CBP to follow its appearance and degradation during embryogenesis and germination. It appears in developing embryos between 15 and 20 days post-anthesis and continues to increase throughout embryo development. At maturity, CBP represents approximately 9% of the soluble protein in the embryo. The amount of CBP decreases by about half on the first day after the onset of germination and is completely absent by the next day. This profile of synthesis and degradation closely resmebles that of a storage protein, however, CBP is always found in association with the embryo and not with the endosperm (Keim et al., 1981). The profile of CBP appearance correlates almost precisely with the profile of cytokinin binding activity in wheat embryos.

Despite past claims made for CBP as a cytokinin receptor, the proponents of this claim have recently agreed with the more widely held view that CBP is not a receptor (Brinegar et al., 1985). Two pieces of evidence speak strongly in favor of this view. First, CBP is estimated to be present in wheat embryos at 2.2-2.7 mg/g fresh weight, which is approximatedly 20 nmol/g fresh weight (Erion and Fox, 1981; Polya and Davis, 1978). This is a much higher concentration than expected for a receptor protein, and almost three orders of magnitude greater than auxin-binding sites in maize coleoptiles, which are on the order of 10-100 pmol/g fresh weight and considered to be high (Jones et al., 1984). Second, a study of the binding of CBP to a number of diverse compounds demonstrated that it has a broad ligand specificity. CBP bound with high affinity to the herbicidal phenyl ureas DCMU and CMU, triazines and carbamates; all of these are PS II electron transport inhibitors. Methylxanthines, uncoupling agents, such as DCCD, and a range of indoles also bound CBP with K_ds in the micromolar range. In many cases, the binding of these compounds inhibited ^{14}C-kinetin binding competitively (Polya and Bowman, 1979). The ability of CBP to bind such a diverse range of compounds with high affinity argues against a role for CBP as a cytokinin receptor.

Proteins immunologically related to CBP have been identified in homogenates of barley and oat embryos by double immunodiffusion techniques (Keim et al., 1981). In addition to this, Reddy et al., (1983) reported the isolation of a protein from barley embryos similar in size, subunit composition, and dissociation constant to CBP. This protein also has a synthesis and degradation profile very much like that of CBP.

Tobacco leaf binding proteins

A small polypeptide (4000 daltons) from tobacco leaves with cytokinin binding activity was identified by Takegami and Yoshida (1975) using a benzyladenine affinity column. This protein binds ^{14}C-benzyladenine with a relatively high dissociation constant, $K_d = 4 \times 10^{-5}$ M. Therefore, it is unlikely to be a cytokinin receptor. Chen et al. (1980) also isolated a glycoprotein from tobacco leaves by affinity chromatography. This protein was 8500 da and exhibited heat sensitive binding to ^{3}H-isopentenyladenine. The K_d for this binding was reported as 8.8×10^{-7} M. However this value has been criticized based on the fact that the binding assays were all done in ammonium sulfate, which can artifactually lower binding constants by as much as 40-fold (Venis, 1985). Such an increase in the K_d of ^{3}H-isopentenyladenine binding to this protein would bring it very close to the K_d for the tobacco leaf protein isolated by Takegami and Yoshida.

Particulate cytokinin binding sites

Gardner *et al*., (1978) identified a particulate fraction in protonemata of *Funaria hygrometrica* which had characteristics of both high-affinity, specific binding and low-affinity, nonspecific binding to ^{3}H-benzyladenine. These sites sedimented between 13,000 x g and 80,000 x g, were heat labile and detergent soluble. However these two components could not be resolved from one another physically or by Scatchard analysis.

Sussman and Kende (1978) reported particulate cytokinin binding sites from tobacco cell cultures which bound ^{3}H-benzyladenine with a K_d of 1.4×10^{-7} M and were heat labile. These sites were found to be few in number in comparison to a high background of heat stable sites which had a K_d of 7.7×10^{-6} M. A comparison of the binding of active cytokinins and inactive analogs (as determined in several cytokinin bioassays) to both the low-affinity and high-affinity sites demonstrated that the high-affinity sites had binding specificity for active cytokinins while the low-affinity sites bound compounds which do not have cytokinin activity as well as those that do. Although no protein has been identified in the high-affinity cytokinin binding fraction of Sussman and Kende, they show more promise for being a cytokinin receptor than any of the other cytokinin binding entities discussed here. These sites have been identified in a tissue with a documented biological response to cytokinins, and the binding of radioactive cytokinins to these sites is saturable. In addition, these sites demonstrate binding specificity for compounds which have biological cytokinin activity.

Cytokinin photoaffinity reagents

A number of groups have synthesized cytokinin photoaffinity compounds and tested their biological activity. Theiler *et al*. (1976) reported the synthesis of 2-azido-benzyladenine and 2-azido-isopentenyladenine, both of which were as active in tobacco cell suspension bioassays as benzyladenine (BA) and isopentenyladenine. Sussman and Kende (1977) reported the synthesis of 8-azidobenzyladenine ($8N_3BA$) and found it to have more biological activity in both the tobacco cell suspension bioassay and the *Funaria* bud formation bioassay than did BA. These workers also tested the activity of $8N_3BA$ after *in vivo* photolysis in the *Funaria* bioassay, and found that the cytokinin activity of $8N_3BA$ was lost when exposed to ultraviolet irradiation. Brandes and Kende (1968) observed that transfer to cytokinin-free medium of *Funaria* protonemata which had been exposed to cytokinin for less than 24 hours and had begun to form buds, will cause the buds to revert to sidebranch development (the process which normally occurs in the absence of cytokinin). This demonstrates that photolysis occurs successfully *in vivo*. Studies of binding of photoaffinity- labelled cytokinins to CBP have shown that these compounds are capable of inhibiting ^{14}C-kinetin binding to CBP (Mornet *et al*., 1979; Keim and Fox, 1980). A number of attempts have also been made to use radio-active photoaffinity cytokinins for *in vivo* studies of cytokinin binding (e.g. Sussman, 1976). However, the problem with this work, as with traditional binding studies using radiolabelled-cytokinins, is the inability to efficiently produce a compound with sufficiently high specific activity to permit detection of high-affinity binding sites over the background of nonspecific or low-affinity binding.

Use of an ABA photoaffinity reagent

Abicisic acid (ABA) contains an unsaturated ketone which can be photoactivated by irradiation with 330 nm light. Hornberg and Weiler (1984) used this as a means of producing (in relatively large quantities) a radiolabelled ABA photoaffinity reagent, [^{3}H]cis-(+)ABA which has been useful in identifying three ABA binding proteins which look promising as candidates for an ABA receptor. The binding of this compound to proteins in mesophyll and guard cell protoplasts was determined by fractionating the proteins on polyacrylamide gels and counting the radioactivity in various factions of the gels. Three regions of the gel containing guard cell protoplast proteins were shown to contain much higher levels of radioactivity than the gel slices containing mesophyll protoplast proteins. The molecular weights of these proteins ranged from 14,000 to 20,000. Binding of [^{3}H]-cis(+)ABA to these proteins was saturable, of high affinity, and correlated with the pH optimum and kinetic induction of stomatal closure by ABA. The level of these proteins is estimated to be approximately 3.2×10^{-15} mol per guard cell protoplast. Based on this information, these workers suggest that they have identified an ABA receptor.

INTRODUCTION

Previous work on cytokinin binding has provided two criteria which must serve as guides in the search for cytokinin receptors. First, the tissue used for such experiments must have a documented physiological response to cytokinins. Although this is an obvious requirement, a great deal of work has been done by Fox and others over the last 10 years in purifying and characterizing a cytokinin binding protein which is very abundant in wheat embryos. However, because there is no documented physiology for cytokinin binding in this system, the biological relevance of this binding is completely unclear. Second, the technique used to detect cytokinin binding must be extremely sensitive while maintaining a high degree of specificity. This criterion arises from work by Sussman and Kende (1978) who used ^{3}H-benzyladenine for binding studies in fractionated tobacco suspension cultures. These workers were able to resolve both low- and high-affinity binding sites by Scatchard analysis. However, identification of the high-affinity sites, which were low in number, could not be accomplished against the background of low-affinity sites using the level of sensitivity which was available to them.

The experiments described in this chapter were undertaken in an effort to identify cytokinin binding proteins with the aim of finding a cytokinin receptor. The approach was to use a photoaffinity cytokinin reagent in combination with immunological techniques which have been very successful in a number of systems because of the very high affinity of antibody binding and the very high degree of specificity which these molecules have for the antigens against which they are directed. We have produced a polyclonal antiserum directed against a BA-bovine serum albumin (BA-BSA) conjugate made by coupling a photoaffinity labelled cytokinin 8-azido-benzyladenine ($8N_3BA$) to BSA *in vitro*. We then attempted to covalently label cytokinin binding proteins *in vivo* by treating tissues with the photoaffinity compound and exposing the tissues to long-wavelength ultraviolet irradiation to activate covalent coupling of $8N_3BA$ to proteins closely associated with it. After isolating proteins from the treated tissues, they were subjected to western blot analysis using the antiserum in an effort to identify proteins which had been labelled covalently with the cytokinin photoaffinity reagent.

Although we were successful in developing an antiserum which contains antibodies specific for cytokinin-like structures, the only successful attempts at detecting proteins covalently labelled with $8N_3BA$ *in vivo* were not convincingly repeated. The experiments attempted are described in the results section of this chapter. Finally, the suspected causes for our lack of success are discussed as well as potential methods for making this kind of approach successful.

MATERIALS AND METHODS

Culture and treatment of moss protonemata

Cultures of Funaria hygrometrica (L.) Sibth. were grown as described by Brandes and Kende (1968) with minor modifications. One spore capsule containing approximately 10^6 spores was aseptically opened to allow as many spores as possible to disperse in a 1 ml solution of 0.01% Tween-20. After mixing vigorously to suspend the spores evenly, this solution was pipetted immediately in 0.5 ml aliquots onto 14-cm Petri plates containing Knop agar (1 g $Ca(NO_3)_2$ $4H_2O$, 0.25 g KH_2PO_4, 0.25 g KCl, 0.25 g $MgSO_4 \cdot 7H_2O$, 43 mg Sequestrene·NaFe, 1 ml Heller's microelements (White, 1963), 15 g Bacto-Agar (Difco, Detroit, MI), made to 1000 ml with distilled water) and dispersed over the agar surface using a bent glass rod. After five days of culture at 22°C with continuous irradiation under cool white fluorescent lamps, which provided 75 μmol s^{-1} m^{-2}, individual protonemata were transferred to 9-cm Petri plates containing Knop agar under a dissecting microscope and overlaid with sterile cellophane discs. This allowed for culture of individual protonemata and facilitated removal of tissues from the medium without agar contamination. Cultures transferred to 9-cm Petri plates were returned to the above growth conditions.

Seventeen-day-old cultures of Funaria hygrometrica were used for experimental treatments. In order to label cytokinin binding proteins in vivo, moss protonemata were treated with a photoaffinity-labelled cytokinin in the dark for 24 hours. The protonemata were floated on liquid media, which contained no cytokinin, $8N_3BA$, BA, or both $8N_3BA$

and BA, the protonemata being floated on the media. Following incubation, $8N_3BA$ was activated by placing the dishes 14 cm below a long wavelength ultraviolet lamp for 30 minutes. The protonemata were then blotted on filter paper and weighed prior to protein isolation.

Culture and treatment of tobacco suspension cell cultures

Cytokinin-dependent cell cultures were originally supplied in June 1974 by Dr. C. Péaud-Lenoel (Faculte des Sciences de Luminy, Laboratoire de Biochimie, Fonctionelle des Plantes, ER 104 CNRS, F-13288 Marseille Cedex 9, France). This was strain #21 (Tandeau de Marsac and Jouanneau, 1972) and was originally isolated from pith of Nicotiana tabacum L. cv. Wisconsin 38. The cells are maintained by subculturing biweekly using a defined liquid medium (Jouanneau and Péaud-Lenoel, 1967) containing 2×10^{-7} M 2,4-D and 5×10^{-7} M kinetin. Cultures were placed in 1-l Erlenmeyer flasks containing 200 ml of medium on a rotary shaker (140 rpm) at room temperature. The cultures were irradiated continuously at a fluence rate of 100 μmol s^{-1} m^{-2} provided by cool white fluorescent lamps. Tissues were harvested for transfer or treatment by filtration using a sterile Büchner funnel. Cells used for experiments were taken 7-10 days following subculture, washed and returned to cytokinin-free medium for 1 week prior to experimental use. For treatment, cells were harvested and redistributed to fresh growth medium containg auxin and various combinations of cytokinins. After incubation with $8N_3BA$ for 24 hours in darkness, the tobacco tissues were transferred to 14 cm Petri dishes (this done in dim light) and the Petri plates were placed 14 cm below a long-wavelength ultraviolet lamp in order to activate the photoaffinity labelled cytokinin. After photolysis, tissues were harvested, blotted dry on filter paper, and weighed prior to protein extraction.

Synthesis and conjugation of a photoaffinity-labelled cytokinin

8-Azido-N^6-benzylaminopurine ($8N_3BA$) was synthesized by Dr. F.R. Lehle (MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824). Dr. Lehle then prepared conjugates of $8N_3BA$ to two different carrier proteins for the purposes of antibody production and analysis of antibody specificity. The proteins used were bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) and ovalbumin (OVA; Sigma). The conjugates were produced by incubating $8N_3BA$ (a light sensitive reagent) in darkness with the appropriate protein, the photoaffinity compound being present in 10- to 20-fold excess on a molar basis. The mixtures were then exposed to long-wavelength ultraviolet radiation in order to create a reactive nitrene group from the photolabile azido group, which could then bind covalently to the carrier protein present in solution. Absorption spectra of the dialyzed products of these reactions prior to and following photoactivation and conjugation were used to determine the approximate amount of ligand which had covalently attached to carrier protein molecules. The estimated ratio of the BA-OVA conjugate was 3.4 BA to 1 OVA molecule. The first conjugation of BA to BSA produced a conjugate with a ratio of 3.3 BA molecules per 1 BSA molecule. This preparation of conjugate was reacted with $8N_3BA$ a second time to produce a batch of conjugate with a ratio of 7 BA molecules per BSA molecule.

Blood collection

One New Zealand white female rabbit (approximately 2 kg) was used for collection of preimmune serum and for antiserum production. All blood was collected from the peripheral vein of the outer ear. Xylene was applied to the tip of the ear prior to nicking the vein in order to dilate the blood vessels. The peripheral vein on the side of the ear opposite the one being bled and the peripheral vein being bled were pinched (the latter was pinched beyond the point of the nick) to increase blood yield. Twenty-five to 30 mls of blood were routinely collected using this procedure.

Immunization and antiserum analysis

One-hundred micrograms of the BA-BSA conjugate in 500 μl of phosphate-buffered saline (PBS; 10mM NaH_2PO_4, pH 7.2, 150 mM NaCl) was sonicated with an equal volume of Freund's complete adjuvant. This was divided into four 250 μl aliquots which were each injected subcutaneously in four locations along the back of the animal. Twenty-one days later blood was collected, and subsequent collections were made at 2- or 4-week intervals. Serum was prepared by allowing the blood to clot for 2-3 hours at room temperature and then overnight at 4°C. The clot was removed from the serum and the remaining cells were removed by centrifugation at 10,000 x g. The serum was then divided into 50 μl volumes and stored at -20°C. Each new serum sample was analyzed for protein content using the Bradford method (Biorad, Richmond, CA) and for antibody (IgG) titer by enzyme-linked immunosorbent assay (ELISA). A good immune response was obtained in the rabbit. Serum protein levels increased from about 80 mg ml^{-1} in preimmune serum to as high as 138 mg ml^{-1} by 11 weeks following immunization. Serum protein levels in subsequent collections were decreased slightly and remained between 110 and 120 mg ml^{-1}. Analysis of IgG titer per unit serum protein using an ELISA showed that IgG levels peaked at 9 weeks following immunization and decreased to 88% of the peak by the 11th week following immunization. At this point, sufficient amounts of serum containing anti-BA-BSA antibodies had been collected, therefore no further immunization or analysis of subsequent serum collections were made.

ELISA

Enzyme-linked immunosorbent assay (ELISA) was performed using an anti-rabbit IgG peroxidase conjugate prepared in goats (GARP; Sigma Chemical Co., St. Louis, MO) and 2,2'-azinodi-(3-ethylbenzthiazoline sulfonic acid) (ABTS; Sigma) for detection. A 96-well Immulon 1 Microelisa plate (Dynatech Laboratories, Inc., Alexandria, VA) was coated with 400 ng of BA-OVA conjugate (corresponding to 30 pmol BA) in 50 µl of 15 mM $NaHCO_3$, pH 9.5, per well by shaking at room temperature overnight. (All stock solutions were sterilized and maintained at 4°C to prevent contamination.) After each step of the assay, the wells were rinsed 4 times with 200 µl of 0.05% OVA in PBS. In order to block all the protein binding sites in the microassay well, 200 µl of 3% OVA in PBS was added to each well and incubated 1 hour while shaking at room temperature. Following the blocking step, 50 µl of antiserum appropriately diluted in PBS was added to each well. In many of the assays, antisera were preincubated in 0.5% BSA in PBS to bind antibodies with specificity for BSA and reduce the possibility of crossreaction with OVA, which would have increased background in the assays. In competitive ELISAs, antisera were preincubated with the approriate ligand to bind any antibodies with specificity for BA. Following incubation with antiserum (or preimmune serum in control wells) and washing, 50 µl of GARP diluted 1:1000 in PBS, 3% OVA was added to each well. This was incubated for 2 hours at room temperature while shaking. After rinsing, the peroxidase reaction was performed by adding 50 µl of 2 mM ABTS in 100 mM $NaCH_3CO_2$, 50 mM NaH_2PO_4, pH 4.2, 0.03% H_2O_2 to each well and incubating for 30 minutes, in darkness at 30°C. The peroxidase reaction was stopped by adding 25 µl of 15% SDS to each well. The reaction was quantitated by measuring absorbance at 405 nm in each well using a Biotek EIA reader.

Protein extraction

Total protein was extracted by grinding tissue in 80% (v/v) ethanol at 4°C. The resulting slurry was spun at 10,000 x gravity and the pellet was extracted three more times with cold 80% ethanol to remove all lipid and pigment materials. The final pellet was lyophilyzed and resuspended in 20 mM NaH_2PO_4, pH 8.5. Protein was analyzed by the method of Bradford (1976) using reagents from Biorad (Richmond, CA).

Polyacrylamide gel electrophoresis (PAGE)

PAGE was done according to the method of Laemmli (1971). A gradient of acrylamide from 8% to 14% (w/v) was used to improve resolution of proteins. Protein samples were suspended in sample buffer (2% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 62.5 mM Tris-HCl pH 6.8, and 0.01% (w/v) bromphenol blue), boiled for 2 minutes, and spun in a microfuge for 3 minutes prior to loading onto the gel. Gels were stained for protein with Coomassie blue (0.1% (w/v) coomassie brilliant blue R-250, 7% (v/v) acetic acid, 50% (v/v) methanol) and destained in a solution of 7% (v/v) acetic acid, 2% (v/v) methanol and 3% (v/v) glycerol.

Electrophoretic transfer of proteins to nitrocellulose filters

Proteins were transferred from SDS-PAGE gels to nitrocellulose filters using the method of Towbin *et al*. (1979). An unstained gel was placed next to a sheet of nitrocellulose and immersed in 192 mM glycine, 25 mM Tris-NaOH, pH 8.3, 20% (v/v) methanol. Proteins were transferred electrophoretically from the gel onto the nitrocellulose filter by applying 60 V for 6 hours across the gel and filter. This was done using a Transphor Electrophoresis Cell (Hoeffer Scientific, San Francisco, CA).

Western blot development

The development of western blots was done using a Vectastain ABC-AP kit (Vector Laboratories, Burlingame, CA). The remaining protein binding sites on the nitrocellulose filter were blocked by incubating the filter in 10% nonfat dry milk (Carnation Co., Los Angeles, CA) in PBS overnight. All incubations and washes of the nitrocellulose filter were made by shaking the filters at room temperature. Rabbit antiserum (or preimmune serum) was preincubated with 0.1% BSA (w/v) at room temperature for 3-4 hours prior to incubation with the nitrocellulose filter. This was done to help reduce background binding of antibodies specific for BSA. The filter was then incubated in a 1:1000 dilution of rabbit serum in PBS, 0.05% Tween-20, 0.1% BSA and 1% OVA for 3-4 hours. Unbound antibody was removed from the filter by washing the blot in three changes of PBS, 0.05% Tween-20, 0.1% OVA for 10-15 minutes each. The blot was then incubated in biotinylated goat anti-rabbit IgG diluted according to kit instructions in PBS, 0.05% Tween-20 for 30 minutes. After washing three times more as above, the filter was incubated in the Vector avidin-biotin alkaline phosphatase (ABC) reagent diluted according to kit instructions in PBS, 0.05% Tween-20 for 30 minutes. The blot was then washed 3 times in PBS to remove Tween-20 and any unbound ABC reagent. Finally the blot was washed twice in 100 mM Tris, pH 8.2. The color substrate for the alkaline phosphatase reaction was prepared according to kit instructions in 100 mM Tris-NaOH, pH 8.2. Color development was performed in the dark for 15-20 minutes. The reaction was stopped by incubating the blot for 10-15 minutes in 10 mM Tris-NaOH, pH 7.5, 1mM EDTA, then for 10-15 minutes in 20 mM Tris-NaOH, pH 9.5, 5 mM EDTA. The blots were dried between sheets of filter paper and photographed.

RESULTS

Characterization of antiserum binding specificity

The binding specificity of a rabbit antiserum made against a BA-BSA conjugate was determined by competitive ELISA. Seven compounds (benzyladenine (BA), trans-zeatin (t-Z), cis-zeatin (c-Z), benzyladenine riboside (BAR), adenine (Ade), guanine (Gua), ATP) were investigated for their ability to inhibit binding of antibodies in the antiserum to a BA-OVA conjugate, which served as the antigen in these assays. Figures 1 through 7 present the results of these experiments. Figure 1 shows the inhibition of antibody binding to BA-OVA by BA. Absorbance at 405 nm is proportional to the amount of antibody binding. This figure demonstrates that there is a population of antibodies within the rabbit serum which have specificity for BA. This is true because no antibody binding was observed when OVA alone was used as the antigen. This figure also shows that the binding of these antibodies to BA-OVA can be completely inhibited by the addition of excess BA to the serum. Similar results were obtained for BAR, t-Z, and c-Z (Figures 2, 3 and 4). Figures 5, 6 and 7 show competitive ELISAs with Ade, Gua and ATP. Ade inhibits antibody binding to BA-OVA by 22% but neither Gua nor ATP inhibit this binding at all. These results demonstrate that there is a population of antibodies within this antiserum which recognize purine-like structures which have sidechains attached at the N_6 position.

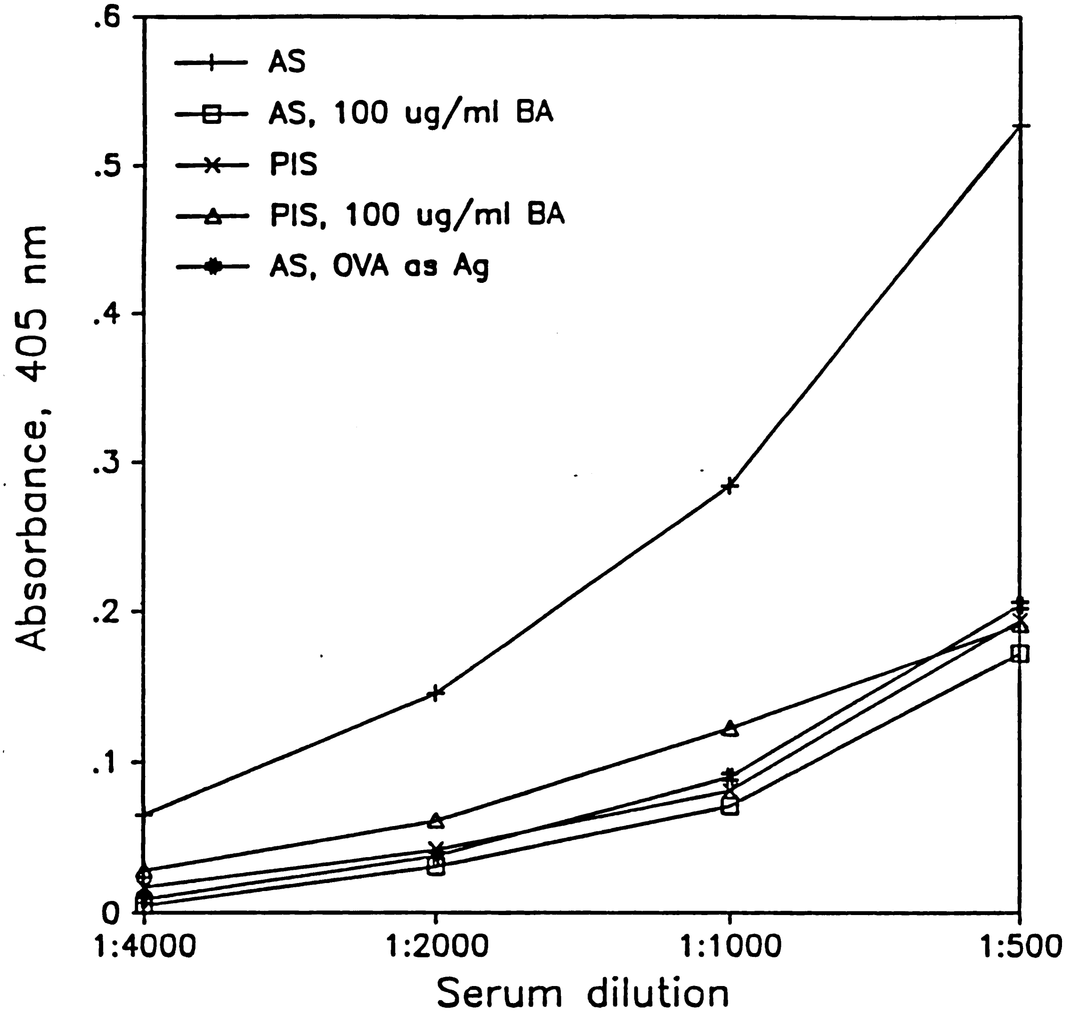

Figure 1 Competitive inhibition of antibody binding by benzyladenine (BA). A BA-OVA conjugate was used as the antigen to test the ability of antibodies within the antiserum (AS) or preimmune serum (PIS) to bind BA. Ova was also used as an antigen in control assays to demonstrate antibody specificity for BA.

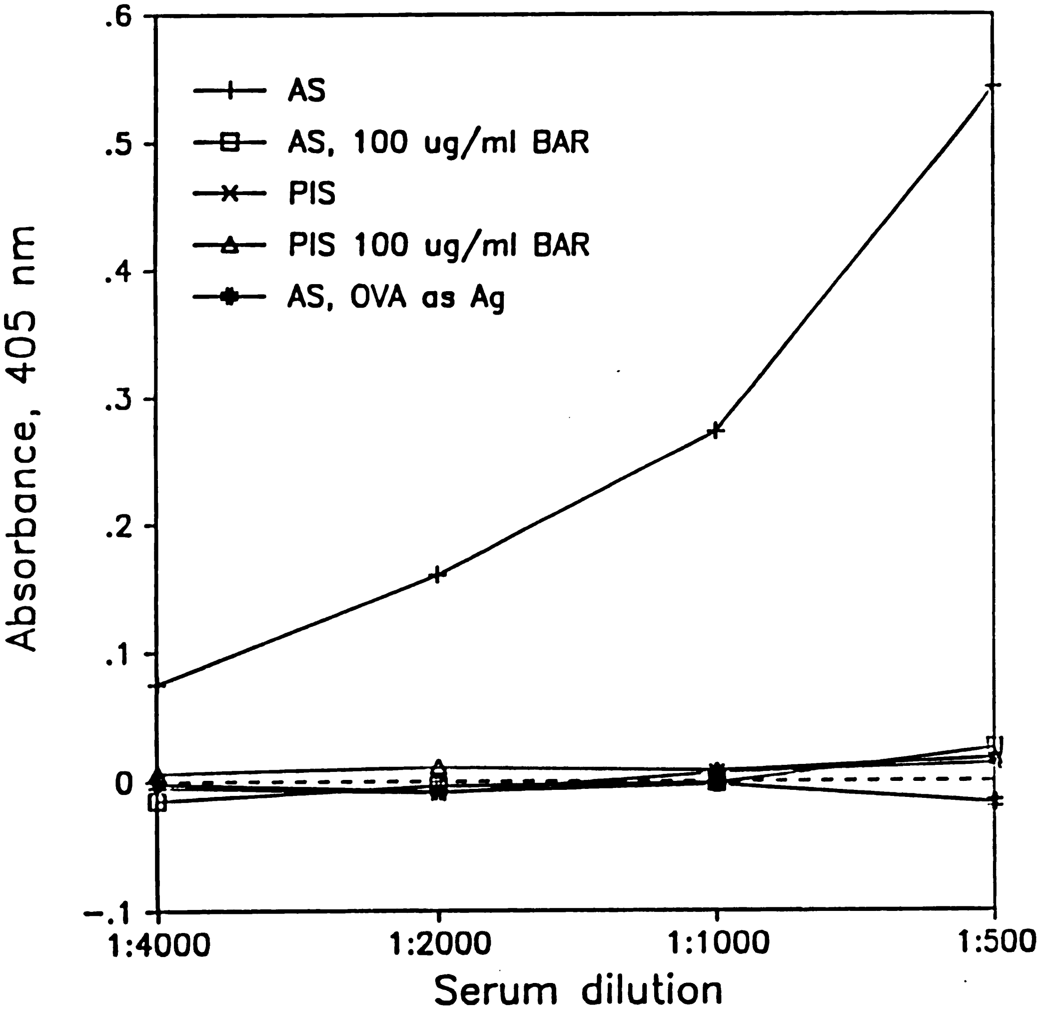

Figure 2 Competitive inhibition of antibody binding by benzyladenine riboside (BAR). A BA-OVA conjugate was used as the antigen to test the ability of antibodies within the antiserum (AS) or preimmune serum (PIS) to bind BA. Ova was also used as an antigen in control assays to demonstrate antibody specificity for BA.

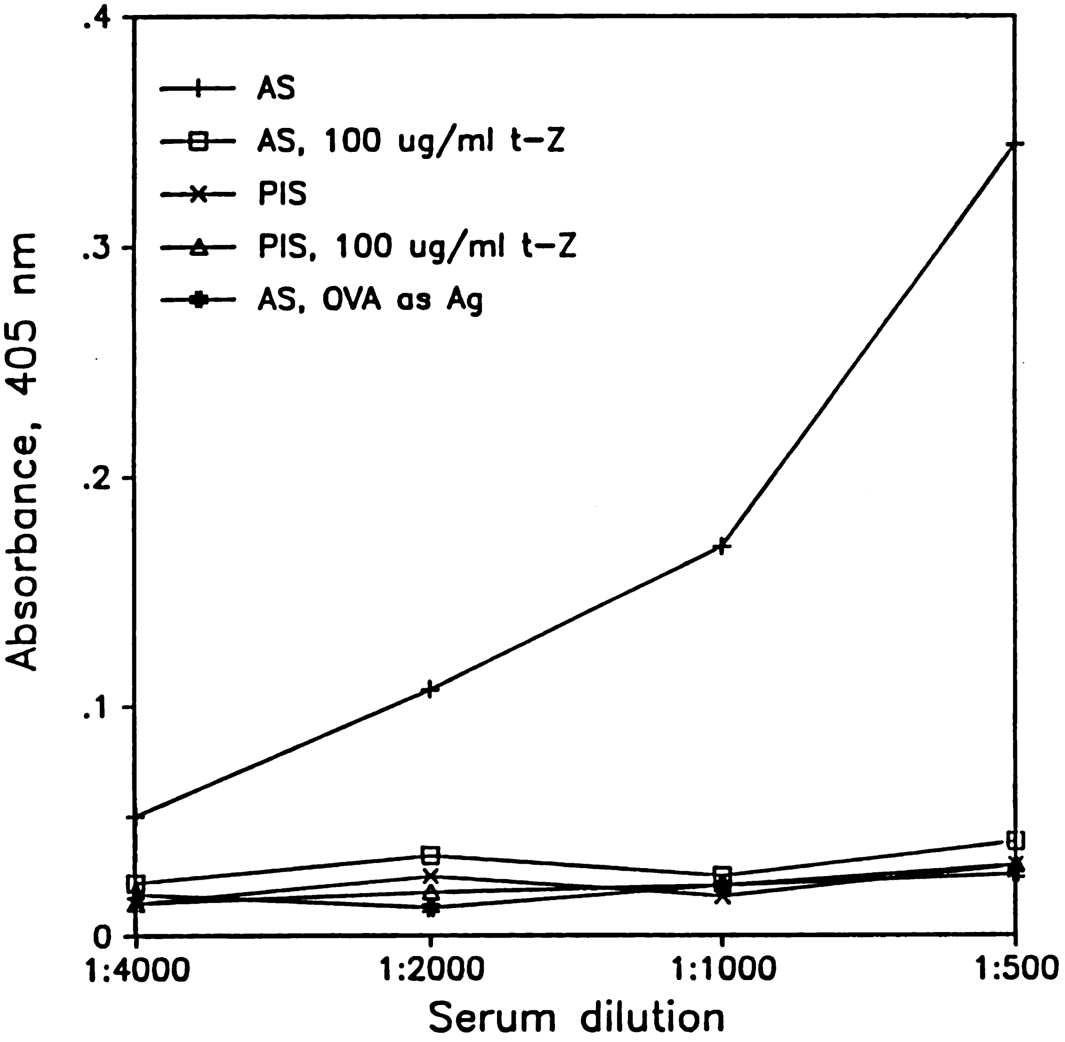

Figure 3 Competitive inhibition of antibody binding by trans-zeatin (t-Z). A BA-OVA conjugate was used as the antigen to test the ability of antibodies within the antiserum (AS) or preimmune serum (PIS) to bind BA. Ova was also used as an antigen in control assays to demonstrate antibody specificity for BA.

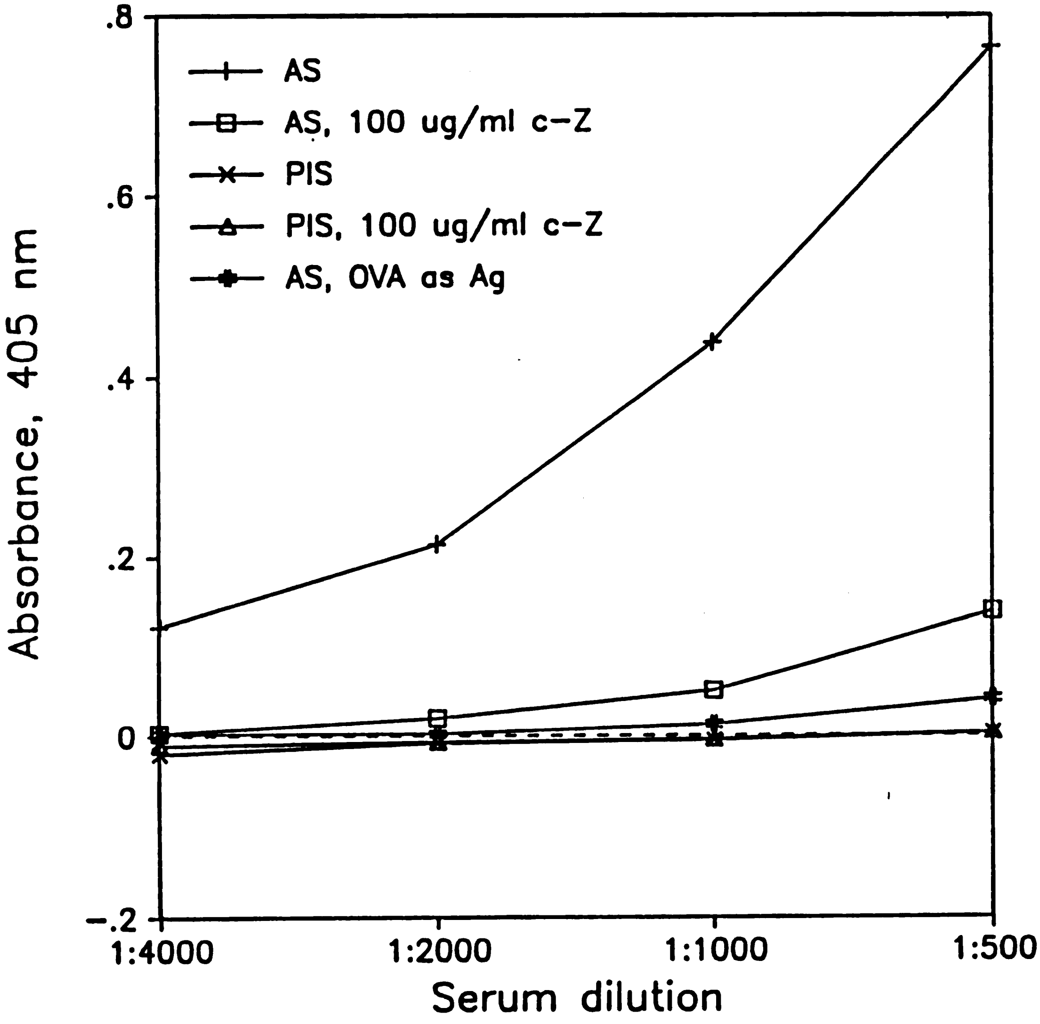

Figure 4 Competitive inhibition of antibody binding by cis-zeatin (c-Z). A BA-OVA conjugate was used as the antigen to test the ability of antibodies within the antiserum (AS) or preimmune serum (PIS) to bind BA. Ova was also used as an antigen in control assays to demonstrate antibody specificity for BA.

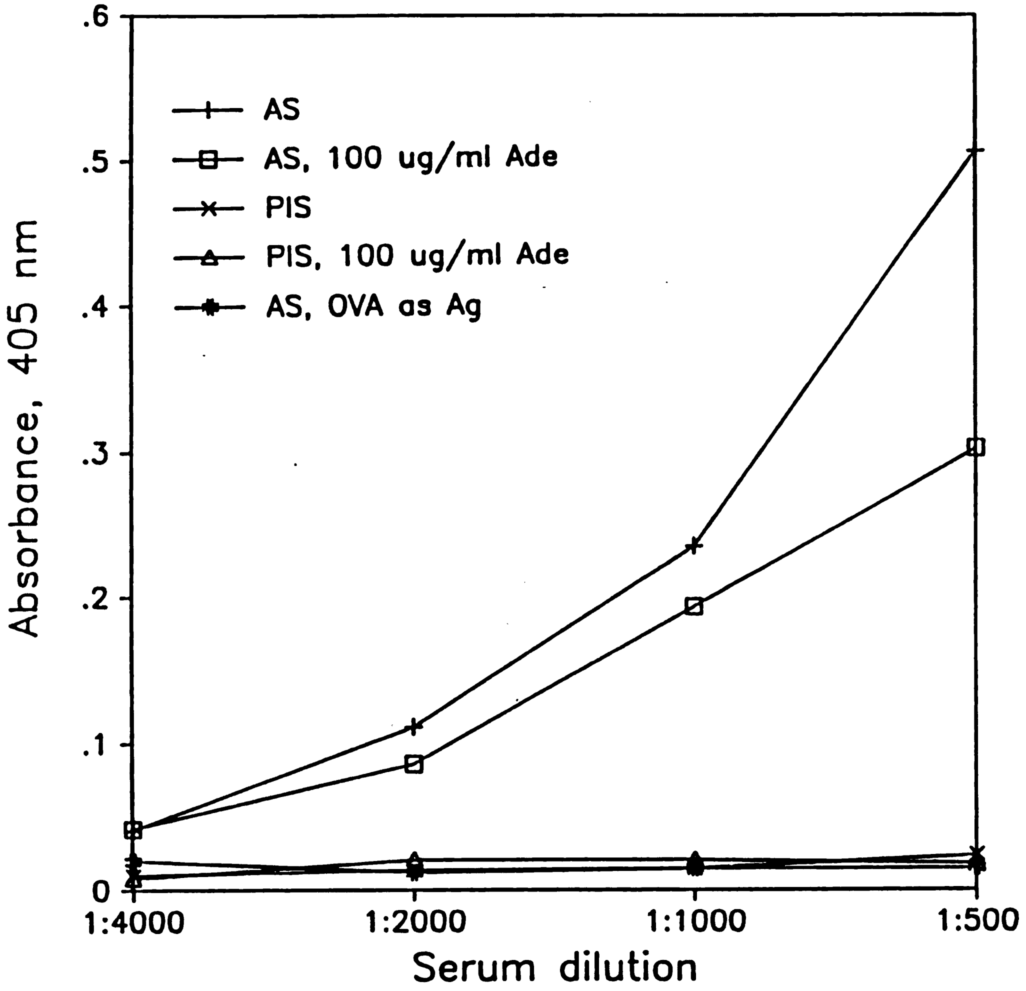

Figure 5 Results of a competitive ELISA testing the ability of adenine (Ade) to inhibit antibody binding to BA. A BA-OVA conjugate was used as the antigen to test the ability of antibodies within the antiserum (AS) or preimmune serum (PIS) to bind BA. Ova was also used as an antigen in control assays to demonstrate antibody specificity for BA. Ade inhibited antibody binding tao BA by 22%.

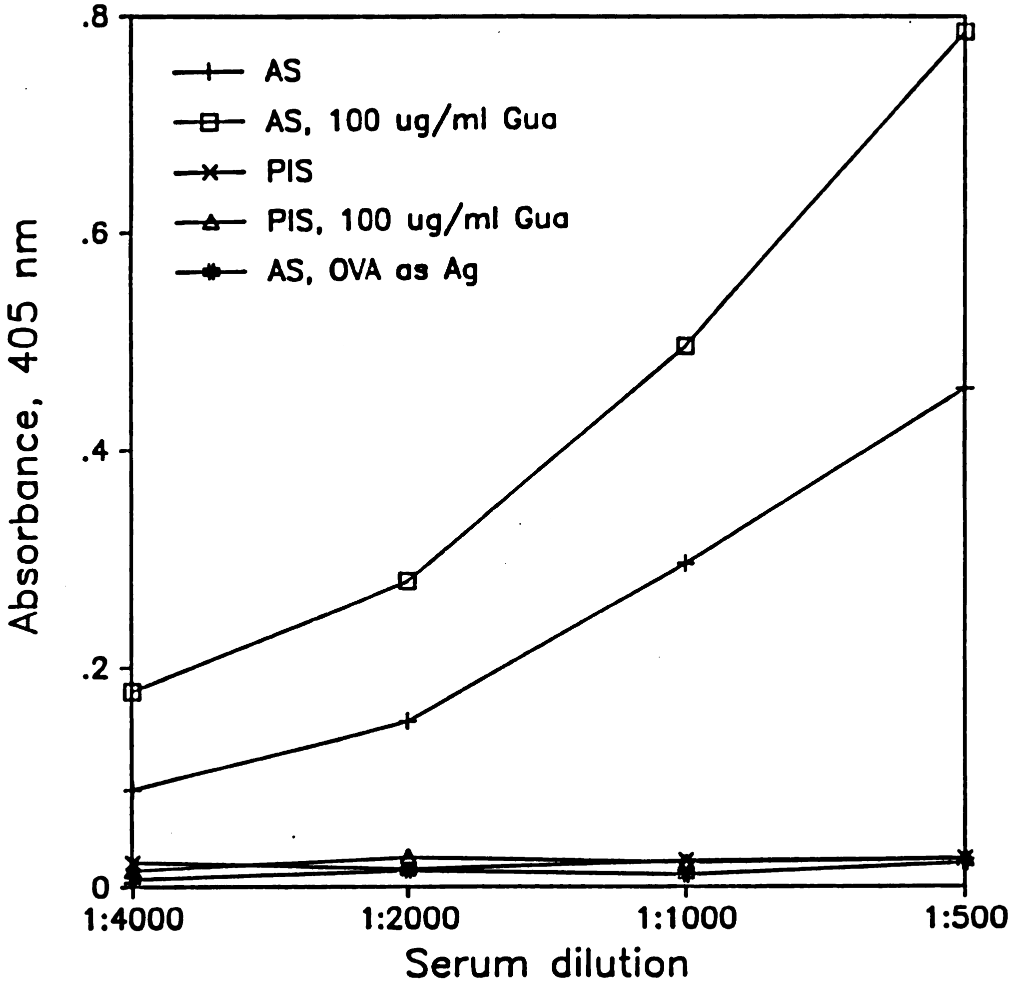

Figure 6 Results of a competitive ELISA testing the ability of guanine (Gua) to inhibit antibody binding to BA. A BA-OVA conjugate was used as the antigen to test the ability of antibodies within the antiserum (AS) or preimmune serum (PIS) to bind BA. Ova was also used as an antigen in control assays to demonstrate antibody specificity for BA. Gua did not inhibit antibody binding to BA.

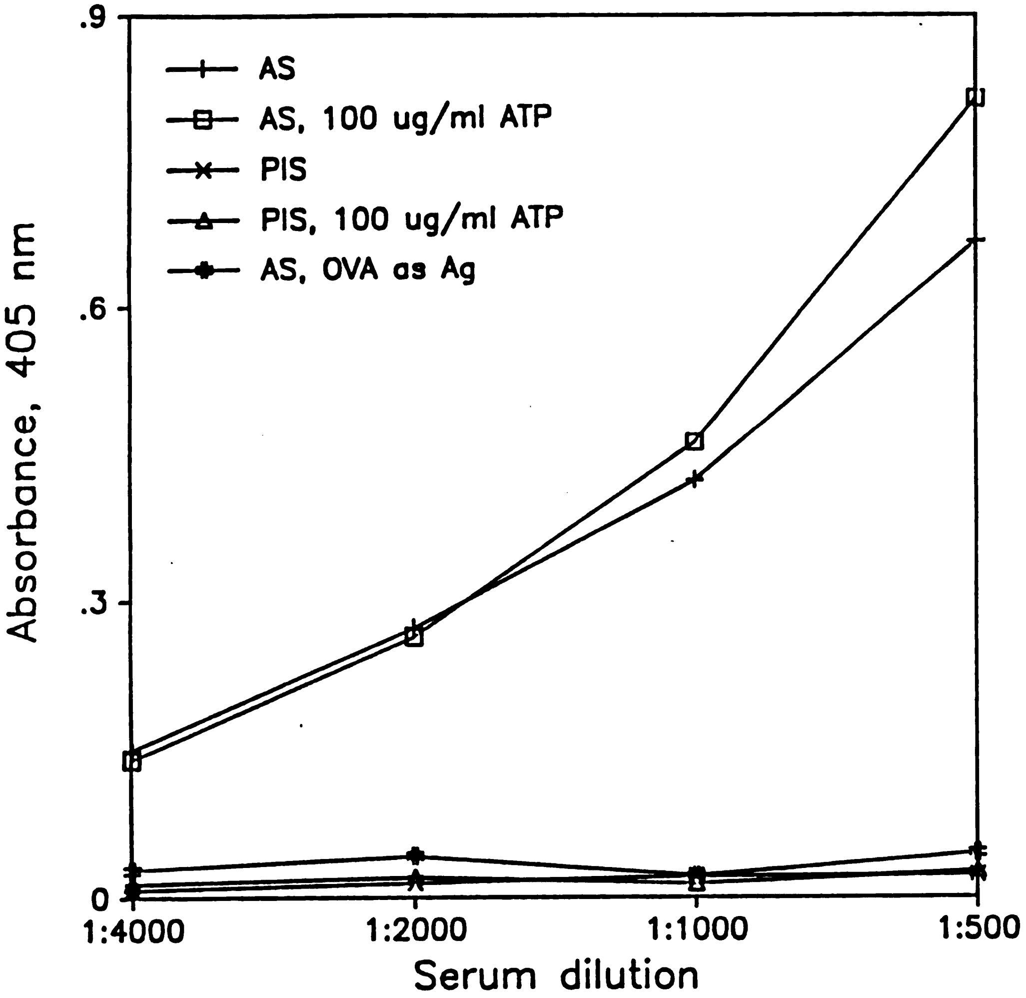

Figure 7 Results of a competitive ELISA testing the ability of ATP to inhibit antibody binding to BA. A BA-OVA conjugate was used as the antigen to test the ability of antibodies within the antiserum (AS) or preimmune serum (PIS) to bind BA. Ova was also used as an antigen in control assays to demonstrate antibody specificity for BA. ATP did not inhibit antibody binding to BA.

Limit of cytokinin detection using western method

The limit of detection of BA by the antiserum described here was determined by spotting known amounts of the BA-OVA conjugate onto a nitrocellulose filter, then subjecting the filter to western analysis using the ABC method. Figure 8 shows the result of such an experiment. The limit of BA detection using this method lies between 6.2×10^{-8} and 6.2×10^{-9} g which corresponds to 1.4×10^{-11} to 1.4×10^{-12} mol of the BA-OVA conjugate. (The molecular weight of this compound is approximately 45,700 da.) The ratio of BA to OVA is approximately 3.4:1, therefore the limits of detection correspond to 4.6×10^{-12} mol BA.

Western analysis of $8N_3BA$ binding in vivo

A number of attempts were made to label cytokinin binding proteins *in vivo* using two different tissues (1) tobacco suspension culture cells which are cytokinin dependent and (2) protonemata of *Funaria hygrometrica*. The tissues were incubated in darkness for 24 hours in solutions containing (A) no cytokinin, (B) 10^{-6} M $8N_3BA$ (C) 10^{-5} M BA or (D) 10^{-6} M $8N_3BA$ and 10^{-5} M BA. BA was added in excess as a competitor to help distinguish physiological binding from any nonspecific binding which might be obtained when using a photoaffinity compound. The tissues were then exposed to long-wavelength ultraviolet light to activate the covalent coupling of $8N_3BA$ to proximal molecules. Total protein was then extracted from the tissues and fractionated by PAGE. After transfer to nitrocellulose filters, the proteins were analyzed by the western blot method using an anti-BA-BSA rabbit antiserum for proteins which had bound $8N_3BA$ *in vivo*.

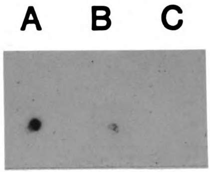

Figure 8 The limit of detection of a BA-OVA conjugate on a western blot using an avidin-biotin amplification system. The conjugate was spotted directly onto the nitrocellulose filter. The filter was then exposed to the standard western procedure. 6.2 x 10^{-7} g (A), 6.2 10^{-8} g (B), and 6.2 x 10^{-9} g (B) of the conjugate were applied to the filter. The limit of detection lies between 6.2 x 10^{-8} and 6.2 x 10^{-9} g of conjugate which corresponds to 1.4 x 10^{-12} mol of conjugate and 4.6 x 10^{-12} mol of BA. (See text p 44 for calculation.)

Figure 9 shows a blot of total tobacco protein whch was probed with anti-BA-BSA antiserum. The proteins were isolated from tissues incubated in 10^{-6} M $8N_3BA$ and 10^{-5} M BA, lane A; 10^{-6} M $8N_3BA$, lane B; or no cytokinin, lane C. Two bands of approximately 54,000 and 58,000 da are labelled in lane b by the anti-BA-BSA antiserum. We presume these bands cannot be resolved in lane a due to competition for binding to these proteins in vivo between $8N_3BA$ and BA. This is the only experiment in which this precise result was obtained. Any attempt to increase resolution of these bands by increasing protein loaded on the gel resulted in a blot as appears in Figure 10. There is a high level of background which increases as a result of increased protein loaded on the gel prior to transfer to nitrocellulose. In another experiment using cultured tobacco cells, we were barely able to resolve a single band of approximately 58,000 da above the background obtained using the ABC detection system (Figure 11, lane A). As in the blot in Figure 9, no bands were detected in the lanes which contained protein from tissues incubated in the absence of $8N_3BA$ or in the presence of $8N_3BA$ and excess BA. If the band in lane A of Figure 10 corresponds to one of the bands detected in lane B of Figure 9, these data suggest that a cytokinin binding protein of approximately 58,000 da is present in cultured tobacco cells. However, the level of this protein is just at the very limits of detection using this technique.

Experiments similar to the experiments conducted with tobacco cultured cells were also performed in Funaria protonemata. The results of one such experiment are shown in Figure 12. A number of discreet bands were detected in western blots analyzed with the anti-BA-BSA antiserum. However, these bands were present in all tissues regardless

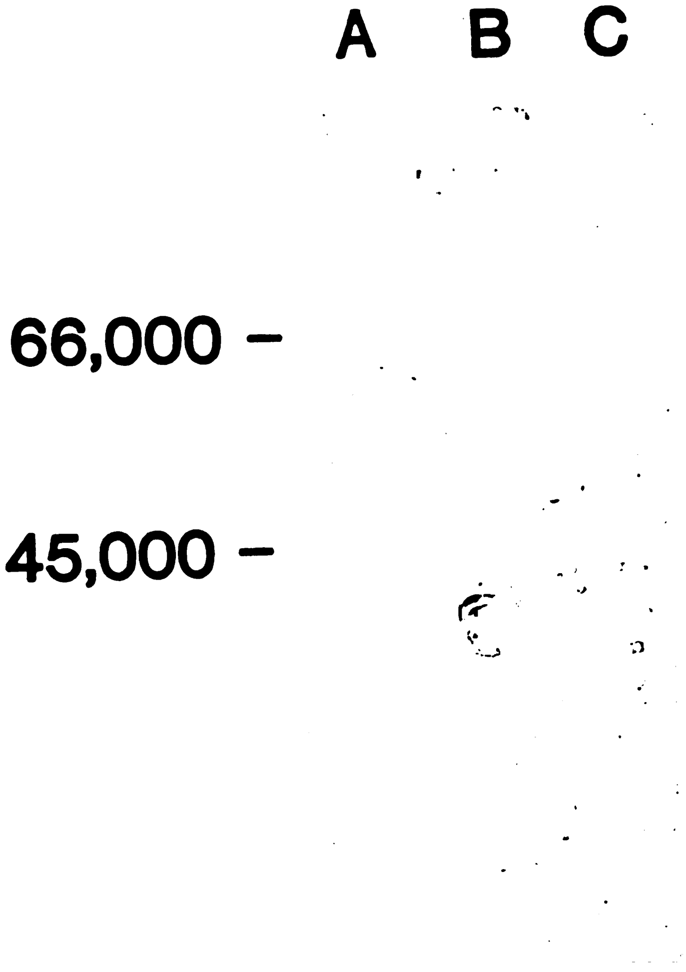

Figure 9 Western blot of tobacco proteins using an anti-BA-BSA antiserum. Protein was isolated from cells treated with 10^{-6} M $8N_3BA$ and 10^{-5} M BA (A), 10^{-6} M $8N_3BA$ (B), or no cytokinin (C). 100 ug of protein was loaded on to each lane.

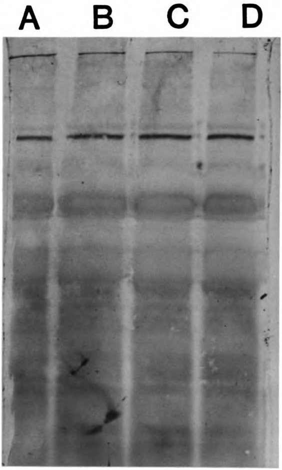

Figure 10 Western blot of tobacco proteins using an anti-BA-BSA antiserum. Protein was isolated from cells treated with 10^{-6} M $8N_3BA$ and 10^{-5} M BA (A), 10^{-6} M $8N_3BA$ (B), 10^{-5} M BA (C) or no cytokinin (D). 200 μg of protein was loaded on to each lane.

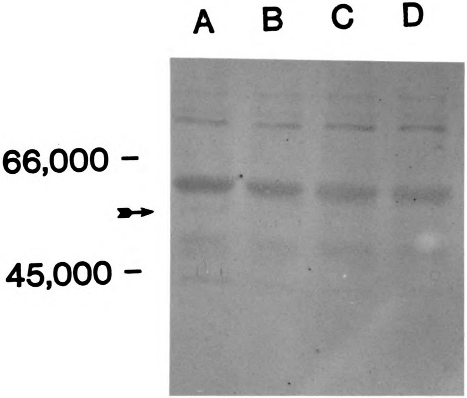

Figure 11 Western blot of tobacco proteins using an anti-BA-BSA antiserum. Protein was isolated from cells treated with 10^{-6} M $8N_3BA$ (A), 10^{-6} M $8N_3BA$ and 10^{-5} M BA (B), 10^{-5} M BA (C) or no cytokinin (D). 150 ug of protein was loaded on to each lane.

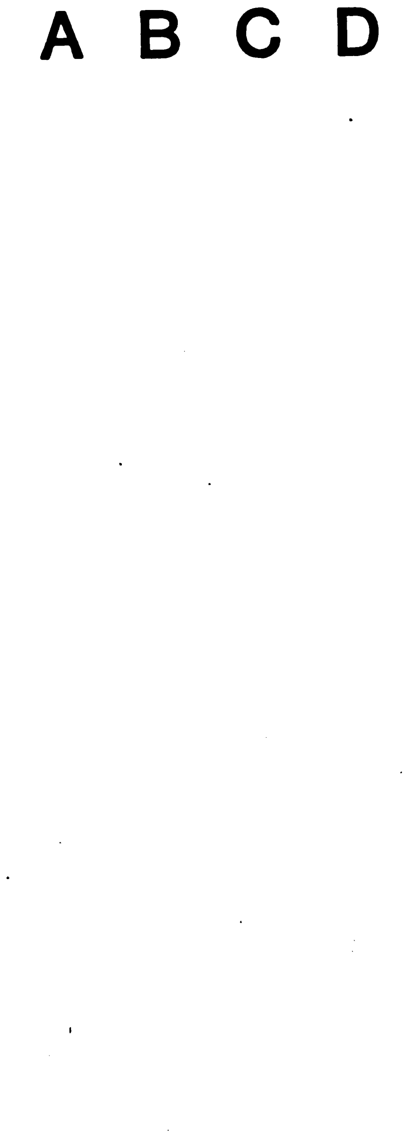

Figure 12 Western blot of <u>Funaria</u> protein using an anti-BA-BSA antiserum. Protein was isolated from tissue treated with 10^{-6} M $8N_3BA$ (A), 10^{-6} M $8N_3BA$ and 10^{-5} M BA (B), 10^{-5} M BA (C) or no cytokinin (D). 20 μg of protein was loaded on to each lane.

of whether or not they were incubated with $8N_3BA$. The meaning of these results is unclear.

DISCUSSION

We were unable to unequivocally detect any proteins which were consistently labelled with $8N_3BA$ using western methods on proteins isolated from treated tissue. An analysis of the sensitivity of this technique provides an explanation for these results. The limit of detection by western technique using an avidin-biotin amplification system of $8N_3BA$ bound to OVA was 4.7×10^{-12} mol BA (Figure 8). This detection limit assumes no background color is present on the filter. Sussman and Kende (1978) calculated the number of cytokinin binding sites present in tobacco cells at 8×10^{-12} mol g FW^{-1}. Total protein was extracted from this tissue at 1-1.5 mg g FW^{-1}. Therefore, the binding sites of Sussman and Kende should be present at 8×10^{-12} mol mg total $protein^{-1}$. The limit of protein which could be loaded onto a single lane of an SDS-PAGE gel without increasing background color on the western blot was about 100 μg (compare Figures 9 and 10). This corresponds to 8×10^{-13} mol of the cytokinin binding sites of Sussman and Kende. Thus we can explain our failure to obtain convincing and repeatable results in tobacco tissues based on the limits of the detection system used in these experiments.

No estimate of cytokinin binding site concentration has been obtained in Funaria hygrometrica. However, one might expect the level of these sites to be much lower on a fresh weight or protein basis than in tobacco cells since a physiological response has been demonstrated in only one of the two cell types present in protonemata. In addition to this, not every cell of this cell type shows the response to cytokinin at any one time, therefore the number of binding sites per unit protein could be expected to be even lower and completely undetectable using this method.

Prospective approaches to improving this technique

Although it is clear that the approach described here does not provide a detection system which is sensitive enough to detect proteins in quantities as low as those expected for a cytokinin receptor, the fact that our antiserum has reasonably good specificity for cytokinin-like structures and the ability of the antiserum to detect relatively low levels of specific proteins suggest that a modification of this type of approach may be useful for detecting cytokinin binding proteins.

There are two ways to improve the method described here: (1) enrich the target proteins in the tissues which are being analyzed or (2) increase the sensitivity of the detection system. The enrichment of a target protein in tissues may be accomplished by using a mutant or variant which has increased levels of the target protein. In the absence of such a variant, enrichment of a target protein might also be achieved by selecting tissues which are known to be homogeneous and uniform in their response to cytokinin. The isolation of ABA binding proteins was accomplished by using guard cell protoplasts which had been dissected out of leaves by hand (Hornberg and Weiler, 1984). The use of the growing zone of coleoptiles has permitted the isolation of auxin transport proteins (Goldsmith, 1982). Fractionation of tissues and analysis of each fraction separately has also assisted in the analysis of auxin binding and transport (Jacobs and Hertel, 1978). The degree of homogeneity of the tobacco suspension cultures used in the experiments described here is unclear. It is possible that analysis of cytokining binding in this system by immunocytological techniques might provide information on the homogeneity of the tissue with regard to cytokinin binding, as well as provide clues as to which subcellular fractions to analyze in searching for cytokinin binding moieties. In Funaria, specific cells which respond physiologically to cytokinin have been identified. These cells are few in number in a given protonema. Isolation of these cells from the remainder of the protonema could be accomplished using a dissecting microscope, however, obtaining enough tissue to analyze for cytokinin binding would likely require a concerted effort on the part of several people.

The only method presently feasible for increasing the sensitivity of antibody detection is to radiolabel antibodies using ^{125}I. Markwell (1982) described a method for labeling antiserum with ^{125}I using nonporous polystyrene beads, which provided a specific activity of 150 $\mu Ci\ \mu g$ protein^{-1}. Weinand et al, 1979 used ^{125}I labeled cDNA to probe recombinant plasmids, and reported detection of approximately 200 positive colonies using 7.5×10^6 cpm. If one assumes a counting efficiency of 50%, this corresponds to approximately 1.5×10^7 dpm or 6.8 μCi. Assuming that the amount of radioactivity in any one colony is 0.5% of the total radioactivity added to the filter, each positive colony on the film corresponds to 34 nCi. Using the specific activity of Markwell, 34 nCi of ^{125}I corresponds to about 200 pg of antiserum. In the experiment shown in Figure 9, 30 μg of protein were added to the nitrocellulose filter as antiserum in the western procedure. If this protein had been labelled with ^{125}I to the same specific activity as that attained by Markwell (150 $\mu Ci\ \mu g$ protein^{-1}), we would have used

1.5 x 10^5 as much radioactivity as required for detection of a single colony in the experiments of Weinand _et_ _al_. Although the use of ^{125}I involves work with a very hazardous substance, it is probably the fastest and most direct method for increasing the probablility of detecting cytokinin binding proteins using antibodies to a cytokinin photoaffinity compound.

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CHAPTER 2

THE SUBMERGENCE RESPONSE IN DEEPWATER AND NONDEEPWATER RICE

INTRODUCTION

Rice is one of the world's primary food and protein sources. It constitutes half the diet of approximately 1.6 billion people and comprises one-fourth to one-half of the diet of another 400 million people. More than 145 million hectares, or about 11% of the world's arable land is devoted to rice production (Swaminathan, 1984). Since 1960, rice production in tropical Asia has increased 60% and yield has increased more than 40%. The increases in yield and productivity are attributable to the development of high-yielding cultivars and a trend toward more scientific crop and soil management. These improvements have been very important in that they have allowed countries such as Indonesia and the Philippines, both formerly rice-importing nations, to become self-sufficient in rice production.

Approximately 11% of Asia's ricelands are dedicated to deepwater rice production. Deepwater (and irrigated cultivars) are distinguished by breeders from upland or lowland cultivars, which are rainfed, and are the primary subsistence crop upon which about 10^8 farmers in the flood plains of southeast Asia depend (Umali, 1981). Flood waters in these regions may reach a depth of up to 6 m (de Datta, 1981). Therefore in order to keep up with the rising water level, deepwater cultivars can elongate as much as 25 cm d^{-1} (Vergara, et al., 1976).

Deepwater rice improvement programs have focused on crop protection methods, improvement of agricultural practices, and traditional breeding. The broad goals of deepwater rice breeders have included incorporation of disease and pest resistance, drought tolerance (at seedling stage), improved grain quality, and (complete) submergence tolerance into new cultivars. One of the more specific goals of deepwater rice breeders is to develop a semidwarf cultivar which will perform (yield) well at depths of 2-3 meters by incorporating elongation genes of deepwater cultivars into high-yielding semidwarf varieties. Such a project requires that one has a simple, objective measure of elongation, preferably one which does not require elaborate equipment. Based on physiological information about the elongation process in deepwater rice, we attempted to develop as assay for this process and to test its validity as a means of distinguishing between cultivars capable of rapid internodal elongation and cultivars lacking that capacity. Through the course of these experiments, we obtained information on the elongation capacity of semidwarfs which may change the way breeders think about so-called elongation genes.

Physiology of deepwater rice elongation

Raskin and Kende (1984a) established that the gas composition in the internodal lacunae of stem sections of deepwater rice is approximately 3% O_2, 6% CO_2, and 1 $\mu l\ l^{-1}$ ethylene after 3 days of submergence. These workers and Métraux and Kende (1983) also demonstrated that the growth response of deepwater rice to submergence is mediated by C_2H_4 and that the submergence response could be reproduced by exposing nonsubmerged stem sections to the above gas mixture in a flow-through system. It has also been shown that submerged or C_2H_4-treated deepwater rice does not elongate if the endogenous gibberellin (GA) level is reduced using tetcyclasis (TCY), a specific inhibitor of GA biosynthesis. Supplying the plant with exogenous GA_3 in the presence of TCY restores the elongation response to submergence (Raskin and Kende, 1984b). Exogenously supplied GA_3 has also been shown to elicit full stem elongation in the absence of exogenous C_2H_4 or in the presence of high levels (2000 $\mu l\ l^{-1}$) norbornadiene (NBD), a reversible inhibitor of C_2H_4 action in deepwater rice (Bleecker et al., 1975) as well as other physiological systems (Sisler and Yang, 1984). Therefore, it is likely that gibberellin is responsible for initiating the elongation process, and that C_2H_4 either increases the level of active gibberellin in the responsive tissue or modulates the tissue sensitivity to GA in some way.

This chapter describes the experiments conducted with 12 different rice cultivars which can be classified as deepwater, short-statured, or semidwarf types. In an attempt to develop an assay for elongation ability, I found that all 12 cultivars were capable of rapid internodal elongation when partially submerged. Therefore, more extensive experiments were conducted with three cultivars, one from each group, in order to determine more specifically what differences exist between these cultivars in their elongation capacities.

MATERIALS AND METHODS

Plant material and growth conditions

The growth of the following 12 cultivars of rice (*Oryza sativa*, L.), was measured and compared under several environmental conditions: Kalar Harsall, Thavalu, Pin Gaew (PG) 56, and Leb Mue Nahng (LMN) 111 (seeds provided by Dr. B.S. Vergara, International Rice Research Institute, Los Banos, Philippines); Labelle and Mars (seeds provided by Dr. T. Johnston, University of Arkansas, Stuttgart, AR); M9 and Calrose 76 (seeds provided by Dr. J.N. Rutger, University of California, Davis, CA); Habiganj Aman (HA) II, III, and VIII (seeds provided by Dr. S.M.H. Zaman, Bangladesh Rice Research Institute, Dacca, Bangladesh); IR8 (seeds provided by Dr. R.S. Bandurski, Michigan State University, East Lansing, MI). The rice plants were germinated in darkness on moist filter paper in Petri dishes at 30° C for 72 h. Seedlings were sown singly in one-quart pots in a soil mixture of 1 part Perlite (coarse grade, Therm-O-Rock, New Eagle, PA), 2 parts sand, 2 parts black peat, 2 parts loam, 1 part ground clay (Terra Green, Oil-Dri Corp. of America, Chicago, IL), and 2 parts triple superphosphate (U.S. Steel Corp.). The pH of the soil was adjusted to 6.2 with lime (about 2 oz of lime per 5.5 quarts of soil). Plants were grown in a glass house from March 14, 1983 through June 20, 1983 and from March 1, 1984 through June 14, 1984 under the following conditions: day temperature, 27-31°C; night temperature, 27°C; 16-h photoperiod. The natural photoperiod was extended to 16 h using sodium lamps. Plants were watered with half-strength Hoagland solution using a drip-irrigation system. The pots were placed in

6-cm-deep flats containing half-strength Hoagland solution to prevent complete drainage.

Measurement of internodal length in whole plants

Prior to treatment, the length of each internode in each tiller of every plant to be tested was determined. Nodes in whole plant stems were detected by holding the culms in front of a high-intensity lamp in an otherwise darkened room and were marked with water-insoluble ink. The length of each internode was then recorded and designated as initial internodal length. Following treatment, each culm was dissected longitudinally, and the length of each internode was recorded and designated final internodal length. For each culm, the sum of the initial internodal lengths was subtracted from the sum of the final internodal lengths to give the internodal elongation. In experiments conducted in 1983, the mean internodal length (initial or final) and the mean internodal elongation were taken as the average of 7-10 tillers for a given cultivar randomly selected from all tillers measured. In experiments conducted in 1984, the mean internodal length and internodal elongation were taken as the average of 3 tillers per plant and of 3 or 4 plants per cultivar. In some cultivars and at some ages, the internodes were nested at the base of the culm making it difficult to determine the initial internodal length using a high-intensity lamp. In these cultivars, 10 or more culms were dissected and measured in order to obtain a mean initial internodal length.

Excision and measurement of stem sections

Stem sections containing the youngest two nodes and the uppermost internode were excised from well developed tillers with a razor blade. Each section was 20 cm long and was cut such that the lower node was 2 cm above the basal cut. Any leaf sheath remaining on the section was removed except for that originating at the basal node of the section (Raskin and Kende, 1984). The initial internodal length of each section was determined by holding the section in front of a high intensity lamp in a dimly lit room. The position of the apical node in the section was marked with water-soluble ink, and the distance between the two nodes (internodal length) was then measured. Sections containing internodes of no more than 80 mm in length were used. Following treatment, each section was dissected longitudinally, and the final length of the internode (or internodes, if any new ones developed) was determined.

Whole plant submergence

Whole plants were suspended in 300-l Nalgene tanks, which were filled with distilled water. Approximately 10-20 cm of leaf material remained above the water level to create a condition of partial submergence. The water in the tanks was replaced on the third day of submergence. The tanks were placed in an environmental growth chamber under the following conditions: day temperature, 28°C; night temperature, 25°C; relative humidity, 60%; 13-h photoperiod; and light intensity of 400 μmol s^{-1} m^{-2} at soil level. Control plants for these experiments were kept in the same environmental growth chamber in flats which contained half-strength Hoagland solution.

Submergence of stem sections

Sections to be submerged were placed upright in a 100-ml glass beaker filled with glass beads to anchor the sections. The beaker was then lowered into a 1-l graduated cylinder, 42 cm deep, which was filled to the rim with distilled water. All experiments were performed at 27 C, in continuous light provided by cool-white fluorescent tubes at a photosynthetic photon flux density of 70 μmol s^{-1} m^{-2}.

Gas treatment of stem sections

All gas treatments were administered in a flow through system. For each treatment, ten stem sections were placed upright in a 100-ml glass beaker containing 20-30 ml of distilled water. Each beaker was then placed in a 2.5-l Plexiglas cylinder, 60 cm deep, which had an inlet port at the base, an outlet port at the top and a gas tight lid. Either air or a gas mixture containing 3% O_2, 6% CO_2, 91% N_2 (all by volume), and 1 μl l^{-1} C_2H_4 was passed through the cylinder at a flow rate of 80 ml min^{-1}. Nitrogen, O_2 and CO_2 were supplied from high-pressure gas cylinders. The gas mixture was prepared with gas-pressure regulators and rotameters containing three calibrated flow-meter tubes equipped with high-accuracy valves and a mixing tube (Matheson Gas Products, Joliet, IL, USA). Compressed laboratory air was used for all flow-through air (control) treatments. Gas mixtures and air were brought to 100% relative humidity by bubbling them through water. They were divided and dispersed to the incubation cylinders with flowmeter boards (Pratt et al. 1960). Ethylene was the last component added to the gas stream. This was accomplished by passing the gasses through a glass tube which was inside an ethylene reservoir. The capillary was discontinuous, within the reservoir, and the two ends of the capillary connected by gas permeable Tygon tubing. The length of the Tygon tubing was adjusted to regulate the amount of ethylene that diffused into the gas stream (Saltveit, 1978). The ethylene concentration in the gas stream was then calibrated to 1 μl l^{-1}.

Statistical analysis

Experiments in 1983 were performed with whole plants and stem sections with 7-10 replicates using a completely randomized design. The effect of air, submergence and gas treatment (the latter in sections only) was tested by least square difference (LSD). Experiments in 1984 were performed with whole plants in a split-split plot design. The main plot was age, the subplot was treatment (air or submergence), the sub-subplot plot was cultivar. The average tiller length per plant was the fundamental unit for statistical analysis. Since each rice plant has a different number of tillers, each at a different physiological age, a way of standardizing information from plant to plant was established. The three longest tillers of each plant prior to submergence were used in the statistical analysis, even though data were acquired for every tiller of each plant. These data were then subjected to analysis of variance (AOV). To determine initial internodal lengths for each cultivar, data from the two treatments, air and submergence were pooled and analyzed by AOV.

RESULTS

Response of 12 rice cultivars to partial submergence

Whole plants of 12 rice cultivars representing three classes of cultivar (deepwater, semidwarf and short-statured) were partially submerged in deionized water or left to grow in air for 5 d. The average internodal length prior to and following treatment as well as the mean internodal elongation of well developed tillers of each cultivar is presented in Table 1. Each value represents the average elongation of 7-10 tillers randomly selected from all tillers measured. A comparison of the internodal elongation between plants of a given cultivar which have been either partially submerged or grown in air shows that the elongation of partially submerged plants was significantly greater than the elongation of air-grown plants. This was true in all cultivars tested.

The data in Table 1 provide several criteria by which these 12 cultivars may be ranked. Four such rankings are presented in Tables 2 and 3. Table 2 presents two rankings of the cultivars based on internodal length following both treatments. Of the four rankings presented, these two correlate best with cultivar classification. The final internodal length of plants grown in air ranged from 654 mm in HA VIII, a deepwater cultivar, to 13 mm in IR8, a semidwarf. In plants which had been submerged, these values ranged from 898 mm in HA III, another deepwater cultivar, to 71 mm in IR8. Table 3 presents a ranking of these 12 cultivars based upon internodal elongation following both treatments. Unlike internodal length, the amount of elongation which occurs over the treatment period correlates poorly

Table 1 Effect of partial submergence on internodal elongation in whole plants of 12 rice cultivars. The initial internodal length of 12- to 13-week-old plants was determined, the plants were treated for 5 days, then final internodal length and internodal elongation were determined. All values are reported in millimeters. [a-b]Means across treatments followed by different letters are significantly different at the 0.05 level, using LSD. [c]Cultivars in which panicle initiation had begun by the time experiments were conducted.

Table 1

		AIR			SUBMERGENCE		
		Internodal	length	Internodal	Internodal	length	Internodal
Cultivar	Classification	initial	final	elongation	initial	final	elongation
				mm			
HA VIII	deepwater	632	654	22^{a}	289	545	256^{b}
HA III	deepwater	509	534	25^{a}	617	898	281^{b}
HA II	deepwater	375	390	15^{a}	423	685	262^{b}
PG 56	deepwater	249	276	27^{a}	134	570	436^{b}
LMN 111	deepwater	243	294	51^{a}	290	550	260^{b}
KH	deepwater	198	230	32^{a}	152	272	120^{b}
Thavalu	tall	162	186	24^{a}	207	291	84^{b}
Mars^{c}	short-statured	186	209	23^{a}	258	467	209^{b}
Labelle	short-statured	103	142	39^{a}	101	295	194^{b}
M9^{c}	semidwarf	147	204	57^{a}	180	451	271^{b}
Calrose 76^{c}	semidwarf	29	36	6^{a}	29	204	175^{b}
IR8^{c}	semidwarf	11	13	2^{a}	11	71	60^{b}

AIR			PARTIAL SUBMERGENCE		
Cultivar	Class	Internodal length	Cultivar	Class	Internodal length
		mm			mm
HA VIII	deepwater	654	HA III	deepwater	898
HA III	deepwater	534	HA II	deepwater	685
HA II	deepwater	390	PG 56	deepwater	570
LMN 111	deepwater	294	LMN 111	deepwater	550
PG 56	deepwater	276	HA VIII	deepwater	545
KH	deepwater	230	Mars	short	467
Mars	short	209	M9	semidwarf	451
M9	semidwarf	204	Labelle	short	295
Thavalu	tall	186	Thavalu	tall	291
Labelle	short	142	KH	deepwater	272
Calrose	semidwarf	36	Calrose	semidwarf	204
IR8	semidwarf	13	IR8	semidwarf	71

Table 2 Two rankings of 12 rice cultivars based on internodal length. The internodal length of plants of each cultivar was determined following 5 days of treatment. These values are presented in millimeters and were used to establish rankings of the cultivars for both air-grown and partially submerged plants.

AIR			PARTIAL SUBMERGENCE		
Cultivar	Class	Elongation mm d^{-1}	Cultivar	Class	Elongation mm d^{-1}
M9	semidwarf	11.4	PG 56	deepwater	87.2
LMN 111	deepwater	10.2	HA III	deepwater	56.2
Labelle	short	7.8	M9	semidwarf	54.2
KH	deepwater	6.4	HA II	deepwater	52.4
PG 56	deepwater	5.4	LMN 111	deepwater	52.0
HA III	deepwater	5.0	HA VIII	deepwater	51.2
Thavalu	tall	4.8	Mars	short	41.8
Mars	short	4.6	Labelle	short	38.8
HA VIII	deepwater	4.4	Calrose	semidwarf	35.0
HA II	deepwater	3.0	KH	deepwater	24.0
Calrose	semidwarf	1.2	Thavalu	tall	16.8
IR8	semidwarf	0.4	IR8	semidwarf	12.0

Table 3. Two rankings of 12 rice cultivars based on internodal elongation. The internodal length of plants from each cultivar was determined prior to and following treatment. The difference in internodal length after 5 days of treatment was determined and is reported as elongation in millimeters per day. The 12 cultivars analyzed were then ranked according to the amount of internodal elongation which occurred during the treatment period.

with cultivar classification. Over a five day period, the increase in the internodal length of air-grown plants ranged from 57 mm in M9, a semidwarf, to 2 mm in IR8. In partially submerged plants these values ranged from 436 mm in PG 56, a deepwater cultivar, to 60 mm in IR8.

Effect of submergence and gas treatments on stem sections

In order to determine whether or not the physiology of the submergence response in the other cultivars was similar, I analyzed the effect of ethylene and submergence on stem sections of each cultivar. Ten stem sections containing the uppermost internode of tillers from each of 12 cultivars were excised from 12- to 13-week-old plants and were exposed to the following treatments for 3 d: a) complete submergence in distilled water, b) a gas mixture composed of 3% O_2, 6% CO_2, 91% N_2 and 1 $\mu l\ l^{-1}$ C_2H_4, or c) air; treatments b and c were administered in a flow through system developed by Raskin and Kende (1984). Table 4 presents the average internodal length of all sections from each cultivar prior to and following treatment, as well as the internodal elongation over the treatment period. In each cultivar, the enhancement of internodal growth observed in whole plants was also observed in stem sections which were submerged or placed in an atmosphere composed of 3% O_2, 6% CO_2, 91% N_2, and 1 $\mu l\ l^{-1}$ C_2H_4. As in whole plants, within each treatment a wide range of elongation occurred between cultivars. Nevertheless, sections from short-statured lowland rice cultivars, such as Mars, or semidwarf cultivars, such as M9, were capable of responding to submergence or to gas treatment in the same fashion as did deepwater rice.

Table 4 Effect of submergence and gas treatment on internodal elongation in excised stem sections of 12 rice cultivars. The uppermost internode from culms of 12- to 13-week-old plants was dissected and measured for internodal length. The plants were either submerged or treated with a mixture of 3% O_2, 6% CO_2, and 1 $\mu l\ l^{-1}$ C_2H_4 or air in a flow through system for 3 days. The final internodal length and internodal elongation were determined. All values are reported in millimeters. $^{a-b}$Means across treatments followed by different letters are significantly different at the 0.05 level, using LSD. ^{c}Cultivars in which panicle initiation had begun by the time experiments were conducted.

Table 4

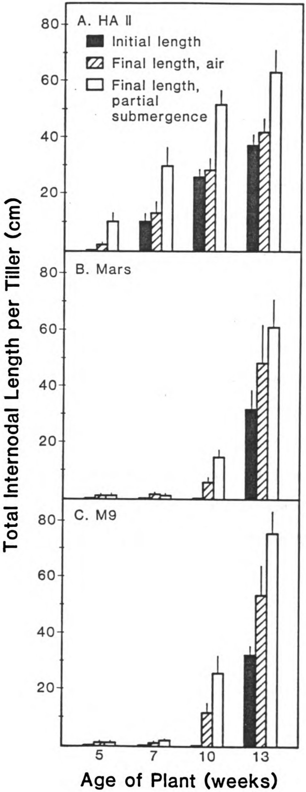
	AIR			SUBMERGENCE			GAS		
	Internodal	length	Internodal	Internodal	length	Internodal	Internodal	length	Internodal
Cultivar	initial	final	elongation	initial	final	elongation	initial	final	elongation
					mm				
HA VIII	44	56	12^a	39	115	76^b	38	153	115^b
HA III	47	65	18^a	47	132	85^b	50	145	95^b
HA II	76	81	5^a	83	152	69^b	73	135	62^b
PG 56	46	48	2^a	42	92	50^b	39	89	50^b
IMN 111	70	92	22^a	59	126	67^b	70	140	70^b
KH	55	64	9^a	60	133	73^b	62	127	65^b
Thavalu	55	61	6^a	49	70	21^b	54	68	14^b
Mars^c	73	88	15^a	66	114	48^b	71	117	46^b
Labelle	—	—	—	25	67	42^b	26	49	23^b
M9^c	—	—	—	42	88	46^b	47	112	65^b
Calrose 76^c	25	28	3^a	26	31	5^a	25	33	8^b
IR8^c	30	34	4^a	12	25	13^b	12	24	12^b

The only exception observed was in Calrose 76 sections subjected to the flow through gas treatment (3% O_2, 6% CO_2, and 1 $\mu l\ l^{-1}$ C_2H_4). No significant difference in the growth of sections under these conditions when compared to the growth of sections placed in air was observed. However, the growth of Calrose sections which were submerged was significantly greater than the growth of Calrose sections treated with flow-through air. Therefore, it is assumed that the lack of significance between gas-treated and air-treated sections is due to variability in plant material and too low a number of replicates, and that the physiology of the growth response to submergence in Calrose involves O_2, CO_2 and C_2H_4 as in all the other cultivars tested.

Effect of age on growth during submergence of three rice cultivars

In order to determine the submergence response over the lifespan of the rice plant, the internodal elongation capacity was analyzed at four ages (5 weeks, 7 weeks, 10 weeks, and 13 weeks of age). Whole plants of HA II, a deepwater cultivar, Mars, a short-statured cultivar, and M9, a semidwarf cultivar were used for these experiments. Figure 1 shows the internodal length per tiller of each cultivar prior to treatment and after 5 d of partial submergence. The same measurements were also made with control plants which were grown in air. Internodal elongation in HA II (Fig. 1A) which had been partially submerged for 5 d was 5-6 times greater than internodal elongation of identical plants grown in air. This was true at all 4 ages tested. In Mars, there was little or no internodal elongation in either partially submerged or control plants at ages five and seven

Figure 13 Effect of partial submergence on the average internodal length per tiller in three rice cultivars. HAII (A) Mars (B) M9 (C). Internodal lengths were measured prior to treatment (solid bars), following 5 d of submergence during which the top 20 cm of the foliage was above the water surface (open bars), or after 5 d of growth in air (striped bars). The plants were 5, 7, 10 or 13 weeks of age on the 1st day of treatment. Each bar represents the average internodal length of 3 tillers per plant and 4 plants per cultivar for each age and teatment (±SD). All treatment effects (age, cultivar, and submergence) and first order interactions (age by cultivar, age by submergence, and cultivar by submergence) were highly significant (AOV; $P=0.01$).


A. HA II
Initial length
Final length, air
Final length, partial submergence
B. Mars
C. M9
80
60
40
20
Total Internodal Length per Tiller (cm)
5
7
10
13
Age of Plant (weeks)

weeks (Fig. 1B). However at both 10 and 13 weeks, internodal elongation of partially submerged plants increased twofold over that of control plants. Like Mars, M9 did not elongate at five and seven weeks of age, but at 10 and 13 weeks, internodal elongation in partially submerged plants was twice that of the air grown plants. In air, elongation of HA II was about 0.5 cm d^{-1}. In contrast, internodal growth in air-grown plants of Mars and M9 did not occur until about 10 weeks of age. Once this growth began, it reached a rate of 4 cm d^{-1}. The difference in the age at which internodal elongation occurs in various cultivars is confirmed by a highly significant interaction between age and cultivar (AOV; $P= 0.01$).

Following dissection, all plants were observed for panicle initiation and internode expansion, and the ages at which each of these processes began were noted. Internodal expansion was not observed in air-grown plants of Mars or M9 until 10 weeks of age. In HA II, the internodes began to telescope at 4-5 weeks of age. Panicle initiation in M9 was observed at 10 weeks of age. Panicle initiation was observed in Mars at 13 weeks of age. No panicle development was seen in HA II up to 13 weeks of age.

DISCUSSION

The elongation response to submergence in 12 rice cultivars

In an attempt to develop an assay which would distinguish deepwater rice from other cultivars based on elongation ability, a comparison of the elongation capacity of 12 rice cultivars under conditions of partial submergence was made. These cultivars included several types of rice with different growth habits, namely, deepwater, tall, short-statured and semidwarf cultivars. Whole plants of all cultivars tested were capable of elongating rapidly when partially submerged at 12 to 13 weeks of age (Table 1). This result demonstrates the ability of semidwarf and short-statured rice types to elongate rapidly when partially submerged. It also implies that the deepwater character involves more than the simple capacity for internodal elongation.

At age 12 to 13 weeks, some of the nondeepwater cultivars had initiated reproductive development. Therefore, the possibility existed that the elongation process might differ physiologically between cultivars undergoing reproductive development and those growing vegetatively. An analysis of the elongation of excised stem sections under several physiological conditions gave similar results for all twelve cultivars tested (Table 5). The increase in internodal elongation observed in whole plants was also seen in stem sections of all rice types which had been submerged or treated with 3% O_2, 6% CO_2, and 1 $\mu l\ l^{-1}$ C_2H_4 in a flow-through system. The data in Table 5 are in agreement with the results of Raskin and Kende (1984a) and indicate that the elongation response of each of these cultivars to partial submergence is mediated by C_2H_4 and that the physiological response of each of these cultivars to partial submergence is similar. Our results with whole plants of the deepwater cultivars agree with those of Métraux and Kende (1983). However, our results with the semidwarf, M9, conflict with theirs in that we obtained rapid internodal elongation in this cultivar when partially submerged and they did not. The difference betweeen our experiment and theirs is that they tested 5- to 6-week-old plants, while we tested 12- to 13-week-old plants. In addition to this discrepancy, some of the nondeepwater cultivars we tested had already begun reproductive development. Therefore it was unclear as to what extent reproductive physiology might influence the elongation process in these cultivars or what role the age of the plant might play in this process.

Development of elongation ability with age

In order to determine how elongation capacity changed with age in different types of rice, a study of internodal elongation in whole plants was conducted at four different ages (Figure 12). The ages at which internodal expansion and panicle development began were also noted. For these experiments, three cultivars were selected, one deepwater cultivar, HA II, one short-statured cultivar, Mars, and one semidwarf, M9. In all three cultivars, the age at which internodal expansion began in air-grown plants correlated with the age at which the capacity for rapid internodal elongation developed in partially submerged plants. In HA II, the deepwater cultivar, both of these characteristics developed at 4 to 5 weeks of age (Figure 12A). In Mars and M9, these characteristics develop between 7 and 10 weeks of age (Figures 12B and 12C). Thus, it appears that rapid internodal elongation in response to submergence (and also a gas mixture containing ethylene) will occur in any rice cultivar provided that internodal growth in air has already begun.

Kihara et al. (1962) made a comparison of "weak floaters" and "pronounced floaters" when exposed to five different rates of increase in water depth. They found that weak floaters would elongate under conditions in which the water level was increased by no more than 3.1 cm per day, whereas pronounced floaters could elongate in water rising by as much as 9.3 cm per day. These experiments were begun on plants that were 9 weeks of age and continued through 19 weeks of age. If the short-statured and semidwarf cultivars of our experiments are compared to the weak floaters it appears likely that internodal expansion in these plants had just begun at the time the experiment began, or began shortly thereafter. The slow increase in water depth would have allowed these plants to develop the capacity for internodal elongation before the water level was high enough to drown them. Whereas weak floaters exposed to waters rising more quickly would have drowned.

The deepwater character

Although it has been observed that nondeepwater rice cultivars as well as the deepwater cultivars are capable of rapid internodal elongation, a number of differences between these cultivars have been noted. Internodal expansion in deepwater cultivars begins much earlier than in nondeepwater cultivars. This allows a longer period in the life-span of the deepwater plants during which elongation may occur. Panicle initiation in deepwater rice is photoperiodically controlled, whereas the other cultivars are photoperiodically insensitive so that flowering occurs at a genetically determined stage in the plant's development. Since flowering terminates vegetative growth, the time at which it occurs will determine the length of the vegetative growth phase. Effectively, this again restricts the duration of the period over which nondeepwater rice is capable of internodal elongation. From our data, it is unclear whether or not elongation capacity in nondeepwater cultivars is strictly linked to flowering. M9 was found to have begun reproductive development at both ages at which it was capable of elongating. Mars, however, had only begun panicle development at the later of the two ages at which it elongated. Perhaps in nondeepwater rice a range of possibilities exist with regard to this particular characteristic. It is very clear, though, that in deepwater rice, the ability to elongate is not physiologically related to reproductive development.

Another difference observed between deepwater and nondeepwater cultivars is the rate at which elongation occurs in plants which have not been submerged (Figure 12). Nondeepwater cultivars grew about twice as fast as deepwater cultivars in air. This indicates that elongation in air must be regulated in some way in deepwater cultivars which does not occur in the other cultivars.

In comparing and analyzing the elongation capacity of nondeepwater and deepwater rice cultivars, many researchers refer to the ability of internodes to elongate rapidly as an "indispensable character" of deepwater rice (Hanada, 1983). Others have referred to elongation genes which may be bred from a deepwater cultivar into higheryielding semidwarf cultivars (Swaminathan, 1984). Our results indicate that there is no difference between deepwater and other rice cultivars in their short-term capacity for internodal elongation. Rather, it is the duration of the period over which this capacity can be expressed which differs between these cultivars. This also means that the cultivars differ in the regulation of elongation capacity.

A model for the regulation of internodal elongation

It has been shown that submerged or ethylene-treated deepwater rice does not elongate if the level of endogenous GA is reduced using TCY, a specific inhibitor of GA biosynthesis. Supplying the plant with exogenous GA_3 in the presence of TCY restores the elongation response to submergence (Raskin and Kende, 1984b). GA is also known to be directly involved in the induction of bolting and flowering in a number of rosette plants (Jones and Zeevaart, 1980; Koorneef and van der Veen, 1980). These results have led to an hypothesis on the regulation of internodal growth in rice plants. We assume that deepwater rice is able to respond to submergence (or ethylene) at an early stage because GA biosynthesis commences early in the vegetative growth phase of the plant. This would be manifested by early internodal elongation in air (Figure 12A). In contrast, other rice types may behave more like rosette plants. For much or all of their vegetative growth phase, internodes are telescoped at the base of the plant. Internodal elongation or "bolting" occurs usually at or just preceding panicle development. According to this model, GA biosynthetic activity should be very low at the "rosette" stage, but should increase prior to internodal elongation or "bolting". Thus, the time at which internodal elongation can be stimulated by submergence or ethylene would be determined by the ability of the plant to produce GA and the age at which this growth phase is ended is determined by reproductive development.

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