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The Sesbania rostrata Early Nodulin Gene SrEnod2 As A Marker For Cytokinin Signal Transduction

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David L. Silver

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THE *SESBANIA ROSTRATA* EARLY NODULIN GENE *SRENOD2* AS A MARKER FOR CYTOKININ SIGNAL TRANSDUCTION

By

David L. Silver

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ABSTRACT

THE *SESBANIA ROSTRATA EARLY* NODULIN GENE *SRENOD2* AS A MARKER FOR CYTOKININ SIGNAL TRANSDUCTION

By

David L. Silver

The *Sesbania rostrata* early nodulin gene *SrEnod2* encodes a proline-rich protein which is expressed tissue-specifically in the nodule. Additionally, the *SrEnod2* mRNA accumulates in roots in response to cytokinin application. This accumulation occurs in the absence of infection by the microsymbiont *Azorhizobium caulinodans*. Nuclear run-on assays using isolated root nuclei indicated that *SrEnod2* mRNA accumulation in response to cytokinin application occurs posttranscriptionally. Analysis of nuclear RNA revealed that this cytokinin enhancement occurs primarily in the cytoplasm and not in the nucleus. Application of the translational inhibitor, cycloheximide, was found to cause its rapid decay. It was also found that okadaic acid and staurosporine, inhibitors of protein phosphatases and kinases, respectively, inhibited cytokinin enhancement of *SrEnod2* mRNA accumulation. *Arabidopsis thaliana* was used to study the mechanism of cytokinin-mediated *SrEnod2* mRNA accumulation. It was demonstrated that a chimeric *SrEnod2* 5'-*gus-SrEnod2* 3'construct can be specifically induced by cytokinin and expressed in *Arabidopsis* roots in the vascular tissue and emerging lateral roots, which closely resembles the pattern seen in the legume *Lotus japonicus*. In addition, expression was found to be localized to the shoot apical meristem, newly expanding leaves, and trichomes of *Arabidopsis*. The observed mode of regulation was shown to be dependent on the *SrEnod2* 3' region. A cross between *Arabidopsis* plants harboring the *SrEnod2* 5'-*gus-SrEnod2* 3' construct and the cytokinin-resistant mutant *cyr*1 (Deikman and Ulrich, 1995) yielded F_2 progeny in which GUS activity could not be induced upon cytokinin treatment. This data provides genetic evidence that the *SrEnod2* 5'-*gus-SrEnod2* 3'construct is

regulated by a conserved cytokinin signal transduction pathway. The underlying hypothesis is that conserved regulatory protein(s) is interacting with the *SrEnod2* 3' region which regulates root/apical shoot meristem-specific expression and regulation by cytokinin. To test this idea genetically, the *SrEnod2* 3' region was overexpressed under the control of the CaMV 35S promoter in *Arabidopsis*, and the resulting transgenic plants were crossed to plants harboring the *SrEnod2* 5'-*gus*-*SrEnod2* 3'construct. The assumption was that if the *SrEnod2* 3'region can titrate out an important regulatory factor(s), then expression of the *gus* reporter construct should be downregulated. Five out of six F_2 plants analyzed from this cross did not show cytokinin-enhanced GUS expression, thus supporting the existence of a factor(s) which interact with the *SrEnod2* 3' region in *Arabidopsis*. In addition, a genetic screen for the isolation of the gene(s) which may encode this factor(s) is presented.

To Irma

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Chapter 1

INTRODUCTION

The plant hormone cytokinin comprises a group of growth substances which are derivatives of adenine first isolated by Miller et al. (1955) from autoclaved herring sperm DNA and later by Letham (1963) from plants. The aim of this chapter is to present information gathered from key studies indicating the roles of cytokinins in plant development and gene expression, with the goal of presenting a perspective on cytokinin action. However, this chapter will not cover the metabolism and biochemistry of cytokinins, as this information can be found in a recent review (Brzobohaty et al.,1994). Here, I will focus primarily on two aspects of cytokinin action in plants, the first being the effects of cytokinins on plant development. This section will focus on studies involving the application of cytokinins and other hormones to plants, which may share common signal transduction pathways with cytokinins. Second, I will discuss the *Agrobacterium* system and transgenic expression studies using *Agrobacterium* cytokinin biosynthetic genes. Third, information will be presented on plant cytokinin response mutants. Fourth, the molecular action of cytokinins will be reviewed. Lastly, nodule development and the roles of cytokinin, as well as other hormones, in nodule organogenesis will be discussed.

The pleiotropic effects of cytokinins on plant development

One mode of studying cytokinin action has been through the application of cytokinin to intact plants or isolated tissues. Cytokinins were first identified by their ability to stimulate cell division in tobacco pith cells (Miller et al., 1955; Miller et al., 1956). In these studies, it was shown by Miller and co-workers that auxin stimulated cell elongation, and that upon the addition of kinetin, a synthetic cytokinin, the pith cells began to divide. This work was the first to demonstrate the involvement of more than one phytohormone in inducing a biological response. This is now recognized as a general feature of plant hormone action. After the original work, demonstrating the influence of cytokinin on cell division, Skoog and co-workers set out to test the effects of cytokinins on plant

development. It was discovered that cytokinins influence the formation of flowers and fruits, activities of enzymes (metabolism), and the appearance of chloroplasts. Cytokinins also delay the onset of leaf senescence, as well as play a role in the resistance to adverse environmental stresses. Even today the mechanisms by which cytokinins influence these processes are not well understood.

For the past 40 years, the sole method available to studying cytokinin action has been the application of cytokinin to excised tissues, cells, and intact plants. Although the results obtained from such direct application experiments are often correlative and circumstantial, these types of experiments have built the framework for current work on cytokinin action and have led to the elucidation of the general characteristics of cytokinin action in plants. The result of the application of cytokinin depends largely on the concentration and type of cytokinin used and the kind of plant tissue it is applied to. In general, effects observed include the release of axillary buds from apical dominance, the accumulation of anthocyanins, the inhibition of root and hypocotyl growth, the greening of etiolated leaves, and a delay in senescence.

The effects of cytokinin application on plant development is not always directly due to cytokinin, but may be through the effects of ethylene and light. It is known that cytokinins stimulate ethylene production (Fuchs and Lieberman, 1968; Radin and Loomis, 1969). Bertell and Eliasson (1992) demonstrated in pea roots that cytokinin application inhibited root elongation, the formation of lateral roots, and stimulated swelling of the root tips. These effects were obtained at a benzylaminopurine (BAP) concentration as low as 0.01 μM. They further demonstrated that BAP caused up to a four-fold increase in ethylene levels in roots. The application of cobalt ions to inhibit ethylene production counteracted both the inhibition of elongation and the swelling at the root tip caused by BAP. In addition, it was shown that BAP treatment increased the levels of IAA per root tip approximately two-fold, whereas treatment with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) caused a 50% reduction in IAA levels. This

study indicates that cytokinins can influence growth processes in roots via multiple pathways, including through ethylene and auxin. Cytokinin-induced radial expansion of hypocotyls has also been shown to be cytokinin-mediated (Corriveau and Krul, 1986). Cytokinins produce effects in dark-grown *Arabidopsis* seedlings which are similar to the effects caused by ethylene, known as the "triple response." The "triple response" is characterized by the inhibition of hypocotyl growth, the curling of the apical hook, and the expansion of the hypocotyl base (Crocker et al., 1913). In addition, ethylene inhibits primary root elongation (Guzman and Ecker, 1990). This "triple response" has been exploited in the isolation of ethylene resistant mutants (Guzman and Ecker, 1990), and mutants which produce a constitutive triple response (Kieber et al., 1993). The constitutive mutants are divided into two groups, based on whether or not the phenotype is repressed by inhibitors of ethylene biosynthesis or action. The *ctr* mutants are not repressed by inhibitors, whereas the ethylene overproducer (*eto*) mutants are repressed (Guzman and Ecker, 1990). Cary et al. (1995) tested genetically, by the use of mutants in ethylene responses and action, the hypothesis put forth by Lieberman (1979) that cytokinin action is coupled to ethylene action in seedlings. They found that the inhibitory effects of BAP on root and hypocotyl elongation were partially blocked by the action of ethylene inhibitors or in the ethylene-resistant mutations *ein1-1* and *ein2*-1 (Guzman and Ecker, 1990). Furthermore, the finding that cytokinin and ethylene responses are coupled was reinforced by the demonstration that the cytokinin-resistant mutant *ckr1* (Su and Howell, 1992) is allelic to *ein2* (Cary et al., 1995).

Cytokinin and light interact in processes such as anthocyanin accumulation (Kasemir and Mohr, 1982), betacyanin synthesis (Koehler, 1972), hypocotyl elongation (Cohen et al., 1991), and chloroplast development (Feierabend and de Boer, 1978). From these studies, it appears that cytokinin can mimic some of the effects produced by light in photomorphogenesis, but it remained unclear whether cytokinin action was dependent on light or vice versa, or whether they act independently. Tong et al. (1983) showed using

mustard plants that the effects of cytokinin and light are additive with respect to increases in cotyledon size, carotenoid contents, levels of glyceraldehyde-3-phosphate dehydrogenase, and anthocyanin formation. These effects were observed regardless of the order of treatment with cytokinin and light, or a simultaneous treatment with both. More recent work has provided genetic evidence for the independence of light- and cytokinin-mediated action on photomorphogenesis (Chory et al., 1994; Su and Howell, 1995). A class of *Arabidopsis* mutants has been identified which shows many characteristics of light-grown plants when grown in complete darkness. These mutants have been designated *det* (de-etiolated) because of the de-etiolated phenotype in the dark, as compared with wild-type seedlings (Chory et al., 1989, 1991b; Cabrera et al., 1993). *det1* and *det2* mutants grown in the light are small and have reduced apical dominance and fertility as compared with wild-type plants (Chory and Peto, 1990; Chory et al., 1991). This indicates that the gene products of the *det1* and *det2* genes play a role in light-grown as well as dark-grown plants. Recently, the *det2* gene has been cloned and shown to have significant homology with mammalian steroid 5α-reductases, which may function in the brassinolide biosynthetic pathway (Li et al., 1996). In support of this idea, the application of brassinolide to dark-grown *det2* mutants partially suppressed the mutant phenotype (Li et al., 1996). Cytokinins applied to wild-type dark-grown seedlings resulted in a phenocopy of the *det1* mutant, which includes inhibition of hypocotyl elongation, promotion of cotyledon expansion and leaf development. In addition, thylakoid-containing plastids are formed in the cytokinin treated seedlings in much the same fashion as those formed in *det1* mutants (Chory et al., 1989). The light-regulated genes *cab*, *chs*, and *rbcS* are also seven- to eight-fold more active in the cytokinin-treated dark-grown seedlings, as compared to untreated seedlings. This enhancement of the expression of light-regulated genes was also found in dark grown *det1* seedlings (Chory et al., 1994). Interestingly, the cytokinin levels in wild-type and *det1* seedlings (dark- or light-grown) were found to be the same. However, in a detached leaf experiment to measure senescence, *det1* and *det2* detached

leaves had a significant delay in senescence, as compared to wild-type leaves. In addition, *det1* and *det2* root and leaf explants in tissue culture continuously produced callus while wild-type explants formed roots under the hormone conditions used, suggesting that *det1* and *det2* have a different requirement for cytokinins to initiate a normal developmental response. This work indicates that cytokinin can partially overcome the requirement of light to induce leaf and chloroplast development, as well as the expression of light-induced genes, and that a simple additive relationship of light and cytokinin may not be true, at least for the process of de-etiolation in *Arabidopsis*, but may take place in other photomorphogenetic processes. Ultimately, the action of light and cytokinin appears to be connected by some signal transduction pathways.

Cytokinin and light both inhibit hypocotyl elongation in a Ca^{2+}-dependent manner (Cohen et al., 1991), and the effects of cytokinin are mediated primarily through the action of ethylene (Cary et al., 1995). It was recently shown by Su and Howell (1995) that the effects of ethylene and cytokinin and light on the inhibition of hypocotyl elongation are independent and additive. This was demonstrated by the use of the *Arabidopsis* hypocotyl elongation mutants (*hy*) (Koornneef et al., 1980; Liscum and Hangarter, 1993) which comprise a group of mutants insensitive to light. A number of genes represented by these mutants have been cloned (Parks and Quail, 1991; Somers et al., 1991; Reed et al., 1993; Ahmad and Cashmore, 1993; Koornneef et al., 1980; Chory et al., 1989; Chory, 1992; Parks and Quail, 1993). The inhibition of hypocotyl elongation by the application of cytokinin was similar in the *hy* mutants as in wild-type plants, indicating that cytokinin acts independently of light. In contrast, the *ckr1/ein2* mutant, which exhibits a normal response to light, did not respond to cytokinin by inhibition of hypocotyl elongation, indicating that the action of cytokinin in this process is coupled to ethylene action.

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A role of cytokinin in flowering and flower development

The identity of the floral stimulus, or "florigen" is unknown. However, many factors are known which influence the transition to flowering, and among them are carbohydrate, light, and cytokinin. The mustard plant *Sinapis alba* has been used as a model to study the transition to flowering (Bernier et al., 1977). It has been demonstrated that a long day treatment will stimulate *S. alba* to flower, but a single low dose of cytokinin to the apical meristem will evoke a partial flowering phenotype (Bernier et al., 1977; Havelange et al., 1986), consisting of an increase in the mitotic index of meristem cells, halving of the size of DNA replication units, and the splitting of vacuoles (Bernier et al., 1977; Havelange et al., 1986; Houssa et al., 1990). In addition to cytokinin, sucrose may play a signaling role in the evocation to flower. During the exposure of *S. alba* plants to a long-day light treatment, sucrose accumulates very early in the apical meristem of induced plants (Bodson and Outlaw, 1985). This increase in sucrose precedes mitotic activation. The mobilization of sucrose stores to the apical meristem may be related to the export of cytokinin from the roots, as suggested by the work of Bernier et al. (1993) who showed that the removal of phloem by girdling at 8 h after the start of the inductive long day treatment inhibited the transition to flowering, but surprisingly a treatment of cytokinin to the apical meristem at 16 h after girdling reversed this inhibition. This result indicates that the mobilization of cytokinin from root to shoot may play a role in the induction of flowering. It was also demonstrated that a transient increase in cytokinin levels in roots occurs 1 h after the long day treatment, and that elevated levels of cytokinin in mature leaves could be detected 16 h after induction with a long-day treatment (Bernier et al., 1981). In an experiment designed to abolish the postulated export of cytokinins from root to shoot, plants were grown in 100% relative humidity to prevent transpiration, which is believed to be the force of cytokinin movement in plants. This treatment completely

abolished the induction to flower (Bernier et al., 1993), although a 100% relative humidity treatment may have many pleiotropic affects.

The application of cytokinin to the developing inflorescence of *Arabidopsis* has been shown to result in increases in floral organ number, formation of abnormal floral organs and production of secondary floral buds in the axils of sepals (Venglat and Sawhney, 1996). These abnormalities resemble the *Arabidopsis* floral mutants *clv1*, *ap2*, *ap3* and *ap1* (Leyser and Furner, 1992; Okamuro et al., 1993). Although cytokinin was applied at a high local concentration for a relatively long time period, this work suggests that cytokinins play a role in normal floral organ development, perhaps by affecting the regulation of floral organ identity genes. Transgenic plant studies will be presented later which further support this idea.

Apical dominance

One of the known effects of cytokinin action in plants, other than effects on cell division, is the role cytokinins seem to play in regulating apical dominance. The application of cytokinin to axillary buds stimulates bud growth in many plant species including apple, *Cuscuta*, *Macadamia*, oats, peas and soybeans (Cline, 1991). This raises the question whether there is a correlation between cytokinin levels in buds and their ability to grow and develop. Sossuountzov et al. (1988) have shown by the use of immunolabelling of cytokinins that in the *Craigella* sideshootless tomato mutant (*Cls*), which does not have axillary bud growth, the highest levels of cytokinins were found in the apical bud closest to the meristem and decreased basipetally in the normal isogenic parental line. The *Cls* mutant has been shown to have strikingly lower levels of cytokinin in the terminal apical bud and in all subapical buds, whereas the root apical meristem had equal levels of cytokinin as compared to the parental line. This work should be interpreted with caution, however, as only the levels of one type of active and two types of inactive

cytokinins were measured. In a separate study using the aquatic fern *Marsilea drummondii* A. Br., it was shown that the apical meristem contained the highest levels of IAA and cytokinins. Upon decapitation of *M. drummondii* A. Br. plants, the subapical bud will be the most rapidly growing bud (Pilate et al., 1989). In non-decapitated plants, the highest levels of iPA, a precursor of zeatin, are found in this bud (Pilate et al., 1989). This suggests that the subapical bud is in a "standby state" for release from apical dominance, and that this process depends on the local cytokinin levels.

Leaf senescence

Cytokinins have also been implicated in the control of leaf senescence (Richmond and Lang, 1957; Nooden and Leopold, 1978). Little is understood of the hormonal involvement in sequential leaf senescence, which is the senescence from older, lower leaves toward the younger leaves near the apex. Singh et al. (1992) have shown that the levels of cytokinin bases and cytokinin ribosides are lower in older than upper younger leaves in tobacco. Moreover, application of cytokinin to leaves was found to be effective in retarding senescence, independent of metabolite mobilization in leaves (Singh et al., 1992). It has also been observed that the application of nitrogenous compounds to tobacco leaves greatly increases cytokinin levels, and retards leaf senescence (Singh et al., 1992). These studies lend support to the idea that endogenous cytokinin levels are involved in the control of sequential leaf senescence. Recent work using transgenic plants indicates that this is indeed the case (see below).

The *Agrobacterium* paradigm

The morphological and physiological effects of cytokinin application have been the basis for understanding cytokinin action in plants. However, in order to better understand

cytokinin action in plants, the need arose to understand the effects of changing endogenous levels of cytokinins on plant processes. The genes responsible for plant cytokinin biosynthesis have not been isolated, even though biosynthetic enzyme activities have been described (Chen and Melitz, 1979; Chen and Leisner, 1984). The discovery that *Agrobacterium tumefaciens* and *A. rhizogenes* produce cytokinins has proven immensely valuable for the *in vivo* manipulation of hormone levels in plants. *A. tumefaciens* is the causative agent of crown gall tumor disease, which is the formation of a hyperplasia, although in some plants shooty tumors are formed (Morris, 1995). It was first shown by Braun (1958) that cell division factors are responsible for the formation of hyperplasias caused by *A. tumefaciens*. Work by Willmitzer et al. (1983) gave the first indication that *A. tumefaciens* contained a gene responsible for hormone production which contribute to alter hormone levels in crown galls. It was subsequently shown that *A. tumefaciens* contains genes for auxin and cytokinin biosynthesis on a large plasmid, termed the Ti plasmid (Akiyoshi et al., 1983; Barry et al., 1984; Klee et al., 1984; Schröder et al., 1984; Kemper et al., 1985; Yamada, et al., 1985). *Agrobacterium* is able to transfer these genes into the plant nucleus. The mechanism of *Agrobacterium*-mediated transformation will not be discussed here as this information can be found elsewhere in more comprehensive reviews (Lessl and Lanka, 1994; Zupan and Zambryski, 1995; Tinland and Hohn, 1995).

It can be seen from Table 1.1 that both phytopathogens and symbionts produce cytokinins. The phytopathogens can be divided into two fundamentally distinct groups based on their mode of forming hyperplasias. One group, comprised of *A. tumefaciens* and *A. rhizogenes* (although rhizogenes does not from hyperplasias, but proliferation of malformed roots on stems), transform dicotyledonous plants (De Cleene, 1988) with a region of the Ti-plasmid DNA, the T-DNA, (Zaenen et al., 1974), harboring the genes necessary for auxin and cytokinin biosynthesis, or genes which enhance the sensitivity of the plant cell to hormones (Spena et al., 1987; Estruch et al., 1991a). Therefore, after T-DNA transfer the presence of the bacteria is no longer required for the

Table 1.1. Cytokinin-Producing Bacteria

Bacteria	Characteristics	Cytokinins	References
Agrobacterium tumefaciens	crown gall tumors	Z, [9R]Z, iP, [9R]iP	Scott and Horgan, 1984
Agrobacterium rhizogenes	hairy root disease	Z	Akiyoshi et al., 1987
Pseudomonas savastanoi	olive galls	Z, [9R]Z, 1'MeZ, 1''Me[9R]Z	Surico et al., 1985
Pseudomonas solanacearum	bacterial wilt	Z	Akiyoshi et al., 1987
Pseudomonas amygdali	almond canker	[9deoxyR]Z, Z, dHZ, iP	Iacobellis et al., 1990
Rhodococcus faciens	witches broom disease	cZ, iP, [9R]iP	Morris et al., 1991
Bradyrhizobium japonicum	symbiont nodules	2ms[9R]Z, [9R]iP, 2ms[9R]iP	Sturtevant and Taller, 1989
Azorhizobium caulinodans	symbiont nodules	Kinetin equivalents	Taller
Rhizobium sp	symbiont nodules	Z, [9R]Z, iP, [9R]iP	Badenoch-Jones et al., 1987
Rhizobium IC3442	symbiont nodules	Z, iP	Upadhyaya et al., 1991
Frankia	symbiont nodules	[9R]iP	Stevens and Berry, 1988
Erwinia herbicola pv *gypsophilae*	gypsophila galls	Z, [9R]Z, iP, [9R]iP	Lichter et al., 1993
Azotobacter vinelandii	rhizosphere associated	Z, iP, [9R]iP	Taller and Wang, 1989
Azotobacter chroococcum	rhizosphere associated	cZ, Z, [9R]Z, [9R]dHZ, iP, [9R]iP	Nieto and Frankenberger, 1989
Vibrio sp	free living, marine	iP, [9R]iP	Maruyama et al., 1988

*Table was adapted from Morris RO 1995 in PJ Davies ,eds, Plant Hormones, Kluwer Academic Publishers, 318-339

maintenance of the grown gall or root proliferation. The second group is comprised of bacteria which do not introduce DNA into the plant cell nucleus, but secrete hormones and therefore need to be present in close association with the plant for the maintenance of the gall tissue. These bacteria include *Erwinia herbicola*, *Pseudomonas savastanoi* and *Rhodococcus fascians*.

Cytokinin biosynthesis by *A. tumefaciens* is controlled by a single gene, called *tmr*. These genes encode proteins catalyzing the rate-limiting step in cytokinin biosynthesis, namely the transfer of an dimethylallylpyrophosphate onto the purine ring of adenine. This gene, also known as the isopentyl transferase gene (*ipt*), is regulated by a plant promoter and has plant termination and polyadenylation sequences. Therefore, the transformed plant is capable of synthesizing cytokinins independent of bacterial control. The *ipt* gene has provided a way to test *in vivo* the effects of altering cytokinin levels in plants, either at the whole-plant level or in individual organs, tissues and cells.

A. rhizogenes is the etiological agent of the hairy-root disease (Riker et al., 1930). This disease develops as a result of expression of several oncogenes located on the T-DNA of Ri plasmids (Chilton et al., 1982). Unlike *A. tumefaciens*, *A. rhizogenes* does not transform the plant cell by transferring auxin and cytokinin biosynthetic genes, but introduces so called *rol* genes (named *rol* for root loci) (White et al., 1985). The *rol A*, *B*, and *C* genes have been shown to be necessary for the induction of hairy roots in tobacco plants (Jouanin et al., 1987; Schmulling et al., 1988). It has been demonstrated *in vitro* that the Rol C protein hydrolyzes cytokinin glucosides (Estruch et al., 1991a). Although recently Faiss et al. (1996) failed to detect changes in the endogenous pool of different cytokinin glucosides *in planta* in *rol C*-expressing tobacco plants. They propose that other low molecular weight signals, such as oligosaccharins, may be the *in vivo* substrate for the Rol C protein, rather than cytokinin glucosides. The expression of the *rol B* gene in plants has been implicated in auxin action (Cardarelli et al., 1987; Filippini et al., 1994). The substrate for the Rol B protein has never been conclusively determined.. However,

recently it has been demonstrated *in vitro* that recombinant Rol B protein has tyrosine phosphatase activity and is localized in the plasma membrane of transformed plants (Filippini et al., 1996). These data indicate a role of kinase/phosphatase cascades in auxin signal transduction. Therefore, the *rol* genes may interfere with plant hormone metabolism rather than *de novo* synthesis, as observed in *A. tumefaciens* transformed plants. Further *rol C* expression studies in transgenic plants will be presented below.

Altering cytokinin levels in transgenic plants

The effect of applied cytokinin on plant processes may not be the same as that of endogenous cytokinin. Problems of cytokinin uptake, transport, metabolism, and tissue/cell-specific hormone concentrations and sensitivities may produce numerous secondary effects. To avoid these problems, many groups have utilized the cytokinin biosynthesis gene from *A. tumefaciens* to manipulate endogenous cytokinin levels in transgenic plants, to test the effects of altered hormone levels on plant physiology, development, and gene expression. Overall, most of these studies in transgenic plants have confirmed the previous results from experiments using applied cytokinins.

In the first of these studies, the *ipt* gene was placed under the control of the CaMV 35S promoter to achieve high levels of endogenous cytokinins (Ooms et al., 1983; Binns et al., 1987b; Smigocki and Owens, 1988; Smigocki and Owens, 1989). All transformants displayed extreme phenotypes correlated with cytokinin action, such as profuse shoot development and little or no root development. Aside from these classic phenotypes, the transgenic tobacco plants constitutively expressing *ipt* also showed auxin-autonomous growth. Tobacco cell lines in which the *ipt* gene was overexpressed were also found to be auxin autonomous, while non-transformed tobacco cells grown in the presence of cytokinin are auxin-requiring (Binns et al., 1987). This indicates that applied cytokinin does not completely mimic the effects of the endogenous production of cytokinin. Smigocki and

Owens (1989) measured an increase of cytokinin levels of up to 300-fold in transgenic tobacco plants harboring a CaMV 35S-*ipt* fusion with no significant increase in IAA levels. In addition, a 24- to over a 2,000-fold increase in cytokinin-to-auxin ratios was observed, which may explain the morphogenic changes and the auxin-autonomous growth of tissues *in vitro*.

To better control the levels of cytokinins *in planta*, which would allow the regeneration of transgenic plants with roots, experiments were conducted using inducible promoters. Medford et al. (1989) placed the *ipt* gene under the control of the maize heat shock promoter *hsp*70 and generated transgenic tobacco and *Arabidopsis* plants harboring this construct. They found that under noninducing conditions, the levels of zeatin riboside, and zeatin riboside 5'-monophosphate increased 3 and 7 times, respectively. After heat induction, the levels of zeatin, zeatin riboside, and zeatin riboside 5'-monophosphate were found to increase 52-, 23-, and 2-fold respectively. The small increase in cytokinin levels in plants under non-inducing conditions already caused dramatic affects on plant development, such as reduction in stature, release of axillary buds, generation of smaller stem and leaf areas, reduced xylem production, and the generation of a reduced root system with short and thicker roots with more root hairs. Heat treatment of the transgenic plants did not lead to further alterations in plant development, despite large increases in cytokinin levels. These results indicate that there is a threshold level at which cytokinins are perceived and act. Similar results were obtained by Schmulling et al. (1989) and Smigocki (1991) using the *Drosophila melanogaster hsp*70 promoter. Moreover, Ainley et al. (1993) performed similar experiments using the soybean heat shock promoter, which was shown to be tightly regulated and mediated a high level of cytokinin production at elevated temperatures (Ainley and Key, 1990). In contrast to experiments using the maize and *Drosophila* heat shock promoters, transgenic plants harboring the *ipt* gene under the control of the soybean heat shock promoter showed no alterations in phenotype under non-inducing temperatures, but exhibited phenotypic alterations only after heat treatment. Heat

treatment had a strong effect only on developing leaves from transgenic plants, but not on fully developed leaves, indicating that certain developing tissues may be susceptible to cytokinin, whereas mature tissues may not be.

One additional phenotypic alteration, which has not previously been reported as a cytokinin effect, is the production of leaf chlorosis. The chlorosis effect is in direct contrast to the observed increase in greening of leaves upon cytokinin treatment (reviewed by Thomas and Stoddart, 1980). The authors explain that chlorosis may be due to the inhibition of vascular tissue development in cytokinin-overproducing transgenic plants, as observed by Medford et al. (1989). This inhibition may lead to a limitation in the transport of assimilates to expanding leaves and could severely limit the development of leaves, as well as cause chlorosis. Smart et al. (1991), using the soybean heat shock promoter fused to the *ipt* gene in tobacco, showed that transgenic plants at non-inducing temperatures did show marked phenotypic differences as compared to untransformed plants. These plants were shorter in stature, had an increase in side shoot production, and remained green for longer time periods than untransformed plants. Differences were more pronounced after several heat shock treatments. These results again suggest a role of cytokinins in the delay of leaf senescence. The developmental and morphological alterations seen in these transgenic plants complicate the interpretation of a direct role of cytokinins in leaf senescence. In an elegant approach to address this problem Gan and Amasino (1995) placed the *ipt* gene under the control of a senescence-specific promoter, SAG12, in transgenic *Arabidopsis* plants. The idea was to autoregulate the production of cytokinin in leaves during senescence. The SAG12 (Senescence-Associated Gene) was isolated as a gene specifically expressed during leaf senescence (Lohman et al., 1994). Transgenic plants did not exhibit any developmental alterations. As wild-type, non-transgenic plants aged, leaf senescence progressed sequentially from the bottom to the top leaves. In contrast, identically aged transgenic plants showed no sign of leaf senescence. In a detached leaf assay, transgenic plant leaves showed no sign of senescence after more than

40 days, whereas wild-type plant leaves began to senescence after 10 days. The autoregulatory nature of pSAG12-*ipt* expression was analyzed by placing the *gus* reporter gene under the control of pSAG12 promoter and monitoring *gus* expression during senescence in plants with or without the pSAG12-*ipt* gene construct. The levels of GUS activity in pSAG12-*gus* plants, increased only in leaves undergoing senescence. In the pSAG12-*gus*/pSAG12-*ipt* plants GUS levels were over 1,000-fold lower. Thus, the pSAG12 promoter is strongly autoregulated. This work confirms that cytokinins delay leaf senescence and that cytokinins are able to negatively affect the senescence program through the regulation of senescence-specific genes.

Altering cytokinin sensitivity

The overexpression of the *rol* C gene in transgenic tobacco and potato plants shows similar effects as the overexpression of the *ipt* gene (Schmulling et al., 1988; Fladung, 1990). Phenotypic changes observed include the reduction of apical dominance, generation of male sterile flowers, reduced leaf pigment content (seen only in the *ipt* study by Ainley and Key, 1990) and, surprisingly, dwarfism. The reduced leaf pigment content has been used as a phenotypic marker to determine whether the Rol C protein acts in a cell-autonomous fashion. Transgenic tobacco plants, harboring the *rol* C gene under the control of the CaMV 35S promoter interrupted by an Ac element, gave rise to sections of yellowing cells on leaves upon Ac excision. This type of clonal analysis indicates that the *rol* C product acts in a cell-autonomous fashion (Spena et al., 1989).

It was shown that the overexpression of *rol* C in transgenic potato, but not in tobacco plants, led to a 4-fold increase in the free cytokinin content, which may explain some of the phenotypic alterations seen in potato plants (Schmulling et al., 1993). Interestingly, alterations in the levels of other hormones were found, such as an up to 50% reduction in ABA content in leaves and a 100% increase in roots. The dwarfism phenotype

could be correlated with a 28-60% reduction in GA_1 in the apical shoots of both transgenic tobacco and potato plants, since this phenotype could be suppressed by the application of GA_1 to the apical shoot of *rol C* transgenic plants (Schmulling et al., 1993). It should also be noted that the *rol C* related phenotype could not be phenocopied by the application of any hormone. The sensitivity of *rol C*-expressing transgenic tobacco seedlings to externally supplied hormones in a germination assay was also different as compared to hormone sensitivity in wild-type seedlings. *rol C*-overexpressing seedlings were found to have an increased resistance to auxins and ABA, and a higher sensitivity to cytokinins, the ethylene precursor ACC, as well as the auxin transport inhibitor TIBA. In addition, Schmulling et al. (1993) crossed *rol C* overexpressing plants with *ipt* overexpressing transgenic plants and found that the phenotype exhibited by *rol C* overexpression is dominant to the phenotypes obtained by overexpression of the *ipt* gene, in that the plants were now able to root, and were dwarfed. The *rolC/ipt* plants as well as the *ipt* plants did have normal chlorophyll levels, indicating that at least this phenotype is dominant to the phenotype of *rol C* overexpressing plants having reduced chlorophyll levels. Overall, this work suggests that, although Rol C acts by releasing active cytokinins from inactive forms *in vitro* (Estruch et al., 1991a), the regulation of free hormone levels in plants is much more complex, as shown by the pleiotropic morphological alterations in *rol*C plants, as well as the tissue-specific changes in various hormone levels and sensitivities.

In an interesting variation to the regulated expression studies of the *ipt* gene in plants, Hewelt et al. (1994) used a promoterless *ipt* gene to utilize endogenous plant promoters to regulate cytokinin production in a developmental and tissue-specific manner. A wide variety of phenotypic alterations were observed. Although, it cannot be determined in this study which tissues do not respond to changes in cytokinin levels since a reporter gene was not included in this work to follow tissue-specific expression in transformed, but phenotypically normal plants. It was seen that not all *ipt* lines showed dosage effects of the

trangene on plant phenotype, indicating that gene dosage effects are dependent on the tissue and/or cell type as well as its developmental state.

Cytokinins in stress responses

Cytokinins may play a role in plant responses to certain physiological stresses. Application of cytokinin to certain plants can mimic salt-induced responses (Thomas et al., 1992; Thomas and Bohnert, 1993), namely the accumulation of proline and an osmotin-like protein, although endogenous cytokinin levels tend to decrease under salt stress (Kupier et al., 1990; Thomas et al., 1992). This effect was reconfirmed *in planta* by expressing the *ipt* gene in tobacco under the control of the light-inducible *rbcS*-3A promoter from pea. Under high light conditions, the transgenic plants accumulated appreciable amounts of proline and osmotin, although the plants also showed dramatic morphological alterations (Thomas et al., 1995). Another stress which may involve modulations of endogenous cytokinin levels is the plant response to pathogen attack. Non-rooting shoot lines of tobacco overexpressing the *ipt* gene were found to exhibit an increase in the expression of defense-related mRNAs (Memelink et al., 1987). The proteins encoded by these genes are coordinately induced by wounding and pathogenic attack (Chen and Varner, 1985; Ward et al., 1991). In a more recent study, the *ipt* gene was placed under the control of the proteinase inhibitor II promoter and introduced into tobacco. Upon attack by the insect larvae *Manduca sexta* , transgenic PI-II-*ipt* plants were 70% less susceptible to consumption by the larva than were control plants (Smigocki et al., 1993). It is not clear what the mode of action of the *ipt* gene in resistance. The authors propose that an increase in endogenous cytokinin levels may cause an increase in the production of secondary metabolites with insecticidal properties (Binns et al., 1987a; Orr and Lynn, 1992; Teutonico et al, 1991).

The effects of *ipt* gene expression on flower development

One of the most intriguing phenotypes resulting from endogenous alteration of cytokinin levles is the production of viviparous leaves and epiphyllous floral bud development (Estruch et al., 1991b; Estruch et al., 1993). In these studies, the *ipt* gene was placed under the control of the CaMV 35S promoter, interrupted by the maize transposable element Ac. Upon somatic transposon excision, the 35S promoter activated the expression of the *ipt* gene. Surprisingly, in one transgenic line, the vascular parenchyma of leaves re-differentiated into vegetative buds at the leaf midrib. This change in cell fate, related to the alteration in cytokinin levels, is the first example of developmental switches caused by alterations of endogenous cytokinin levels. In a more striking example of this type of developmental change, Estruch and co-workers (1993) showed that the same epiphyllous bud-producing tobacco plants were capable of producing both normal and abnormal epiphyllous floral buds. Epiphyllous floral buds developed only after the normal apical vegetative buds underwent the transition to floral bud development. Epiphyllous buds which were produced prior to this developmental switch remained vegetative. The abnormal epiphyllous floral buds had fused organs and were characterized by a local activation of the *ipt* gene, resulting in a 100- to 1000-fold increase in zeatin riboside equivalents. In contrast, the normal epiphyllous floral buds had cytokinin levels equivalent to those found in normal apical floral buds. In addition, there was a decrease in the mRNA steady-state levels of the tobacco homologues of the homeotic genes *DEFA* (Sommer et al., 1990), *GLO* (Schwarz-Sommer et al., 1992)and *PLENA* (Bradley et al., 1993) of *Antirrhinum majus*. This study lends support to the physiological evidence showing that the application of cytokinin provokes floral development (see above), as well as alters normal floral development. It is not known how cytokinins act to induce epiphyllous vegetative and floral bud development. However, what is clear is that the development of

either type of bud relies on the same signals that the normal apical buds respond to for vegetative and floral bud development. This suggests that cytokinins are not acting only to reactivate the cell cycle, but are triggering a complex ectopic developmental program.

Cytokinin-altered mutants

Two approaches have been taken toward the isolation of mutants altered in cytokinin production or action. One has been to screen for mutants altered in sensitivity to cytokinins, while the other has been based on a screen for plants with characteristic changes in morphology and development that have been correlated with cytokinin action.

A classic result of cytokinin application to seedlings is the inhibition of root growth, increase in root hair production, and root tip swelling. Using these criteria, Blonstein et al. (1991) isolated a cytokinin-resistant mutant of *N. plumbaginifolia*. This mutant was originally named *ckr1* and is characterized by a reduction in root development, cytokinin resistance during seedling development, and wiltiness of the shoot. The wiltiness was found to be caused by a defect in stomatal closure. In a study it was shown that the *ckr1* mutant is deficient in abscisic acid biosynthesis (Rousselin et al., 1992), and that the mutation affects the conversion of ABA-aldehyde to ABA, the final step in the ABA biosynthetic pathway (Parry et al., 1991). Thus, the mutant was renamed *Aba1*.

In a similar study by Su and Howell (1992), *Arabidopsis* mutants were isolated based on resistance to low levels of cytokinin, in order to avoid the isolation of mutants in general stress responses. Five independent mutants were isolated which comprise a single complementation group, named *ckr1*. In a later study it was shown that the *ckr1* mutant is allelic to the ethylene insensitive mutant, *ein2* (Cary et al., 1995). The isolation of *ckr1* indicates that the pathways for ethylene and cytokinin responses overlap. These studies underscore the problem associated with isolating cytokinin-response mutants, namely that cytokinin action is very pleiotropic and may be mediated through other factors. In a more

recent effort to isolate cytokinin-resistant mutants, Deikman and Ulrich (1995) found the *Arabidopsis cyr1* mutant (cytokinin-resistant 1). The *cyr1* mutant is characterized by a 10-fold reduction in sensitivity to benzyladenine in a root-elongation assay, but not to ACC, IAA or ABA. Rather, *cyr1* has an increased sensitivity to ABA. The phenotype of *cyr1* includes abbreviated shoot development, limited leaf production, reduction in cotyledon and leaf expansion, reduced chlorophyll accumulation, failure to accumulate anthocyanins in response to cytokinin treatment (a typical response to cytokinins; Pecket and Bassim, 1974; Ozeki and Komamine, 1981), and the formation of a single infertile flower. All of these traits are consistent with a mutation in cytokinin perception rather than biosynthesis. In support of the idea that *cyr1* is a true mutant in cytokinin perception, it was demonstrated that expression of an *SrEnod2*-GUS chimeric construct, which is cytokinin-enhanced in wild-type *Arabidopsis*, was found not to be cytokinin-enhanced in the *cyr1* mutant (see Chapter 4). The complex phenotype exhibited by *cyr1* appears to be due to a mutation in a single gene, indicating that a single gene required for normal cytokinin responses can have diverse effects on plant growth and development.

An *Arabidopsis* mutant deficient in adenine phophoribosyltransferase (APRT) activity (*apt*), was originally isolated in a screen for purine metabolism mutants (Moffatt et al., 1991). The *apt* mutant has approximately 1% of the APRT activity found in wild-type *Arabidopsis* plants. This mutant has normal vegetative morphology, grows more slowly than wild type, and is male sterile. In both *in vivo* and *in vitro* tests, the *apt* mutant was found to be unable to convert benzyladenine (BA) to benzyladenine-monophosphate (BAMP), indicating that APRT is the main enzyme which converts BA to its nucleotide form in young *Arabidopsis* plants. It is not known whether the slow growth and the male sterility are caused by the alteration in cytokinin metabolism in the *apt* plants.

A group of tobacco mutants originally isolated on the basis of their resistance to cytokinin define three complementation groups, *zea1*, *zea2*, and *zea3* (Jullien et al., 1992). The *zea3* mutant has a particularly complex phenotype in that it is highly sensitive to a high

carbon/nitrogen ratio, as well as to cytokinin, but only during germination at the jointed-cotyledon developmental stage (Faure et al., 1994). Under low nitrate conditions, *zea3* accumulates three times more sucrose and 5 times more amino acids than wild-type seedlings. The *zea3* mutant is able to germinate under high cytokinin concentrations, whereas the wild-type is completely inhibited. In addition, cytokinin causes the development of leaf hypertrophies in *zea3* mutant plants. Faure et al. (1994) have proposed that in *zea3* export or translocation of photoassimilates is perturbed causing the cotyledons to act as a sink instead of a source organ, thereby competing with the apical meristem for import of sucrose and amino acids. Also, the authors suggest that the cytokinin-induced hypertrophies may be related to the large import of photoassimilates into cotyledons, resulting in an increase of turgor pressure. This cytokinin-induced hypertrophy has been recently shown to be specific to cytokinins in the *zea1* group of mutants, and proposed as a bioassay for cytokinins (Nogue et al., 1995).

Using an entirely different approach, Chaudhury et al. (1993) screened for *Arabidopsis* mutants with novel developmental phenotypes. Their objective was to test developmentally altered plants for changes in the levels of plant growth regulators. The result was the isolation of the *amp1* mutant (altered meristem program). The *amp1* mutant is characterized by the frequent occurrence of polycoty (20% of total plants), bushiness of shoots, increased life span, floral abnormalities, such as siliques made of three or four carpels, and semi-sterility. Other abnormalities include a four-fold increase in rosette leaves formed before flowering as compared to wild-type, a significant decrease in the time to flower, and de-etiolation in the dark. The effect of a lack of phytochrome on *amp1* mutation was investigated in the double mutant *hy2 amp1*. *hy2* mutants lack the phytochrome chromophore and are deficient in the production of all phytochromes (Parks and Quail, 1991). The *hy2* mutant has a longer hypocotyl and exhibits an increase in apical dominance over wild-type plants. The *amp1 hy2* double mutant displays an intermediate phenotype with respect to hypocotyl length in the dark and apical dominance. This

suggests that the AMP1 product is required for the *hy2* phenotype. Another interesting characteristic of the *amp1* mutant is that it displays a 6-fold increase in cytokinin levels. Therefore, *amp1* represents the only known cytokinin overproducing mutant of *Arabidopsis*. The observed 6-fold increase in cytokinin levels is well within the range measured in transgenic plants expressing the *ipt* gene, which gave rise to similar phenotypic variations (Ainley et al., 1993; Hewelt et al., 1994). Therefore, the pleiotropic phenotypes of *amp1* can most likely be explained by an elevated level of cytokinin, although some of the phenotypes of the *amp1* mutant have not been reported before in *ipt* expressing transgenic plants. The authors further propose that AMP1 may be a regulator of cytokinin biosynthesis or metabolism. It is known that several genes whose expression is enhanced by application of cytokinin are, in fact, constitutively expressed in the *amp1* mutant, and that the *amp1* mutation appears to affect primarily the shoot and not the root (E. Dennis, personal communication). The determination of the identity of AMP1 promises to yield exciting information, and map-based cloning efforts of the corresponding locus are in progress (J.-D. Faure, personal communication).

Molecular responses to cytokinin

Cytokinin is capable of modulating the expression of a wide variety of genes, as shown in Table 1.2. It can be seen in Table 1.2 that cytokinin can act either at a transcriptional or posttranscriptional level, depending on the gene in question. Unfortunately, little is known about the molecular mechanisms of cytokinin action. Great progress has been made in understanding ethylene signal transduction through the isolation of *Arabidopsis* mutants and the identification of the corresponding genes. One of these genes *ETR1* possesses all

Table 1.2. Examples of Cytokinin-Regulated Genes

Gene name	level of gene expression	mode of regulation	time of response	other influencing factors	reference
wheat protein kinase, wpk4	mRNA↑	ND	24 h	light, nutrients	Sano and Youssefian, 1994
L. gibba rbcS	mRNA↑	P	24 h	light	Flores and Tobin, 1988
L. gibba cab	mRNA↑	P	24 h	light	Flores and Tobin, 1988
tobacco defense-related genes	mRNA↑	ND	3 weeks	ND	Memelink, et al., 1987
maize PEPC; C4ppc1	mRNA↑	T/P	2 h	light, nitrogen	Suzuki et al., 1994
barley nr	mRNA↑	T	15 min	light, nitrogen, ABA	Lu et al., 1990
soybean pollen allergen cim1	mRNA↑	ND	4 h	auxin	Crowell, 1994
Arabidopsis chs	mRNA↑	T	3 h	light	Deikman and Hammer, 1995
Arabidopsis pal1, chi	mRNA↑	P	10 d	light	Deikman and Hammer, 1995
Arabidopsis dfr	mRNA↑	T	10 d	light	Deikman and Hammer, 1995
Arabidopsis cyclin D homolog δ3	mRNA↑	ND	4 h	sucrose	Soni et al., 1995
Arabidopsis cdca	GUS↑	T	72 h	auxin, wounding	Hemerly et al., 1993
19 unidentified soybean cDNAs	mRNA↑	ND	4 h	auxin	Crowell et al., 1990
tobacco multiple stimulus response gene pLS216	mRNA↑	ND	< 10 h	auxin	Dominov et al., 1992
S. rostrata Enod2	mRNA↑	P	2 h	none found	Dehio and de Bruijn, 1992
Alfalfa Enod12, Enod40	mRNA↑	ND	6 h	nod factor	Hirsch and Fang, 1994
rice β-glucanase Gns1	mRNA↑	ND	> 24 h	ethylene, wounding salicyclic acid,fungal elicitors	Simmons et al., 1992
tobacco msr gene, str 246C	GUS↑	T	18 h	pathogen attack, auxin, salicyclic acid	Gough et al., 1995
Spirodela polyrrhiza L basic peroxidase	mRNA↓	ND	24-72 h	ABA	Chaloupkova and Smart, 1994
pumpkin hpr	mRNA↑	T	1.5 h	ND	Anderson et al., 1996
tobacco class I B-1,3-glucanase glb	GUS↓	T	4 d	auxin, ethylene, pathogen attack	Vogeli-Lange et al., 1994
cucumber catalase, HMGR, Lectin	mRNA↓	ND	1-4 h	ND	Toyama et al., 1995

of the characteristics of an ethylene receptor (Schaller and Bleecker, 1995). In addition, there are multiple genes which have been genetically determined to code for proteins which lie downstream of *ETR1* in the ethylene response pathway (Kieber et al., 1993; Hua et al., 1995). In analogy, it has generally been assumed that cytokinin also interacts with a specific receptor protein. There are reports of cytokinin-binding proteins, although there is little evidence that any act a receptor (Mitsue et al., 1993; Mitsue and Sugiura, 1993; Palme, 1993). The question remains as to why cytokinin receptor mutants have not been isolated to date. One possibility, other than lethality caused by such mutations, is that there are no specific receptor proteins, but rather that cytokinin interacts with multiple receptors, which feed into multiple signal transduction pathways. The identification of the genes involved in cytokinin-resistance (Deikman and Ulrich, 1995; Nogue et al., 1995) and overproduction mutants (Chaudhury et al., 1993) promises to shed light on this topic.

A confounding problem when using a specific gene as a probe or reporter to study cytokinin action is that most genes which are regulated by cytokinin are co-regulated by other factors, such as light, nutrients, and other hormones. Many of these cytokinin-regulated genes are regulated by auxin and light, which is supported by data showing that cytokinin, auxin and light interact to affect plant development (Miller et al., 1955; Miller et al., 1956; Tong et al., 1983; Su and Howell, 1995). The complex interaction of cytokinin and other factors in gene expression is best exemplified by the nitrate reductase gene. Treatment of etiolated barley leaves with light, nitrate, and cytokinin greatly enhances the accumulation of the *nr* mRNA, primarily through transcriptional activation (Lu et al., 1990), and ABA negatively regulates this enhancement (Lu et al., 1992). The requirement for both nitrate and light for *nr* mRNA accumulation has not been found in the case of the *nr* gene from *Agrostema githago*, in which nitrate reductase activity, as well as mRNA accumulation, are enhanced solely upon cytokinin treatment, and seems to occur primarily at the posttranscriptional level (Kende et al., 1974; White, 1996). In addition, the accumulation of the *A. githago nr* mRNA upon cytokinin treatment appears to be inhibited

by ethylene (White, 1996). The cytokinin-specific enhancement of the *A. githago nr* mRNA accumulation occurs only in embryos and not at any other stage of plant development. It is interesting to note that although the expression of both *nr* genes from barley and *A. githago* is enhanced by cytokinin, the mechanisms by which this occurs appear to be quite different, as are the tissues in which the *nr* genes are expressed. More recently, it has been shown that the genes of the anthocyanin biosynthesis pathway also are cytokinin-induced in a light dependent manner (Deikman and Hammer, 1995), which now explains old observations of anthocyanin accumulation as a typical effect of cytokinin application. One gene which may be an exception to this complex interaction of cytokinin and other factors in regulating gene expression is the *SrEnod2* gene from *Sesbania rostrata*. The expression of the *SrEnod2* gene appears to be enhanced solely by cytokinin (Dehio and de Bruijn, 1993). The *SrEnod2* gene may be a candidate marker gene for studying cytokinin signal transduction (Silver et al., 1996; and Chapter 4).

One of the best known affects of cytokinin is its effect on cell division. It has been shown that the *Arabidopsis cdc2a* gene is transcriptionally induced by cytokinin after long exposures to the hormone (Hemerly et al., 1993), although its expression is not directly coupled to cell division, but always precedes it. Hemerly et al. (1993) propose that multiple signals may be involved in the triggering of cells to divide or to be competent for division, and that cytokinin, acting through the cdc2a protein, may be one of these signals. The cyclin gene *δ3* from *Arabidopsis*, which is homologous to the human D-type cyclins, is expressed at the G1/S transition and is proposed to play a role in regulating this transition in a similar way as the CLN1 and CLN2 cyclins in yeast (Richardson et al., 1989; Wittenberg et al., 1990; Soni et al., 1995). Interestingly, the expression of the *δ3* gene is rapidly induced by cytokinin after 4 h of treatment. In a separate study by John et al. (1993) using tobacco pith cells, a tissue classically known to require cytokinin and auxin for cell division, it was shown that *cdc2* expression was induced by treatment with auxin alone, but that the protein was nonfunctional unless cytokinin was also present in the

growth medium. They speculate that the cytokinin-induced component required for the activation of cdc2 in G1 cells might be cyclin δ3. The activation of the cell cycle is critical for the development of new plant organs, such as lateral roots and nodules (see below), and determining how cytokinin acts on the cell cycle will be of great importance to understanding the mechanisms that regulate cell division during plant development.

Overall, these studies reveal that, in some instances, the effects of cytokinin on plant development and physiology can now be partially explained at the molecular level.

A role for cytokinin in nodule development?

In the case of most plant organogenesis events, a role for cytokinin has been postulated, although in several cases it has been very difficult to prove this hypothesis directly. This also appears to be the case for the involvement of cytokinin in the development of nitrogen-fixing nodules on legume plants. Nodule development has been recently extensively reviewed (Verma, 1992; Mylona et al., 1995), and will only be reviewed briefly here. The development of nitrogen fixing nodules involves the highly specific interaction of rhizobia with the legume plant root, or stem as in the case of the tropical legume *Sesbania rostrata*. At the onset of this interaction, rhizobia are induced by plant phenolics to produce a specific chito-lipooligosaccharide molecule, known as the Nod factor (Peters and Verma, 1990). The Nod factor, in turn, seems to interact with an as yet unidentified receptor at the root epidermis, resulting in root hair deformation or curling. This root hair curling is part of the uptake mechanism of the bacteria into the plant cell. Following, is the production of an infection thread produced by the plant harboring the bacteria, and the initiation of cortical cell division in the inner or outer cortex, depending on the type of legume infected (determinate versus indeterminate nodules). Once the bacteria make their way to the dividing cortical cells, via the infection threads, they are taken up into large plant cells known as infected cells, in which they will differentiate into nitrogen-fixing

bacteroids. During this entire process, the expression of specific plant genes is being induced. These plant genes are termed nodulins, since they are induced or their expression is enhanced in nodules, although some are known to be expressed elsewhere in the plant as well. The nodulins have been classified based on their time point of expression. Those which are expressed early during nodule development are termed early nodulins, and those which are expressed in fully developed nodules are called late nodulins. The early nodulins, such as *Enod5*, 12, 40, and 2 are believed to play a role in nodule ontogeny, although no exact function has yet been assigned to most of them. The late nodulins such as glutamine synthetase, sucrose synthetase, and leghemoglobin all play a role in nodule functioning. A growing number of other early and late nodulin genes are being isolated, for example by the differential display of mRNAs (Goormachtig et al., 1995; K. Szczyglowski and F. J. de Bruijn, unpublished data), and these genes promise to yield exciting novel information regarding nodule development and functioning.

Nodule development is primarily determined by a plant genetic program and not by the presence of the infecting bacteria, as evidenced by the discovery of spontaneous nodulating alfalfa plants (Truchet et al., 1989). In addition, alfalfa plants can be induced to form nodule-like structures upon treatment with auxin transport inhibitors (Allen et al., 1953; Hirsch et al., 1989), or purified Nod factor (Truchet et al., 1991; Mergaert et al., 1993; Stokkermans et al., 1994). It has been postulated that the ability of the rhizobial Nod factor to induce the plant nodule ontogony program is related to hormone action, although the evidence for the involvement of hormones in nodule development is circumstantial. Thimann (1936) first proposed a role of auxin in nodule development, and postulated that nodule development may be related to lateral root development. Libbenga et al. (1973), using an *in vitro* approach, treated pea root cortical explants with auxin and found that cell division took place in the pericycle, the location of lateral root initiation. However, division of cortical cells, the location of nodule initiation, occurred upon the addition of both auxin and cytokinin to the media. It has been shown that rhizobia secrete cytokinins

into the culture medium (Morris, 1986; Sturtevant and Taller, 1989; Taller and Sturtevant, 1991; Upadhyaya et al., 1991), but again the significance of bacterially produced cytokinin on nodule development remains unclear. Since cytokinin biosynthesis genes have not yet been identified in rhizobium to date, the effects of mutations in these genes are unknown. Probably the most conclusive evidence for a role of cytokinins in nodule development comes from the work by Cooper and Long (1994). They expressed the *Agrobacterium* cytokinin biosynthetic gene *tzs* in a *Rhizobium meliloti* strain carrying a mutation in the *nod* structural genes, preventing the synthesis of the Nod factor. This *tzs* expressing *Rhizobium* was capable of inducing nodule-like structures on alfalfa, supporting the idea that localized cytokinin production may be involved in nodule development and may be able to "substitute for" Nod factor action. This work does not prove that cytokinin secreted from wild-type rhizobia is involved in triggering of nodulation. In fact, it seems more likely that the source of cytokinin is plant derived, given the existence of spontaneously nodulating alfalfa plants.

One of the first events in nodule development involves cortical cell divisions. It has been shown that purified Nod factor is capable of eliciting cortical cell divisions (Spaink et al., 1991; Truchet et al., 1991; Relic et al., 1993). This process of cortical cell division has been extensively studied by Yang et al. (1994). It has been determined that cells susceptible to Nod factor are arrested in the G0/G1 stage of the cell cycle, and not the G2/M stage as previously believed. Therefore, Nod factor-susceptible cortical cells are arrested in the same stage as all other cortical cells. Those cortical cells which divide are opposite protoxylem poles. Interestingly, a positive regulator of nodulation has been isolated from the xylem and shown to be able to replace cytokinin in an *in vitro* pea cortical cell assay (Libbenga et al., 1973). This factor, called stele factor, has been identified to be uridine (H. Spaink, personal communication). As reviewed above, cytokinin also clearly plays an important role in cortical cell divisions, as exemplified by the classical experiments of Miller and Skoog (1957), as well as more recently by Yang et al. (1994). At the

molecular level, it has been shown that the expression of the early nodulin genes *Enod*12 and *Enod*40 are induced by both Nod factor and cytokinin in alfalfa, whereas the *Enod2* gene from *Sesbania rostrata* and an *Enod*2-like gene of alfalfa are induced only by cytokinin (Dehio and de Bruijn, 1992; Hirsch and Fang, 1994). It is not known whether, in fact, cytokinin is the direct regulator of the expression of these genes during nodule development. It has been proposed that Nod factor does not act directly on nodule development but acts through altering the endogenous cytokinin/auxin ratio (Mylona et al., 1995). Support for this idea comes from the expression of the rhizobial *nodA* and *nodB* genes in transgenic tobacco plants. *nodA* and *nodB* encode proteins involved in the production of Nod factor in *Rhizobium* (John et al., 1993; Rohrig et al., 1994). These transgenic tobacco plants displayed phenotypes similar to plants with an imbalance in hormone levels, such as epinastic leaves, and increased apical dominance (Schmidt et al., 1993). Another line of evidence is that the non-nodulating alfalfa line MN1008 can be induced to form nodule-like structures expressing early nodulin genes upon treatment with auxin transport inhibitors, independent of Nod factor application (Hirsch and Fang, 1994). The corollary to this idea is that the Nod factor mimics the action of a plant Nod-like factor which, in turn, triggers the nodule developmental signal transduction pathway. Although active plant Nod-like factors have not been isolated, with the exception of Nod-like factor molecules in plant secondary cell walls (Spaink et al., 1993), application of Nod factor to non-legume plants has yielded a surprising result. For example, a mutant carrot cell line arrested in development can be complimented by the application of Nod factor (De Jong et al., 1993). In addition, Nod factor have been observed to cause suspension-cultured tomato cells to trigger the alkalization of the culture medium (Staehelin et al., 1994).

It can be observed from the above discussion that the role of cytokinin in nodule development is still poorly understood. There is considerable evidence for a role of auxin in nodule development. This idea goes back to Thimann (1936) in which he proposed that a relationship exists between lateral root development and nodule development. The

primary evidence for this comes from the nodule forming non-legume *Parasponia*. *Parasponia* nodules, which are true nodules harboring *Rhizobia*, form from pericycle cells, as do lateral roots, rather than from cortical cells (Marvel et al., 1987). In further support of a role for auxin, an auxin-sensitive alfalfa line A2 (Borgre et al., 1990) was shown to form significantly more nodules and undergo earlier nodule initiation than the genetically related wild-type line R15 (Kondorosi et al., 1993). In addition, Alfalfa plants transgenic for the *rol*B gene, expression of which is correlated with auxin-sensitivity (Shen et al., 1988), produced considerably more nodules and in a shorter time than non-transformed alfalfa plants (Kondorosi et al., 1993). The *rolB* expressing plants also had an increase in root production, which correlates with the effects of *rolB* expression on root production in tobacco (Schmulling, 1988). Interestingly, expression of *Enod*40 in tobacco, which is induced by nod factor in both root pericycle cells and in dividing cortical cells, caused a phenotype similar to a hormone affect. Expression of *Enod*40 in tobacco protoplasts caused auxin-insensitive growth at concentrations which are inhibitory to non-transformed protoplasts (Van de Sande et al., 1996). Mylona et al. (1995) proposes that *Enod*40 expression causes a change in the auxin/cytokinin ratio in cortical cells leading to mitotic reactivation. Therefore, Nod factor may act through certain nodulin genes, such as *Enod*40, to trigger nodule development and or regulation of development.

At the present, an effort is being made to understand the relationship between lateral root development and nodule development, and the involvment of hormones in these processes. K. Szczygwoski and F. J. de Bruijn (unpublished data) have isolated a *Lotus japonicus* mutant *har1* which forms a hyper-amount of root nodules when inocculated with *Rhizobia* similar to the soybean supernodulating mutant nts (Carroll et al., 1985). Surpisingly, in the absence of *Rhizobia, har1* forms a profuse amount of lateral roots. It is proposed that *har1* represents a gene which is a negative regulator of both nodule initiation/development as well as lateral root initiation/development (K. Szczygwoski, personal communication). The superroot mutant, *sup1*, of *Arabidopsis* forms an excess of

lateral roots and contains increased levels auxin (Boerjan et al., 1995). The *alf1* mutant of *Arabidopsis* is also characterized by a profuse production of lateral roots, and is believed to be due to the overproduction of auxin (Celenza et al., 1995). It remains to be determined whether the phenotypes of the *har1* mutant is caused by an increase in auxin levels or sensitivity, and does *har1* represent the legume equivalent of *alf1* or *sup1*. In a distinct approach to these questions, another group is studying the *Arabidopsis* mutants *rml1* and 2 which fail to form lateral roots but instead form nodule-like structures (Cheng et al., 1995) in the hope of elucidating the mechanisms underlying lateral root and nodule developmental programs (R. Wilson, personal communication). The further characterization of nodulation mutants, such as *har1*, as well as non-legume root mutants, will shed light on the understanding of the involvement of hormones in nodulation and the relationship with lateral root development. Towards this goal, it is proposed in this thesis that the *SrEnod2* gene can be utilized in *Arabidopsis* for the isolation of *trans*-acting factors involved in regulating the cytokinin-enhancement of *SrEnod2* gene expression. Any genes which code for such *trans*-acting factors can be tested for their relevance in regulating nodulin genes during nodule development.

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Chapter 2

POSTRANSCRIPTIONAL REGULATION OF THE *SESBANIA ROSTRATA* EARLY NODULIN GENE *SRENOD2* BY CYTOKININ

The contents of this chapter have been published in Plant Physiol, 1996 (Silver, Pinaev, and de Bruijn)

ABSTRACT

The mRNA from the *Sesbania rostrata* early nodulin gene *SrEnod2* accumulates in response to cytokinin application. Nuclear run-on assays using isolated root nuclei have shown that this accumulation occurs posttranscriptionally, and northern blot analysis of nuclear and total RNA levels revealed that it occurs primarily in the cytoplasm and not in the nucleus. After cytokinin enhancement of *SrEnod2* mRNA accumulation and the subsequent removal of cytokinin, the levels of *SrEnod2* mRNA did not return to basal levels, but oscillated over a 36-h time course. Application of the translational inhibitor cycloheximid, was found to inhibit the enhancement of *SrEnod2* mRNA accumulation by cytokinin and to cause its rapid decay. Okadaic acid and staurosporine, inhibitors of protein phosphatases and kinases, respectively, also inhibited cytokinin enhancement of *SrEnod2* mRNA accumulation. In addition, okadaic acid was found to cause a decrease in *SrEnod2* mRNA levels. These results provide evidence for a posttranscriptional mechanism of cytokinin enhancement of *SrEnod2* mRNA accumulation, which appears to require concurrent protein synthesis, to involve protein phosphatases and kinases, and to occur primarily in the cytoplasm of the plant cell.

INTRODUCTION

The plant hormone cytokinin comprises of a group of plant growth substances that are derived from adenine. It has been shown that cytokinins induce cell division and organogenesis in cell cultures (Skoog and Miller, 1957), and also affect other physiological and developmental plant processes (Evans, 1984; Brzobohaty et al., 1994; Davies 1995). Cytokinin and auxin have been shown to play a central role in photomorphogenesis and elongation growth (Hobbie et al., 1994). The available information on auxin signal transduction has been accumulating rapidly with the identification of auxin-induced mRNAs and auxin-binding proteins, the cloning of putative auxin receptors, and auxin-responsive DNA elements, as well as the characterization of mutants in auxin responses (for review see Hobbie et al., 1994), and the cloning of a gene responsible for one of the auxin resistant mutant phenotypes (Leyser et al., 1993).

Only a limited number of cytokinin response mutants have been isolated (Moffatt et al., 1991; Chaudhury et al., 1993; Deikman and Ulrich., 1995), and the genes corresponding to these mutant loci have yet to be identified. Putative cytokinin-binding proteins have been purified (Brzobohaty et al., 1994), but the demonstration of their biological activity is still lacking. Overall, little is known about the molecular mechanisms of cytokinin signal transduction.

At the molecular level, cytokinin has been shown to modulate enzyme activities (Treharne et al., 1970; Chatfield and Armstrong, 1986), and transcript levels of a variety of genes. mRNAs whose accumulation is enhanced by cytokinins, including those encoded by a wheat protein kinase gene (Sano and Youssefian, 1994), the gene for the small subunit of Rubisco (*RbcS*) (Flores and Tobin, 1988), the chlorophyll a/b binding protein gene (*Cab*) (Flores and Tobin, 1988), defense-related genes (Memelink et al., 1987), the PEP carboxylase gene (*PepC)* (Suzuki et al., 1994), nitrate reductase genes (Lips and Roth-Bejerano, 1969; Dilworth and Kende, 1974; Lu et al., 1990), the pollen allergen gene

Cim1 (Crowell, 1994), the multiple stimulus response gene pLS216 (Dominov et al., 1992), genes of the anthocyanin biosynthetic pathway (Deikman and Hammer, 1995), cyclin D homologs (Soni et al., 1995), and genes for a number of unidentified cDNAs (Crowell et al., 1990). It was shown by nuclear run-on assays that the expression of genes encoding PEP carboxylase (Suzuki et al., 1994), nitrate reductase (Lu et al., 1990), chalcone synthase, and dihydrofolate reductase (Deikman and Hammer, 1995) are enhanced primarily at the transcriptional level by cytokinin. On the contrary, nuclear run-on assays show that some genes are regulated by cytokinin primarily at the post-transcriptional level. These genes include those encoding the chlorophyll a/b binding protein, the small subunit of Rubisco (Flores and Tobin, 1988), chalcone isomerase, and Phe ammoniumlyase 1 (Deikman and Hammer, 1995).

The genes involved in the cytokinin-induced accumulation of mRNA are diverse, as are the mechanisms of cytokinin induction. Cytokinin appears to enhance the transcription of genes or enhance mRNA accumulation posttranscriptionally. It is important to note that in all the examples reported thus far, cytokinin enhancement of gene expression is never exclusively the result of cytokinin action, but generally co-mediated by other environmental factors, such as light, nitrogen, carbon, or other plant hormones. This has complicated the analysis of the molecular basis of cytokinin action. One exception is the *Sesbania rostrata* early nodulin gene *SrEnod2*. The *SrEnod2* gene encodes a Pro-rich protein expressed in a cell-specific manner in nodules of legumes (van de Wiel et. al., 1990). This cell layer, which surrounds the cells infected with nitrogen-fixing bacteria, is called the nodule parenchyma. It has been previously shown by Dehio and de Bruijn (1992) that the *SrEnod2 mRNA* accumulates in *S. rostrata* roots in the absence of rhizobia in a time- and concentration-dependent manner in response to cytokinin treatment. The root cell type in which the *SrEnod2* gene is expressed has not been determined. This accumulation of *SrEnod2* mRNA in unnodulated roots occurs primarily in the primary root, and to a lesser extent in fully developed lateral roots, which correlates well with the observed GUS

expression pattern in transgenic *Lotus japonicus* plants harboring *SrEnod2-GUS* fusions (see Chapter 4 of this thesis). *SrEnod2* is a good gene with which to study cytokinin signal transduction, since its mRNA accumulation is stimulated solely by cytokinin (Dehio and de Bruijn, 1992; Hirsch and Fang, 1994). We sought to determine the most important parameters affecting this process. We demonstrate that *SrEnod2* mRNA accumulation is posttranscriptionally enhanced by cytokinin, and that this mechanism requires ongoing protein synthesis, involves protein phosphatases and kinases, and occurs primarily in the cytoplasm.

METHODS

Plant material and treatments

Sesbania rostrata seeds were germinated and seedlings grown in soil composed of Metromix (Hummert International, Earth City, MO) and sand (2:1) at 30°C, with a 18-h light, 28°C/6-h dark, 22°C regime for 2 weeks in growth chambers with 75% RH. For all chemical treatments, plants were washed free of soil and incubated in a 1:2 dilution of Murashige-Skoog minimal organic medium (Murashige-Skoog, Gibco-BRL), along with the appropriate chemicals, under normal growth conditions. 6-BAP (Sigma), cycloheximide (Sigma), okadaic acid (Gibco-BRL), and staurosporine (Sigma) were used at concentrations of 10 μM, 140 μM, 0.5 μM, and 10 μM, respectively. Following chemical treatments, root tissues were excised, frozen in liquid nitrogen, and stored at -80°C.

Isolation of nuclei and analysis of nuclear run-on transcripts.

Frozen roots were ground in liquid nitrogen to a fine powder. The powder was resuspended in nuclei isolation buffer (20 mM MES, pH 6.5; 2.5% Ficoll 400; 2.5% Dextran 40000 [Sigma]; 50 mM KCl; 0.44 M sucrose; 0.1% thiodiglycol; 0.5 mM spermidine; 0.1 mM spermine; 0.5 mM EDTA; 0.5% Triton X-100; 5 μg/ml aprotinin, leupeptin, and leupeptin [Sigma]). The resuspended material was passed through four layers of cheesecloth, two layers of Miracloth (Calbiochem), 1 layer of 100-μm mesh, and spun at 2,500 rpm for 15 min (HB4 rotor, Sorvall). The pellet was resuspended in nuclei isolation buffer, passed through a 20-μm mesh, and spun at 2,000 rpm for 15 min. The pellet was resuspended in nuclei isolation buffer. Nuclei were counted using the DNA stain 4',6-diamidino-2-phenylindole dihydrochloride, and aliquots of 2×10^6 nuclei were

frozen at -80^{0}C. Nuclear run-on assays were performed as described by DeRocher and Bohnert (1993). Slot blot filters containing 5 µg of linearized plasmid DNA containing the *SrEnod2* coding region (Dehio and de Bruijn, 1992), pUC19 (New England BioLabs), and the β*ATPase* gene (Boutry and Chua, 1985) on nitrocellulose membrane (Bio-Rad) were used to hybridize with equal counts of transcripts (5 x 10^7 cpm). Filters were washed at 65°C in 2.0 x SSC (0.3 M NaCl, 0.03 M sodium citrate), 0.1% SDS for 20 min; 0.5 x SSC, 0.1% SDS for 20 min; 0.1 x SSC, 0.1% SDS for 30 min. The signals were quantified using phosphorimager analysis (Model 400B, Molecular Dynamics, Sunnyvale, CA)

Isolation of nuclei for RNA extraction.

Nuclei were isolated as described by Peters and Silverthorne (1995) with the following modification: β-mercaptoethanol was replaced by 10 mM of the RNase inhibitor ribonucleoside-vanadyl complex (Gibco-BRL). Nuclei were resuspended in RNA extraction buffer, and RNA isolated as described by Verwoerd et al. (1989).

Northern blot analysis.

10 µg of RNA was electrophoresed in 1.2% (w/v) agarose gels in Mops buffer (20mM MOPS, 1.0 mM EDTA, 5.0 mM sodium acetate, pH 7.0) containing 5.4% (v/v) formaldehyde. Gels were blotted onto 0.22 µM NitroPlus nitrocellulose membrane (Micron Separation Inc., Westborough, MA). Membranes were probed with a [^{32}P] dATP labeled DNA fragment containing the *SrEnod2* coding region generated with random priming (Boehringer Mannheim). Filters were reprobed with an 18S rRNA DNA probe as a loading control. The use of the *βATPase* gene (Boutry and Chua, 1985) and the soybean

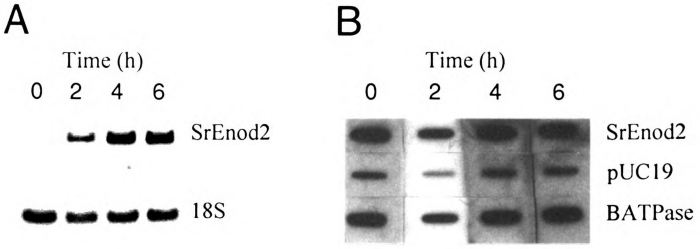
actin gene, pSAC3 (Shah 1982) as further controls are indicated in the Results section. All filters were washed at 65°C in 2.0 x SSC, 0.1% SDS for 20 min; 0.5 x SSC, 0.1% SDS for 20 min; and 0.1 x SSC, 0.1% SDS for 15 min. The signals were quantified using phosphorimager analysis (Model 400B, Molecular Dynamics, Inc.).

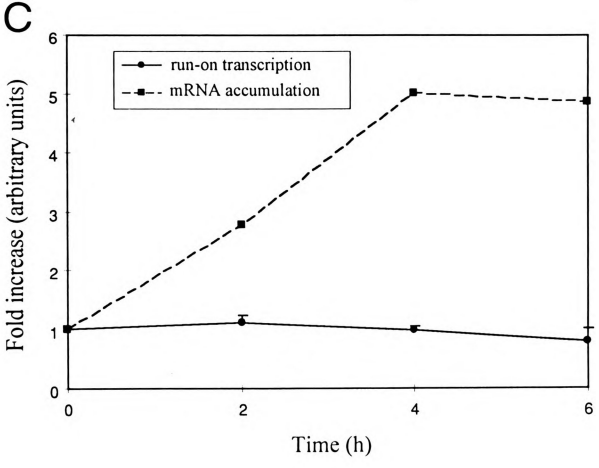
RESULTS

Transcription of the *SrEnod2* gene and accumulation of its mRNA in response to cytokinin

Run-on transcription assays were performed using isolated nuclei to determine whether *SrEnod2* mRNA is regulated at the transcriptional and/or post-transcriptional level by cytokinin. Two-week-old *S. rostrata* seedlings were treated with the cytokinin benzylaminopurine (BAP) for time periods of 0 (no BAP), 2, 4, and 6 h, after which nuclei and RNA were isolated from root tissues. A northern blot analysis of the RNA samples is shown in Figure 2.1A. *SrEnod2* mRNA accumulated to levels approximately 4-fold higher than those of the control over the time course of BAP treatment, as shown in Figure 2.1A. A second *mRNA* smaller than *SrEnod2* was frequently observed on northern blots (Figure 2.1A), the origin of which is unclear. This second *mRNA* probably does not represent a second *SrEnod2* gene, since Southern blot analysis indicates that *SrEnod2* exists as a single-copy gene in *S. rostrata* (Dehio, 1989), and the northern blots were washed at high stringency. More likely, this smaller RNA may be a processing product derived from the *SrEnod2* mRNA. Radiolabelled transcripts from the nuclei were hybridized with an immobilized *SrEnod2* DNA probe on slot blots. No change in transcription of the *SrEnod2* gene was observed over the time course of BAP treatment (Figure 2.1B), as indicated by the *SrEnod2* signal relative to the *βATPase* signal using phosphorimager analysis. A comparison of the northern blot and nuclear run-on data (Figure 2.1C) showed that although *SrEnod2* mRNA accumulates over time, no detectable change in transcription was evident. These data suggest an involvement of posttranscriptional processes in *SrEnod2* mRNA accumulation in response to cytokinin. To examine the possibility that *SrEnod2* mRNA stability was altered in the presence of cytokinin, we examined *SrEnod2* mRNA half-life using the cellular RNA synthesis inhibitor actinomycin-D. However, the detection

Figure 2.1. Comparison of nuclear run-on transcription with mRNA accumulation. A, Northern blot analysis of *SrEnod2* mRNA accumulation enhanced by cytokinin. Two-week-old *S. rostrata* seedlings were incubated in the presence of 10 μM BAP for time periods of 0 h (untreated), 2 h, 4 h, and 6 h, and the total RNA was then extracted from roots. B, Radiolabeled run-on transcripts from root nuclei isolated from the same seedlings treated in A were hybridized with immobilized *SrEnod2*, pUC19 as background plasmid control, and *βATPase* probes on slot blots. C, Signals from northern blot and run-on transcription assays were quantified by phosphorimager analysis and plotted as the increase (-fold) in signal as standardized to *βATPase* mRNA levels. The SD of two independent nuclear run-on transcription assays is shown.
Circle, Run-on transcription; Square, mRNA accumulation.


A
Time (h)
0 2 4 6
SrEnod2
18S
B
Time (h)
0 2 4 6
SrEnod2
pUC19
BATPase

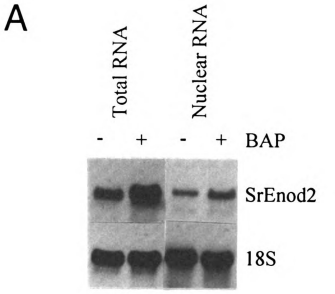

C
run-on transcription
mRNA accumulation
Fold increase (arbitrary units)
0
1
2
3
4
5
6
0
2
4
6
Time (h)

of differences in *SrEnod2* mRNA stability in response to cytokinin treatment has so far not been possible, because it was found that actinomycin-D stabilizes the *SrEnod2* mRNA (Silver and de Bruijn, unpublished results), making this type of analysis impossible. The effect of *mRNA* stabilization due to transcription inhibitors has also been observed in the case of the *PhyA* (Seeley et al., 1992), *rbcS* (Fritz et al., 1991), *PvPrP1* (Zhang et al., 1993), and Fd mRNAs (Dickey et al., 1994).

SrEnod2 mRNA accumulation in response to cytokinin occurs primarily in the cytoplasm

The nuclear run-on data suggest that *SrEnod2* mRNA accumulates posttranscriptionally, but do not provide insight into the question whether *SrEnod2* mRNA accumulation is a nuclear and/ or cytoplasmic event. To better understand the cytokinin signal transduction pathway, it was important to determine if *SrEnod2* mRNA accumulated in the nucleus or in the cytoplasm. Events which occur in the nucleus after transcription include pre-mRNA processing, turnover, and transport to the cytoplasm. To investigate the fate of *SrEnod2* mRNA in the nucleus, nRNA was isolated from *S. rostrata* roots treated and untreated with BAP, and analyzed by northern blot hybridization (Figure 2.2A). There was only a 1.2-fold increase in *SrEnod2* mRNA accumulation in the nucleus, compared with an approximately 4-fold increase in total cellular RNA (Figure 2.2B). It is important to note here that the levels of *SrEnod2* mRNA in the nucleus constitute approximately 38% of the total *SrEnod2* RNA, indicating that most of the *SrEnod2* transcripts are located in the cytoplasm. Therefore, the accumulation of *SrEnod2* mRNA appears to occur primarily in the cytoplasm and not in the nucleus.

Figure 2.2. Abundance of *SrEnod2* mRNA derived from total RNA and nRNA. A, Representative northern blot analysis of total RNA and nRNA isolated from roots of *S. rostrata* seedlings treated with or without 10 μM BAP for 4 h. B, Quantification of *SrEnod2* mRNA accumulation in both total and nRNA. Signals from two independent experiments were quantified using phosphorimager analysis. *SrEnod2* signals were standardized to 18S rRNA signals. The SD is shown.


A
Total RNA
Nuclear RNA
- + - + BAP
SrEnod2
18S
B
Fold induction (arbitrary units)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Total RNA
Nuclear RNA

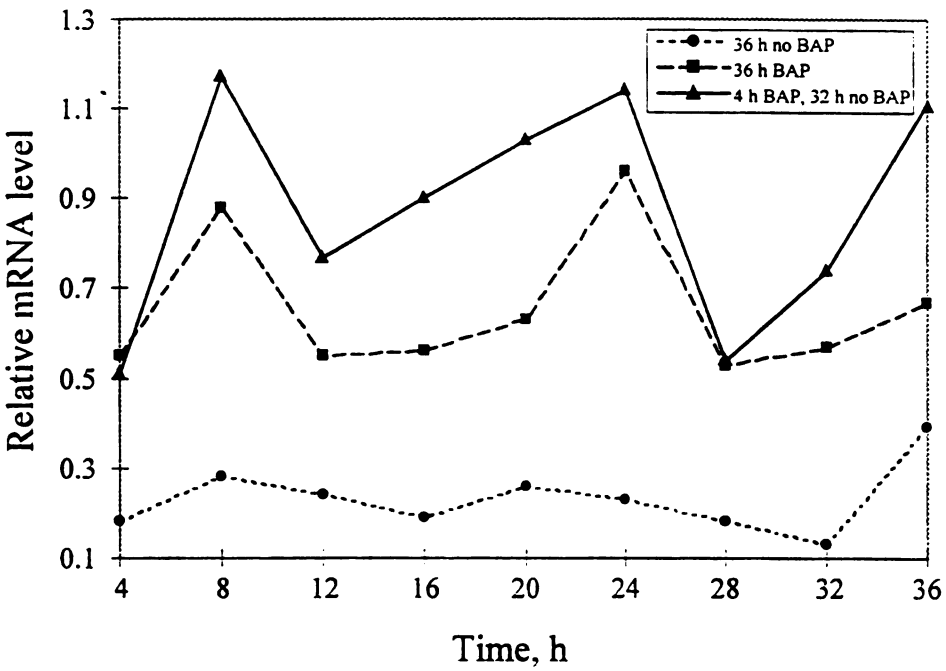
SrEnod2 mRNA accumulation appears to be a long-lived process

To better define the link between *SrEnod2* mRNA accumulation and cytokinin application, we determined the level to which *SrEnod2* mRNA would increase, and to what extent the *mRNA* would be present over time after the removal of cytokinin to determine if this process was a short-lived or a long-lived response to cytokinin. *S. rostrata* seedlings were incubated in the presence of BAP for 4 h, then divided into two groups. One group was washed extensively in water to remove BAP and was placed in BAP-free medium. Another group was further incubated in media with BAP. A third group of *S. rostrata* seedlings was not exposed to BAP and was placed continuously in BAP-free medium. All three groups of seedlings were incubated for 36 h under these conditions. Roots were harvested at 4-h intervals, and RNA was isolated and analyzed by northern blot hybridization. Neither group of seedlings exposed to BAP exhibited *SrEnod2* mRNA decay below induced levels during the 36 h time course (Figure 2.3). In addition, a rhythmic oscillation with essentially the same pattern of oscillation was observed in both groups treated with BAP. However, the level of *SrEnod2* mRNA in samples treated with BAP for only 4 h appeared to be slightly higher than in samples treated for 36 h. The reason for this apparent difference is unclear, but may be due to signal attenuation in samples continuously exposed to cytokinin. These data may indicate that the process by which *SrEnod2* mRNA accumulates is long-lived. Whether the observed oscillation is due in part to a change in stability or synthesis of the *SrEnod2* mRNA is unknown.

Cycloheximide inhibits *SrEnod2* mRNA accumulation in response to cytokinin

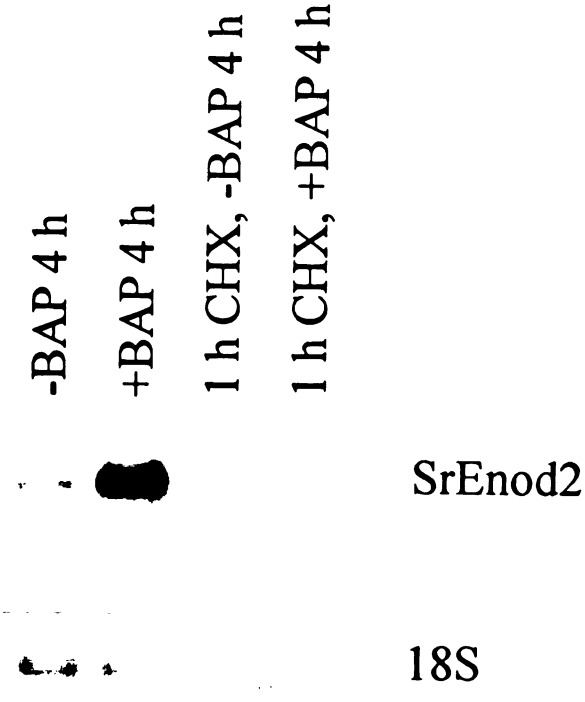
It is a common observation that the levels of mRNA, which is unstable, is increased by the translational inhibitor cycloheximide (Zhang et al., 1993; Dickey et al., 1994; Gil et

Figure 2.3. Fluctuation in *SrEnod2* mRNA accumulation during a 36-h period. Two-week-old *S. rostrata* seedlings were divided into three groups according to the following treatments: 36 h no BAP (circle); 36 h 10 μM BAP (square); 4 h 10 μM BAP then washed free of BAP and incubated for 32 h (triangle). RNA from roots of 5 seedlings were isolated every 4 h for 36 h. Signals were quantified using phosphorimager analysis. *SrEnod2* signals were standardized to *βATPase* mRNA signals.


1.3
1.1
0.9
0.7
0.5
0.3
0.1
Relative mRNA level
4
8
12
16
20
24
28
32
36
Time, h
36 h no BAP
36 h BAP
4 h BAP, 32 h no BAP

al., 1994). There are three possible reasons for this. Cycloheximide may inhibit the accumulation of unstable components of the mRNA degradation machinery (Brewer and Ross, 1989), or translation of the specific mRNA in question may be required for its degradation (Aharon and Schneider, 1993; Byrne et al., 1993; Tanzer and Meagher, 1994). Another possibility is that cycloheximide, through a secondary effect, increases transcription (Edwards and Mahadevan, 1992). Therefore, it was of interest to determine if cytokinin could directly stimulate *SrEnod2* mRNA accumulation in the absence of translation, and/or if the *SrEnod2* mRNA exhibited properties common to unstable transcripts in the presence of cycloheximide. A typical concentration of cycloheximide used in plants is between 50 to 70 μM in cell culture(Heinhorst et al 1985; Gil et al 1994), and up to 300 μM (for 2-h pretreatment) in intact plants (Satoru et al, 1995). In our system 70 μM cycloheximide inhibited the accumulation of *SrEnod2* mRNA, but required slightly more than 1 h of pretreatment to be effective. A concentration of 140 μM was effective after a pretreatment of between 30 min to 1 h (data not shown). It may be that a higher concentration of cycloheximide is needed to elicit an effect in whole plants than is needed in cell culture due to the timing of uptake of the substance. Two-week-old *S. rostrata* seedlings were pretreated for 1 h with 140 μM cycloheximide. The seedlings were then treated with BAP for 4 h, after which RNA was isolated and *SrEnod2* mRNA levels examined by northern blot hybridization. It was observed that 1 h of cycloheximide pretreatment prevented subsequent stimulation of *SrEnod2* mRNA accumulation by BAP (Figure 2.4), suggesting that protein synthesis may be involved in the observed cytokinin stimulation of *SrEnod2* mRNA accumulation. However, one must be cautious when interpreting results from experiments involving cycloheximide, because of its effect on global translation.

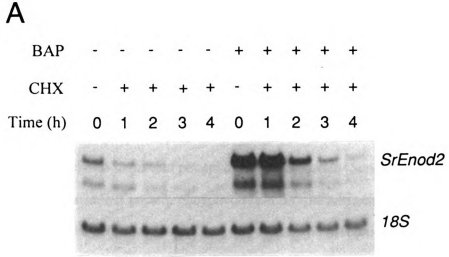
Figure 2.4. Effect of cycloheximide on *SrEnod2* mRNA accumulation. Two-week-old *S. rostrata* seedlings were pretreated for 1 h with 140 μM cycloheximide (CHX), and then extensively washed and further incubated for an additional 4 h with or without 10 μM BAP. Control seedlings were untreated with cycloheximide. Following the treatments, RNA from roots were isolated and analyzed by northern blot hybridization.


-BAP 4 h
+BAP 4 h
1 h CHX, -BAP 4 h
1 h CHX, +BAP 4 h
SrEnod2
18S

In addition to the inhibition of *SrEnod2* mRNA accumulation, a slight decrease in *SrEnod2* mRNA was observed in samples treated with cycloheximide with or without BAP present. Because of the observed slight decrease in levels of *SrEnod2* mRNA, it was subsequently examined whether cycloheximide could cause the decay of *SrEnod2* mRNA over time after an initial BAP treatment of 4 h. Plants were treated with BAP for 4 h, after which cycloheximide was added, and RNA was harvested every hour for 4 h. Control plants were not treated with BAP, but were treated with cycloheximide. Northern blot analysis revealed that the *SrEnod2* mRNA from control plants and from plants treated with BAP decayed upon treatment with cycloheximide (Figure 2.5), whereas the *SrEnod2* mRNA from plants treated with BAP but not with cycloheximide increased approximately 2-fold over this same time period of 4 h (refer to Figure 2.3 between 4 h and 8 h).

An interesting observation was made after analyzing the samples from BAP-treated plants at the 0- and 2-h time points after cycloheximide addition (Figure 2.5B). Between the 0- and 2-h time points, the *SrEnod2* mRNA increased slightly, and then decayed dramatically. The apparent increase between 0 and 1 h may be explained by a lag time required for cycloheximide to be effective, but the reason for the rapid decay after the 1-h time point is not known. In addition, between 2 and 4 h after cycloheximide application, *SrEnod2* mRNA from both BAP-treated and untreated samples decayed similarly. To exclude the possibility that cycloheximide altered the levels of gene expression in general, we reprobed the northern blots with the *βATPase* gene. We found that the levels of *βATPase* mRNA were not affected by cycloheximide, suggesting that the observed effects of cycloheximide on *SrEnod2* mRNA accumulation may be specific to the *SrEnod2* gene (data not shown). Although direct evidence for an inhibition of cellular translation, and/or translation of the *SrEnod2* mRNA by cycloheximide has not been demonstrated under the conditions used in these experiments, these data may suggest that ongoing protein

Figure 2.5. Decay of *SrEnod2* mRNA in the presence of cycloheximide. A, Two-week-old *S. rostrata* seedlings were initially treated with (+) or without (-) 10 μM BAP for 4 h after which 140 μM cycloheximide (CHX) was added and RNA from roots of 5 seedlings isolated every hour for 4 h and analyzed by northern blot hybridization. B, Signals were quantified using phosphorimager analysis. A value of 100% on the ordinate represents the maximum mRNA levels detected, as standardized to 18S rRNA signals. Square, With BAP; circle, without BAP.

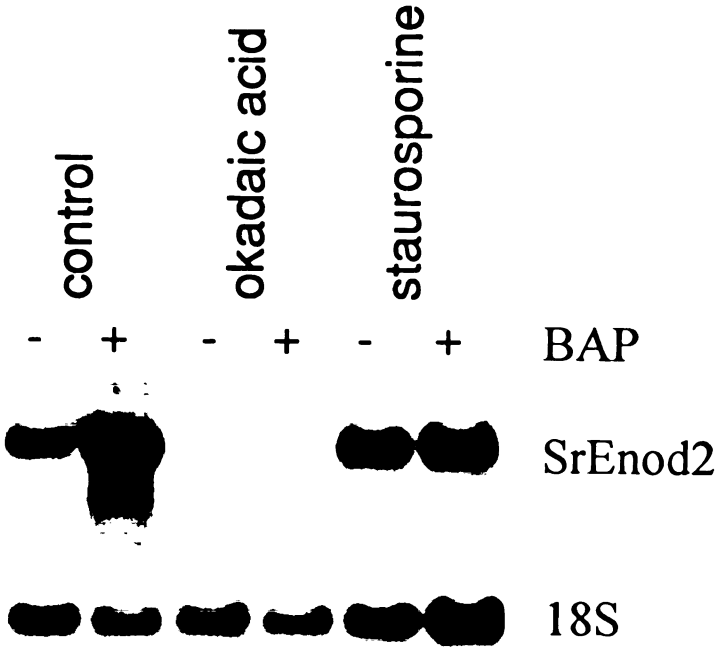

A
BAP - - - - - + + + + +
CHX - + + + + - + + + +
Time (h) 0 1 2 3 4 0 1 2 3 4
SrEnod2
18S
B
% mRNA remaining
120
100
80
60
40
20
0
0
1
2
3
4
Time (h)
- BAP
+ BAP

synthesis is required for the continued accumulation of both basal and cytokinin-stimulated levels of *SrEnod2* mRNA.

Both cellular protein phosphatases and protein kinases may be required for the accumulation of *SrEnod2* mRNA by cytokinin

Since cycloheximide was found to inhibit the enhancement of *SrEnod2* mRNA accumulation by cytokinin, we postulated that cytokinin may act far upstream of the actual event that actually causes the accumulation of *SrEnod2* mRNA. It has been shown that cytokinins are involved in protein phosphorylation cascades (Sano and Youssefian, 1994). We sought to determine if protein phosphatases and protein kinases, the mediators of signal transduction pathways (Bowler and Chua, 1994), may be involved in this mechanism, as has been documented for ethylene signal transduction pathways (Raz and Fluhr, 1993). To test this hypothesis, the pharmacological agents okadaic acid and staurosporine were used. Okadaic acid is an inhibitor of protein phosphatases type PP1 and PP2B at submicromolar concentrations and of PP2A at higher concentrations (Hunter, 1995). Okadaic acid and staurosporine, a broad spectrum protein kinase inhibitor (Tamaoki, 1991), were applied at concentrations previously used for inhibitor studies in plants (Dominov et al, 1992; Raz and Fluhr, 1993; Crowell, 1994) to two-week-old *S. rostrata* seedlings in the presence or absence of BAP for 4 h. Although okadaic acid and staurosporine are useful tools for studying signal transduction, it should be noted that the nature of their *in vivo* action is not known, so results obtained using these inhibitors should be interpreted with caution. Okadaic acid was found to abolish *SrEnod2* mRNA accumulation in response to BAP and to cause a dramatic decrease in the levels of *SrEnod2* mRNA in both BAP-treated and untreated *S. rostrata* seedlings below levels observed in seedlings not treated with BAP (basal level; Figure 2.6). Staurosporine was also found to inhibit *SrEnod2* mRNA accumulation in response to BAP, but did not reduce the amount of *SrEnod2* mRNA below

Figure 2.6. Effect of okadaic acid and staurosporine on *SrEnod2* mRNA accumulation. Two-week-old *S rostrata* seedlings were preincubated for 1 h with 0.5 μM okadaic acid or 10 μM staurosporine, then treated with (+) or without (-) 10 μM BAP. RNA was isolated from roots and analyzed by northern blot hybridization.


control
okadaic acid
staurosporine
- + - + - +
BAP
SrEnod2
18S

basal levels. Levels of both *βATPase* and *actin* mRNA were found to be unaffected by the inhibitor treatments (data not shown).

The observation that both inhibitors of protein phosphatases and kinases also inhibit the accumulation of *SrEnod2* mRNA in the presence of cytokinin suggests the involvement of these classes of proteins in the cytokinin signal transduction pathway.

DISCUSSION

In this paper we present evidence for a posttranscriptional mechanism of enhancement of *SrEnod2* mRNA accumulation in response to cytokinin. Nuclear run-on experiments demonstrated that the *SrEnod2* transcription rate cannot account for the increase in *SrEnod2* mRNA accumulation in response to cytokinin treatment. In addition, the northern blot hybridization analysis of nuclear versus total RNA accumulation showed that cytokinin enhancement of mRNA accumulation appears to occur primarily in the cytoplasm. There is an approximately 4-fold enhancement in *SrEnod2* mRNA accumulation in total RNA and an approximately 1.2-fold enhancement in nRNA populations, which may be due to a small increase in transcription and/or altered mRNA stability in the nucleus. It has been documented that mRNA stability in the nucleus plays an important role in gene expression (Belgrader and Maquat, 1994; Peters and Silverthorne, 1995). Of course, it also cannot be ruled out that the observed small enhancement in nuclear *SrEnod2* mRNA accumulation is due to contamination from cytoplasmic RNA. Nevertheless, these data correlate well with the nuclear run-on data, suggesting the involvement of mRNA stability changes in response to cytokinin treatment in the cytoplasm (Jackson, 1993; Sullivan and Green, 1993; Beelman and Parker, 1995).

Cytokinin seems to be acting indirectly on *SrEnod2* mRNA levels, since it was found that the inhibition of translation by cycloheximide counteracted the enhancement of *SrEnod2* mRNA accumulation by cytokinin. Cytokinin enhancement of the light-dependent expression of the nitrate reductase gene has been shown by nuclear run-on assays to be partially regulated at the transcriptional level. In addition, nitrate reductase mRNA levels do not appear to be affected by cycloheximide (Lu et al., 1990). Lu et al. (1990) proposed that concurrent protein synthesis is not required for cytokinin enhancement of the gene for nitrate reductase (*NR*) mRNA accumulation. This appears to be in direct contrast to the enhancement of *SrEnod2* mRNA accumulation by cytokinin

observed here, which appears to require concurrent protein synthesis and to occur primarily at the posttranscriptional level. Surprisingly, cycloheximide was found to cause the rapid decay of *SrEnod2* mRNA from plants treated with BAP and from those that were not treated with BAP. This could be the result of an the inhibition of translation of a labile protein required for *SrEnod2* mRNA accumulation, or translation of the *SrEnod2* mRNA itself may be required for its stabilization.

In contrast to the observed rapid decay of *SrEnod2* mRNA caused by cycloheximide application, the *SrEnod2* mRNA levels of plants treated with BAP and then washed free of the hormone did not return to basal levels, but oscillated for 36 h in a manner similar to those of plants continuously treated with BAP for 36 h. A circadian fluctuation of mRNA accumulation enhancement was recently reported in genes of the anthocyanin biosynthesis pathway (Deikman and Hammer, 1995). It that study, cytokinin treatment caused a dramatic dampening in the diurnal fluctuations of mRNA accumulation compared with plants grown without cytokinin, which suggests that cytokinin acts independently of the mechanism responsible for the circadian rhythm. This is in contrast to our observation that an increase in amplitude of *SrEnod2* mRNA occurs in the presence of cytokinin. It remains to be determined whether this oscillation in *SrEnod2* mRNA is regulated in a circadian manner. In a study by Pilgrim et al. (1993) on nitrate reductase mRNA, expression of which is known to be induced by cytokinin (Dilworth and Kende, 1974; Lu et al., 1990), it was found that nitrate reductase mRNA accumulation also oscillates in a circadian fashion. These oscillations in mRNA accumulation were shown by nuclear run-on assays to occur at a posttranscriptional level; therefore, the involvement of posttranscriptional mechanisms for the oscillation in *SrEnod2* mRNA accumulation may, also be plausible.

Okadaic acid and staurosporine were found to cause different effects on *SrEnod2* mRNA accumulation. Okadiac acid inhibited cytokinin enhancement of *SrEnod2* mRNA accumulation, causing a dramatic decrease in its accumulation. We postulated that if

okadaic acid was able to inhibit cytokinin enhancement of *SrEnod2* mRNA accumulation, then an inhibitor of protein kinases would probably cause an enhancement of *SrEnod2* mRNA accumulation in the absence of cytokinin. This was found not to be the case, since staurosporine inhibited *SrEnod2* mRNA accumulation in response to cytokinin, but did not cause a reduction in basal levels, as okadaic acid did. These data suggest the involvement of both dephosphorylation and phosphorylation processes, perhaps acting on different proteins of the cytokinin signal transduction pathway. The observation that okadiac acid and cycloheximide cause a dramatic decrease in *SrEnod2* mRNA accumulation in samples treated or untreated with BAP may indicate that both inhibitors act on the same protein(s). In addition, these data suggest that both basal and cytokinin-enhanced levels of *SrEnod2* mRNA may be due to the same mechanism of mRNA accumulation. It may be that basal levels of *SrEnod2* mRNA are caused by a small flux through the signal transduction pathway. The idea of a "flux", as described by Bowler and Chua (1994), relates to basal level activity of signaling intermediates, for example, protein kinases or phosphatases. In the case of *SrEnod2*, endogenous cytokinin levels could be responsible for maintaining a basal level of *SrEnod2* mRNA, as observed by northern blot analysis, and this increase in cytokinin levels above a threshold could cause an increase in *SrEnod2* mRNA accumulation.

Crowell (1994) showed for cytokinin-enhanced expression of the soybean allergen gene, okadaic acid inhibits cytokinin induction, whereas for the cytokinin-enhanced accumulation of *pLS216* RNA, staurosporine inhibits enhancement of mRNA accumulation by cytokinin, and okadaic acid only slightly enhances mRNA accumulation (Dominov et al., 1992). These studies, along with the observations presented here, suggest that the phosphorylation state of proteins are important for cytokinin signal transduction. However, differences may exist in the requirement for phosphorylation among different pathways for accumulation of mRNAs enhanced by cytokinin, as is the case for the signal transduction pathways for other plant hormones (Bowler and Chua, 1994). The evidence

presented in this study strongly suggests that *SrEnod2* is stabilized in the cytoplasm in response to cytokinin. However, direct proof of regulated mRNA stability of *SrEnod2* mRNA by cytokinin remains to be provided, as does the regulated mRNA stability of any mRNA by a plant hormone. The use of a posttranscriptional mechanism of regulation of the *SrEnod2* gene may provide a more rapid means of controlling gene expression than the use of transcriptional regulation (Green 1993).

There are questions that remain to be answered. For example, what is the role of the Enod2 protein in nodulation and the significance of cytokinin regulation of *SrEnod2* gene expression? It has previously been postulated by van de Wiel et al. (1990) that the Enod2 protein may play a role in creating an oxygen diffusion barrier in the parenchyma cell layer necessary for protecting the oxygen-sensitive nitrogenase enzyme in the infected cells of the nodule. A direct test of this hypothesis has not yet been concluded, so the role of the Enod2 protein in nodule development and functioning remains unclear. With regard to cytokinin regulation of *SrEnod2* gene expression, previous studies aimed at understanding the involvement of cytokinin in nodule development have indirectly shown an involvement of cytokinin in nodule development. One such study by Cooper and Long (1994) demonstrated the partial rescue of a nonnodulating *Rhizobium* strain (Nod^-) by the overexpression of the *Agrobacterium tumefaciens* cytokinin biosynthesis gene(*tzs*). The Nod^- *Rhizobium* overexpressing the *tzs* gene was capable of eliciting the production of nodule-like structures on alfalfa roots, indicating, at least in part, that a localized production of cytokinin can produce a phenocopy of nodule morphogenesis.

Preliminary transgenic work has shown the requirement of the *SrEnod2* 3' downstream region for tissue-specific expression in uninfected roots of the legume *Lotus japonicu* (D.L. Silver and F.J. de Bruijn, unpublished data), which may also point to a posttranscriptional mechanism for the regulation of *SrEnod2* gene expression by cytokinin. It now remains to be determined whether DNA elements of the *SrEnod2* gene required for

cytokinin regulation are the same as those required for tissue-specific expression in the nodule. This type of analysis may shed some light on the relationship between cytokinin regulation and the tissue-specific expression pattern of the *SrEnod2* gene in nodules and in roots. In addition, we are trying to develop the use of the *SrEnod2* gene as a model system for understanding cytokinin signal transduction, and to isolate *trans*-acting factors responsible for cytokinin enhancement of plant gene expression.

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Chapter 3

REGULATION OF *LJENOD2* GENE EXPRESSION BY ETHYLENE

ABSTRACT

The early nodulin gene *Enod2* encodes a Pro-rich protein which is expressed in the nodule parenchyma. Expression of most *Enod2* genes is restricted to the nodule. However, expression of the *SrEnod2* gene from *Sesbania rostrata* is enhanced in roots upon application of cytokinin. Here, we present evidence indicating that the *Lotus japonicus LjEnod2* gene is not regulated by cytokinin, but by ethylene. Ethylene gas or submergence of *L. japonicus* roots in water caused an enhancement of *LjEnod2* mRNA accumulation. The accumulation in submerged roots was partially inhibited by AVG, and strongly inhibited by cobalt chloride. In addition, a comparison was made between the effect of ethylene on *SrEnod2* and *LjEnod2* gene expression.

INTRODUCTION

The early nodulin gene *SrEnod2* from the tropical legume *Sesbania rostrata* encodes a Pro-rich protein which is expressed in a cell-specific manner in the nodule parenchyma. The nodule parenchyma is composed of cytoplasmically dense cells which surround the cells harboring the nitrogen-fixing bacteroids (Van de Wiel et al., 1990). The function of the *SrEnod2* gene remains to be determined. However, it has been proposed to play a role in establishing an oxygen diffusion barrier (Tjepkema and Yocum, 1974; Witty et al., 1986; Van de Wiel et al., 1990). The *SrEnod2* gene is regulated both in a tissue-specific manner and by the plant hormone cytokinin (Dehio and de Bruijn, 1992; Chen et al., 1996). *SrEnod2* tissue-specific expression in the nodule parenchyma has been recently shown to require sequences located in the 3'UTR of the *SrEnod2* gene, and not in its promoter (Chen et al., 1996).

The expression of the *SrEnod2* has been shown to be specifically induced in roots in a time- and concentration-dependent manner by the application of cytokinin, in the absence of inoculation by the microsymbiont *Azorhizobium caulinodans* (Dehio and de Bruijn 1992). The mechanism underlying this induction appears to involve posttranscriptional events (Silver et al. 1996). The tissue-specific expression of *Enod2* genes from several legumes is conserved. However, the phenomenon of cytokinin regulation is not. The only two legumes known in which *Enod2* gene expression in roots is induced by cytokinin are *Sesbania rostrata* and alfalfa (Hirsch and Fang, 1994), although the alfalfa *Enod2* message which accumulates in roots upon cytokinin treatment is of a different molecular weight than is the *Enod2* message from nodules of the same plant (A. Hirsch, personal communication).

The effects of cytokinin on plant development have been shown in some instances to mediated through the action of ethylene. For example, Bertell and Eliasson (1992) demonstrated in pea roots that the application of cytokinin inhibits root elongation and

formation of lateral roots and stimulates swelling of the root tips. They further demonstrated that BAP caused an up to a 4-fold increase in ethylene levels in roots. These effects were found to be counteracted by the application of cobalt ions to inhibit ethylene production. Although there is no direct evidence that cytokinin induces the synthesis of ACC synthase mRNA or ACC activity, it is known that cytokinins act to increase auxin levels, which, in turn, can induce ACC mRNA accumulation (Yu et al., 1979; Yoshii and Imaseki, 1981; Bertell and Eliasson, 1992). Su et al. (1995) genetically tested the hypothesis that cytokinin action is coupled to ethylene action (Lieberman, 1979). They demonstrated that the inhibitory effects of BA on root and hypocotyl elongation were partially blocked in the ethylene-resistant mutants *ein1-1* and *ein2-1*, as well as by inhibitors of ethylene action (Cary et al., 1995). Furthermore, the finding that cytokinin and ethylene responses are coupled was reinforced by the demonstration that the cytokinin-resistant mutant *ckr1* (Su and Howell, 1992) is allelic to *ein2* (Cary et al., 1995). Reports of the effects of ethylene on the process of nodule development are limited (Lee and LaRue, 1992a, 1992b). In general, ethylene appears to be inhibitory to early stages of nodule development (Lee and LaRue 1992b).

In a search for other legumes in which the *Enod2* gene is inducible by cytokinin, and to determine if ethylene is indeed involved in cytokinin-mediated regulation, I initiated a study of the *Enod2* gene of the model legume *Lotus japonicus*. In this report I show that expression of the *LjEnod2* gene is not regulated by cytokinin, but, surprisingly, is regulated by ethylene. In addition, I present data comparing the effects of ethylene on the expession of the cytokinin-induced *SrEnod2* gene to the ethylene-induced *LjEnod2* gene. To our knowledge this is the first report of an ethylene-inducible nodulin gene.

METHODS

Plant material and growth conditions

Lotus japonicus GIFU seeds were germinated on B5 media (Sigma)/ 0.8% agar for one week and then grown for two weeks in pots containing a sterilized vermiculite mix (fine vermiculite 2: coarse vermiculite 3: sand 1) in a growth chamber. The growth chamber conditions were 18 h light/ 6 h dark; 23^0 C day/ 18^0 C night. Plants in pots were watered with Hoaglands media once a week. *S. rostrata* seeds were germinated on wet gel blot paper (Schleicher and Schuell, Keene, NH) and seedlings grown in soil composed of Metromix (Hummert International, Earth City, MO) and sand (2:1) at 30^0C, with a 18 h light, 28^0C/6 h dark, 22^0C regime for two weeks in growth chambers with 75% RH. For all chemical treatments, plants were washed free of soil and incubated in water containing 1 µM Benzylaminopurine (BAP) [Sigma], 0.5 mM $CoCl_2$ (Sigma), and/or100 µM Aminoethoxyvinyl-glycine (AVG) [Sigma]. Ethylene treatments were performed as follows: Seedlings of *L. japonicus* plants in pots were placed in desiccating jars injected with 40ppm ethylene gas. *S. rostrata* seedlings were washed free of soil, placed upright in a glass beaker lined with gel blot paper (Schleicher and Schuell, Keene, NH) soaked in water or water containing 1 µM BAP. Seedlings in beakers were placed in desiccating jars containing 40 µL/L ethylene gas. Control seedlings of *L. japonicus* and *S. rostrata* were treated in the same manner, and placed in desiccating jars containing solid $KMnO_4$ (Sigma) to absorb endogenously produced ethylene from plants. After all chemical treatments, roots were isolated and frozen in liquid nitrogen for RNA isolation.

Ethylene gas measurements

Root tissue was isolated from seedlings, and individual roots were placed in 10 ml glass tubes sealed with rubber stoppers. The roots were incubated for 1 h, then 1ml of gas volume was removed and injected into a gas chromatograph (Varian 3400 gas chromatograph, Sugar Land, TX). Measurements were expressed as nL of ethylene evolved *per* hour *per* gram of fresh weight of tissue.

Northern blot analysis.

10 µg of RNA was electrophoresed in 1.2% (w/v) agarose gels in Mops buffer (20mM Mops, 1.0 mM EDTA, 5.0 mM sodium acetate, pH 7.0) containing 5.4% (v/v) formaldehyde. Gels were blotted onto 0.22 µM NitroPlus nitrocellulose membrane (Micron Separation, Inc., Westborough, MA). Membranes were probed with a [^{32}P] dATP labeled DNA fragment containing the *SrEnod2* coding region generated by random priming (Boehringer Mannheim). Filters were reprobed with a *Sesbania rostrata* ubiquitin coding gene (*ubi-1*) DNA probe as a loading control. All filters were washed at 65°C in 2.0 x SSC, 0.1% SDS for 20 min; 0.5 x SSC, 0.1% SDS for 20 min; and 0.1 x SSC, 0.1% SDS for 15 min. The signals were quantified using phosphorimager analysis (400B model PhosphorImager, Molecular Dynamics, Inc., Sunyvale, Ca.).

RESULTS

Water treatment induces *LjEnod2* mRNA accumulation in *L. japonicus* roots via ethylene production

Twenty-one-day-old *L. japonicus* plants were removed from the soil and incubated in water for 4 and 8 h in the presence of 1 uM BAP; control plants were incubated in water without BAP for 8 h, *LjEnod2* mRNA from roots of plants treated with BAP accumulated to the same levels as in the control plants (Figure 3.1). In order to determine if incubation in water alone was the cause of *LjEnod2* mRNA accumulation, 21-day-old *L. japonicus* plants grown in soil were incubated for 4 and 8 h in water, and RNA was isolated from their roots. Control plants were not incubated in water, and RNA was isolated from roots immediately after removal from soil. As shown in Figure 3.2, over an 8 h period, *LjEnod2* mRNA accumulated to a level nearly 17-fold higher than in the control plants. Therefore, accumulation of *LjEnod2* mRNA appears to be the result of submergence of roots in water, rather than to cytokinin action, as seen in *S. rostrata* (Dehio and de Bruijn, 1992).

Next I sought to determine whether ethylene production during flooding (Bradford and Dilley, 1978; Bradford and Yang, 1980) could be responsible for the enhanced accumulation of *LjEnod2* mRNA. To test this hypothesis, roots were treated with aminoethoxyvinylglycine (AVG) to inhibit the activity of ACC synthase, the rate limiting enzyme in ethylene biosynthesis. In addition, cobalt chloride was used to inhibit the activity of ACC oxidase. Plants were incubated in water containing 100 μM AVG for 4 and 8 h. Separate plants were incubated in water containing 0.5 mM cobalt chloride. The concentrations of inhibitors correspond to those reported in the literature (Yu and Yang, 1979). Control plants were incubated in water without inhibitors for 4 and 8 h. As shown in Figure 3.2, AVG caused a greater than 2-fold reduction in *LjEnod2* mRNA

Figure 3.1. *LjEnod2* mRNA levels in roots treated with cytokinin. Northern blot analysis showing *LjEnod2* mRNA accumulation after the indicated treatments. 21-day-old *L. japonicus* plants were treated with 1 μM BAP for 4 and 8 h by submergence of roots in water. Roots of intact control plants were incubated in water for 8 h without BAP. The blot was reprobed with the *ubi-1* gene as a loading control.

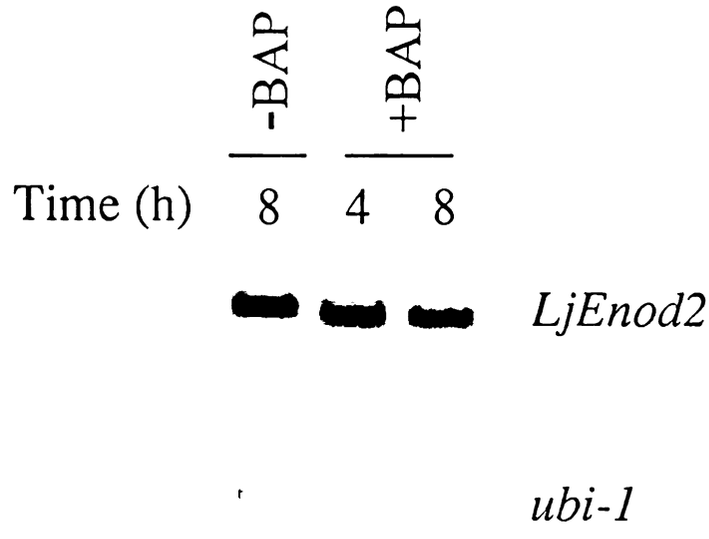
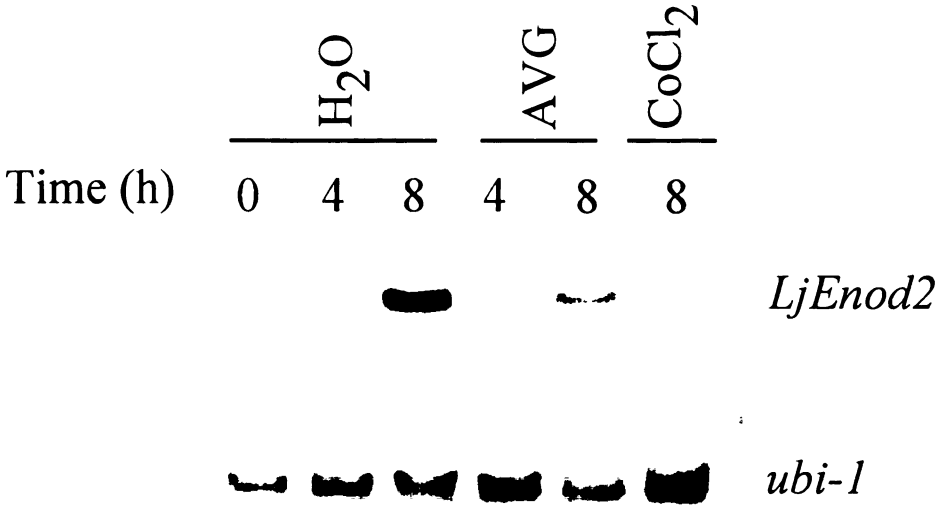
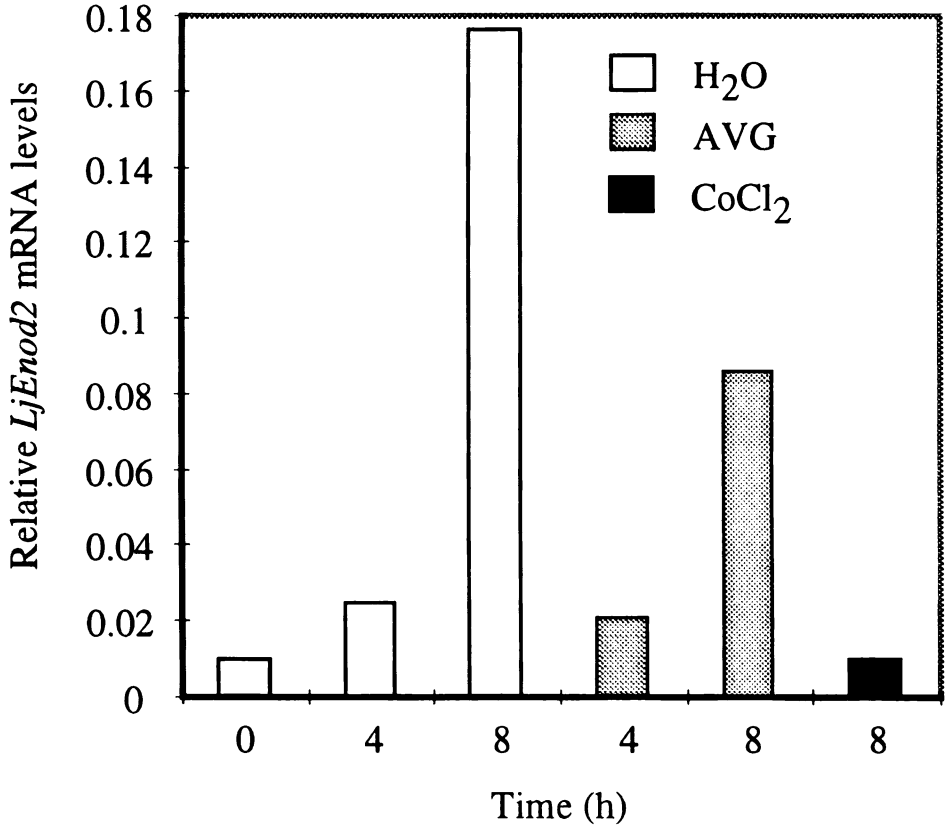

-BAP
+BAP
Time (h)
8
4
8
LjEnod2
ubi-1

Figure 3.2. *LjEnod2* mRNA levels in roots treated with H_2O, AVG and $CoCl_2$. 100 μM AVG and 0.5 mM $CoCl_2$ for the times indicated, then total RNA was isolated from roots. Treatment with water alone was carried out as a control. A, Northern blot analysis showing *LjEnod2* mRNA accumulation after the indicated treatments. The blot was reprobed with the *ubi-1* gene as a loading control. B, Quantification of the northern blot shown in A using phosphorimager analysis. Values are reported as the ratio of *LjEnod2/ ubi-1* signals.

A


H2O
AVG
CoCl2
Time (h)
0 4 8 4 8 8
LjEnod2
ubi-1

B


Relative LjEnod2 mRNA levels
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
H2O
AVG
CoCl2
0 4 8 4 8 8
Time (h)

accumulation in response to flooding. This remaining level of *LjEnod2* mRNA accumulation at 8 h in the presence of AVG was probably due to the fact that AVG did not completely abolish the synthesis of ethylene, as verified by gas chromatography (Figure 3.3). In contrast, cobalt chloride drasticly reduced the accumulation of *LjEnod2* mRNA to levels comparable to the 0-h control (Figure 3.2).

To determine whether ethylene could induce the accumulation of *LjEnod2* mRNA directly, *L. japonicus* plants grown in soil were treated with 40 µL/L ethylene gas for 4 and 8 h, respectively. Control plants were treated under similar conditions for 8 h without ethylene. As shown in Figure 3.4 *LjEnod2* mRNA was found to accumulate in ethylene treated plants to a level 4-fold higher than that in control plants. The level of accumulation was found to be lower than that seen in plants removed from soil and incubated in water (Figure 3.2).

Ethylene effects on *S. rostrata SrEnod2* gene expression

To compare the effects of ethylene on *SrEnod2* to *LjEnod2* gene expression, two-week-old *S. rostrata* seedlings were removed from the soil and treated for 8 h with 40 µL/L ethylene gas with or without the addition of 1 uM BAP. The controls consisted of *S. rostrata* seedlings treated on wetted bloting paper (air control) or with their roots submerged in water. Treatment with ethylene in the absence of BAP resulted in a 2-fold increase of *SrEnod2* mRNA levels, as compared to the air control (Figure 3.5). Seedlings which were submerged in water with or without BAP, accumulated overall lower levels of *SrEnod2* mRNA when compared to the air controls. Treatment of two-week-old *S. rostrata* plants with 100 µM AVG showed no significant effect on *SrEnod2* mRNA accumulation (Figure 3.6), although ACC synthase activity was nearly abolished, as indicated by ethylene gas measurements (Figure 3.3). However, treatment of *S. rostrata* plants with 0.5 mM cobalt chloride significantly reduced *SrEnod2* mRNA levels to below control levels, which is likely an effect by the non-specific action of cobalt ion.

Figure 3.3. Ethylene levels in *S. rostrata*, and *L. japonicus* roots treated with AVG. Plants were incubated with or without 100 μM AVG for the times indicated. Roots were isolated, and ethylene production was determined by gas chromatagraphy. Ethylene levels are reported as the average ethylene produced (nL ethylene *per* h *per* g fresh weight) from three independent root systems. The error bars represent the SD.

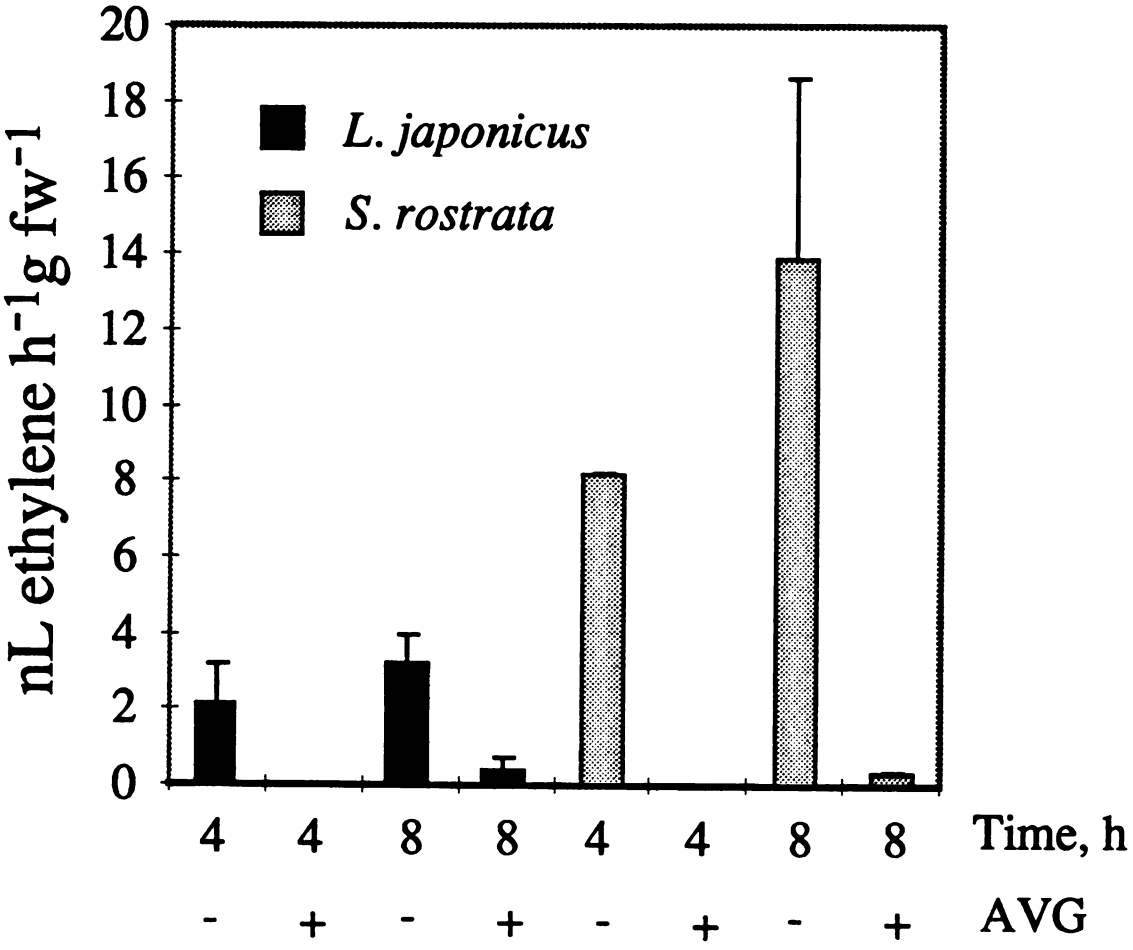
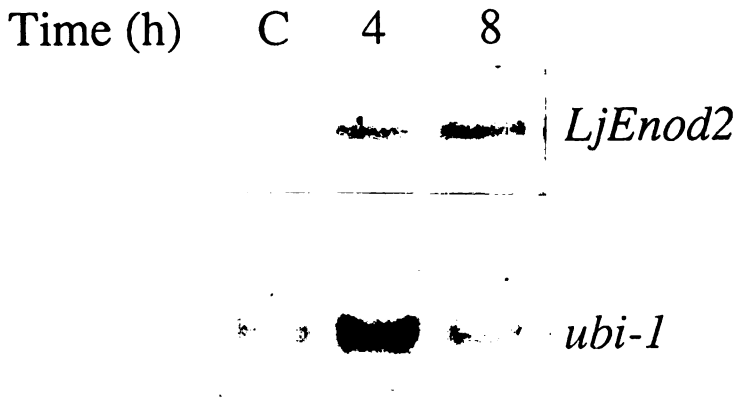

20
18
16
14
12
10
8
6
4
2
0
nL ethylene h-1g fw-1
L. japonicus
S. rostrata
4 4 8 8 4 4 8 8
Time, h
- + - + - + - +
AVG

Figure 3.4. *LjEnod2* mRNA levels in roots from seedlings treated with ethylene. 21-day-old *L. japonicus* plants were treated with 40 nL/L ethylene gas for the times indicated. Control (C) seedlings were incubated under similar conditions for 8 h without ethylene gas. A, Northern blot analysis showing *LjEnod2* mRNA accumulation after the indicated treatments. The blot was reprobed with the *ubi-1* gene as a loading control. B, Quantification of northern blot shown in A using phosphorimager analysis. Values are reported as the fold increase in the ratio of *LjEnod2/ ubi-1* signals over the control level, which has been set at zero.

A


Time (h)
C
4
8
LjEnod2
ubi-1

B

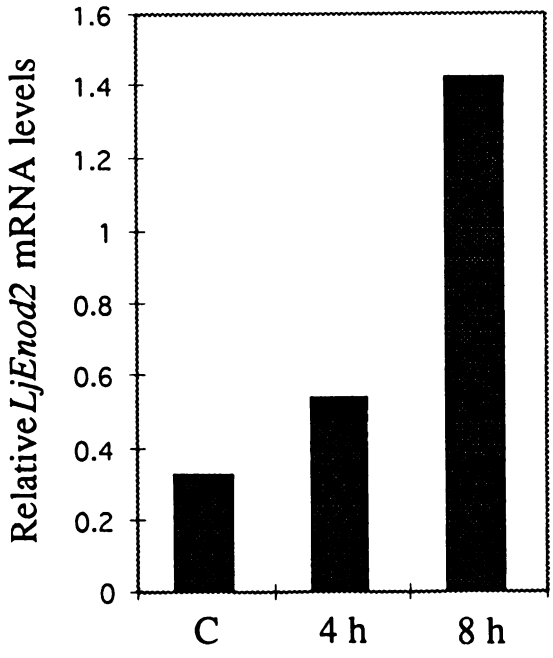
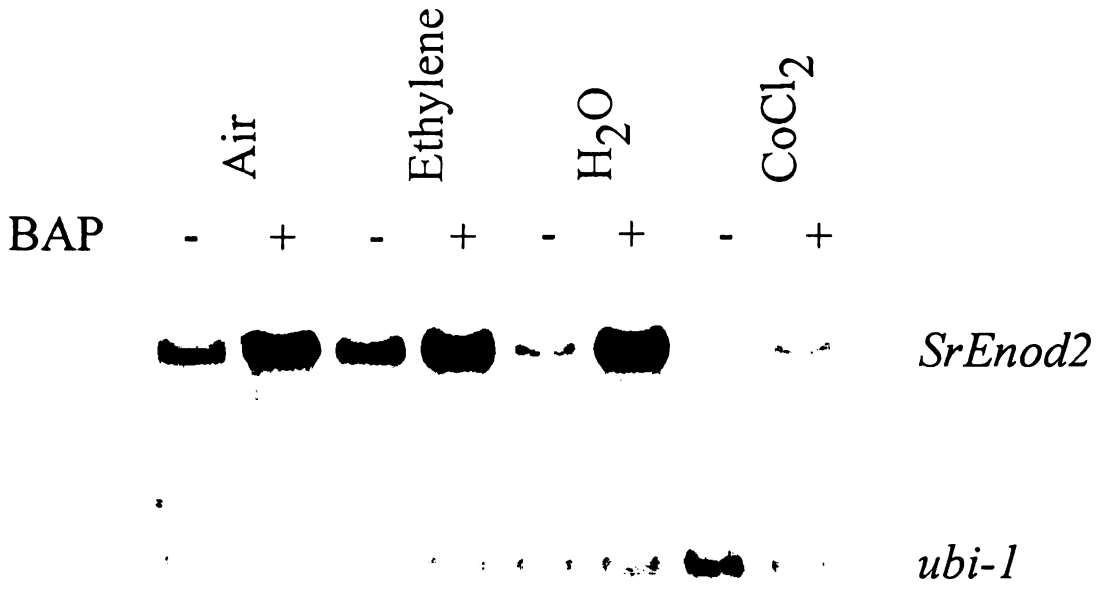

Relative LjEnod2 mRNA levels
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
C
4 h
8 h

Figure 3.5. *SrEnod2* mRNA levels in roots of seedlings treated with H_2O, BAP, ethylene and $CoCl_2$. Two-week-old *S. rostrata* seedlings were incubated for 8 h with or without 1 μM BAP in the presence of 40 nL/L ethylene gas, and/or 0.5 mM $CoCl_2$. Air and water treatments for 8 h served as controls. A, Northern blot analysis showing *SrEnod2* mRNA accumulation after the indicated treatments. The blot was reprobed with the *ubi-1* gene as a loading control. B, Quantification of the northern blot shown in A using phosphorimager analysis. Values are reported as the ratio of *SrEnod2/ ubi-1* signals.

A


Air
Ethylene
H_2O
$CoCl_2$
BAP - + - + - + - +
SrEnod2
ubi-1

B

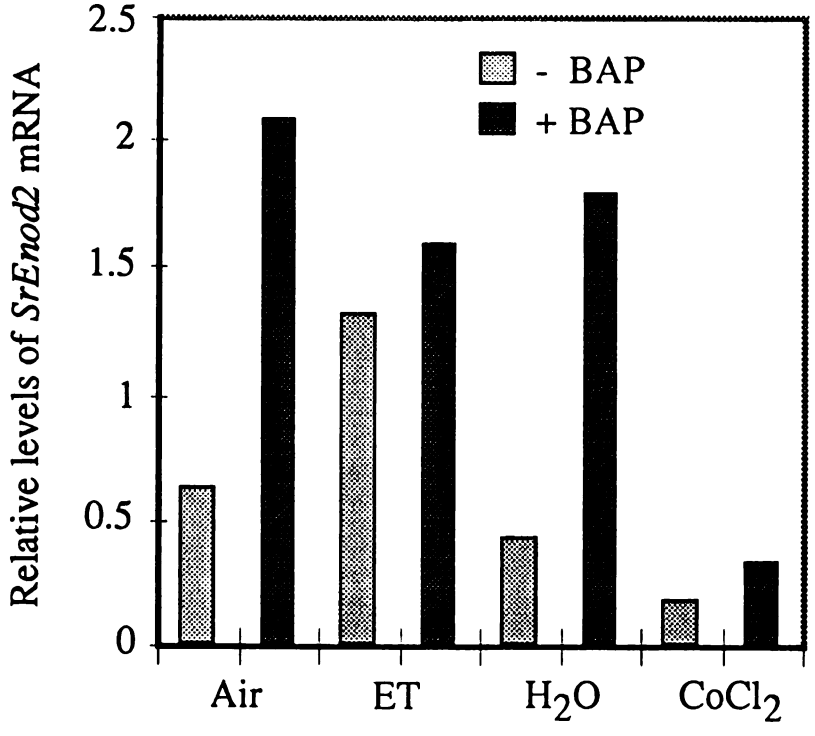
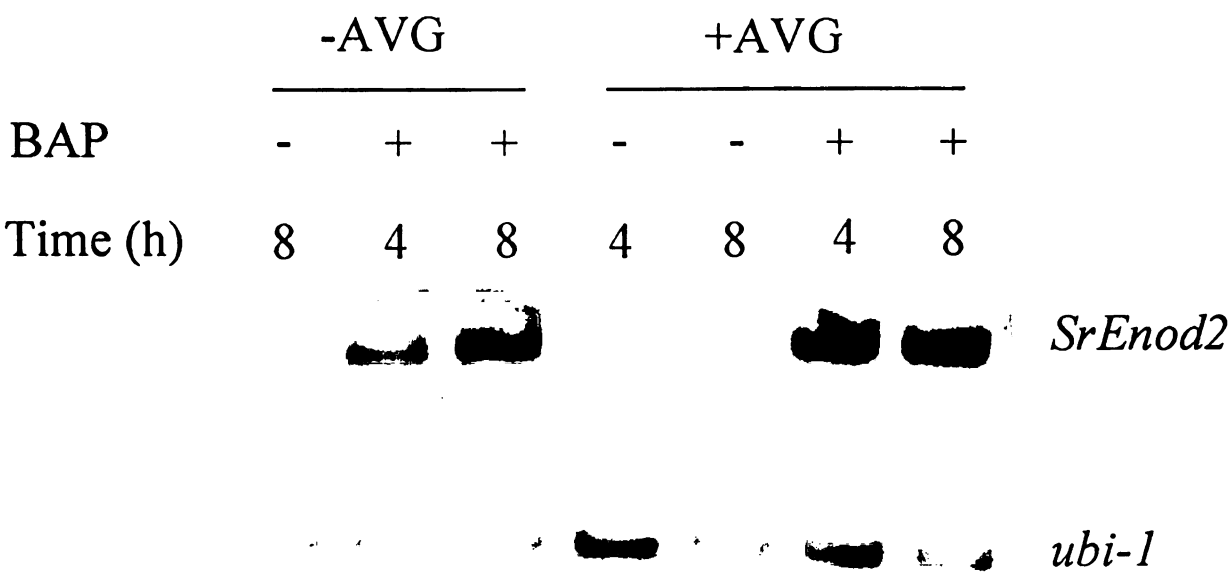
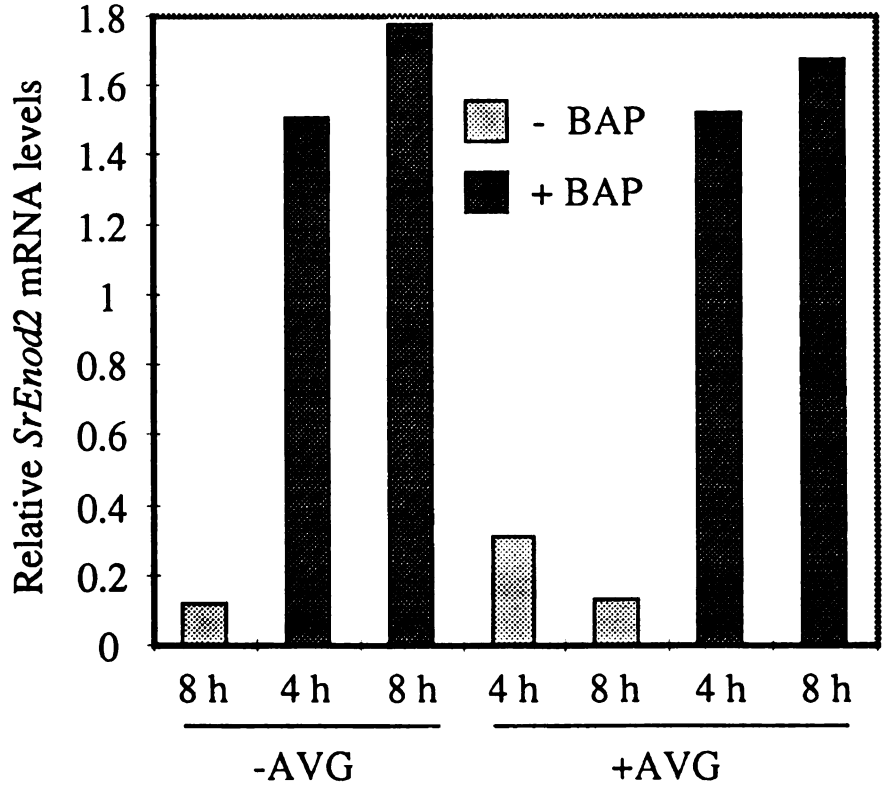

Relative levels of SrEnod2 mRNA
2.5
2
1.5
1
0.5
0
- BAP
+ BAP
Air
ET
H_2O
$CoCl_2$

Figure 3.6. *SrEnod2* mRNA levels in roots treated with AVG. Two-week-old *S. rostrata* seedlings were incubated with or without 100 μM AVG for the times indicated, then total RNA was isolated from roots. A, Northern blot analysis showing *SrEnod2* mRNA accumulation after the indicated treatments. The blot was reprobed with the *ubi-1* gene as a loading control. B, Quantification of the northern blot shown in A using phosphorimager analysis. Values are reported as the ratio of *SrEnod2/ ubi-1* signals.

A


-AVG
+AVG
BAP
- + + - - + +
Time (h)
8 4 8 4 8 4 8
SrEnod2
ubi-1

B


Relative SrEnod2 mRNA levels
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
- BAP
+ BAP
8 h 4 h 8 h 4 h 8 h 4 h 8 h
-AVG
+AVG

DISCUSSION

The results presented here demonstrate that expression of the *LjEnod2* gene is, at least in part, regulated by ethylene. However, the level of increase in *LjEnod2* mRNA by ethylene treatment was lower compared to levels observed after submergence in water. One explanation for this difference may be that the higher increase in ethylene production in the roots may be caused by stress from removal from the pots (wounding) and flooding of the roots. Another possible explanation is that the roots beneath the soil do not encounter high enough levels of ethylene gas due to low diffusion of ethylene gas through soil. Still another explanation may be that some other factor related to water stress is causing a higher accumulation of *LjEnod2* mRNA. Nonetheless, these results show that ethylene gas can indeed cause the accumulation of *LjEnod2* mRNA. This raises important questions regarding the involvement of ethylene in nodulation, since a role for ethylene as a positive regulator of nodulin gene expression has never been reported.. In fact, all reports thus far indicate an inhibitory role of ethylene in nodulation of pea, but not of other legumes (Fearn and LaRue, 1991; Lee and LaRue, 1992; Guinel and LaRue, 1992).

The tissue-specific expression pattern of the *LjEnod2* gene is similar to that seen for the *SrEnod2* gene (D. Silver and F. J. de Bruijn, unpublished data), as well as for other *Enod2* genes (Hirsch et al., 1989; van de Wiel et al., 1990). However, the response of *Enod2* genes from different legumes to hormones is very different. The expression of most *Enod2* genes is not regulated by cytokinin, but only induced during the process of nodulation, while expression of the *SrEnod2* and the *LjEnod2* genes appears to be mediated by two different plant growth regulators. Why is the expression of the *LjEnod2* gene inducible by ethylene, and the *SrEnod2* gene by cytokinin? The difference in habitat of these plants may present an explanation. *S. rostrata* naturally grows in water-logged soil, conditions which can induce ethylene production, while *L. japonicus* grows in semi-dry soil. Therefore, *S. rostrata* may have adopted the cytokinin signal transduction

pathway to regulate its *Enod2* gene, since it may have a high tolerance for ethylene, or a decreased sensitivity to ethylene.

Fearn and LaRue (1991) have shown that the low level of nodulation by the *sym* 5 mutant of pea can be restored to nearly normal levels by treatment of the plants with Co^{+2}, AVG and Ag^{+}. However, the plants' ACC and ethylene levels in *sym 5* were not found to be significantly different from wild-type plants. Ethylene inhibitors were also found to partially restore nodulation by another nodulation mutant of pea, E 107 (Guinel and LaRue, 1992). Moreover, application of ethylene to wild-type pea plants inhibits nodulation on the primary and lateral roots, and this inhibition is relieved by application of Ag^{+} (Lee and LaRue, 1992). On close examination, it was determined that ethylene may inhibit very early stages of nodulation, particularly the advancement of infection threads through the root cell epidermis and outer cortex (Lee and LaRue, 1992). However, it is not known if ethylene plays an inhibitory role in other legume plants. Recently, it was found by Heidstra et al. that ACC oxidase is expressed in cells opposite protophloem poles in pea roots (S. Peck, personal communication). They postulate (S. Peck, personal communication) that during the early events of nodulation, in which cortical cells opposite protoxylem poles begin to divide, expression of ACC oxidase in adjacent cells opposite protophloem cells will set up an ethylene gradient. In turn, the ethylene gradient will reflect an inhibition gradient for the formation of nodule primordia. Nodule primordia which reach a certain stage of development may then become insensitive to ethylene. In order to better understand the roles ethylene may play in nodulation of pea and in other legumes, it will be important to determine the expression pattern of ACC oxidase during nodulation of different species of legumes, and to identify other ethylene-regulated nodulin genes.

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Chapter 4

THE *SRENOD2* GENE IS CONTROLLED BY A CONSERVED CYTOKININ SIGNAL TRANSDUCTION PATHWAY

The contents of this chapter have been submitted for publication in Plant Physiol, 1996 (Silver, Deikman, and de Bruijn)

ABSTRACT

Here we report the use of *Arabidopsis thaliana* to study the mechanism of cytokinin-mediated mRNA accumulation from the *Sesbania rostrata* early nodulin gene *SrEnod2*. A chimeric *gus* gene, comprised of 1.9 kb of the *SrEnod2* 5' promoter region fused to the *gus* reporter gene and terminated by 2.5 kb of the *SrEnod2* 3' downstream region, was found to be expressed in roots of transgenic *Arabidopsis* plants in an identical tissue-specific pattern as in roots of the legume *Lotus japonicus*. Moreover, the expression of this construct was found to be specifically enhanced by cytokinin, but repressed by ethylene, in roots of transgenic *Arabidopsis* plants. Auxin, GA_3 or ABA, did not affect the expression of the chimeric *SrEnod2 5'-gus-SrEnod2 3'* construct. The *SrEnod2* 3' downstream region was found to be essential for both root-specific and cytokinin enhancement of expression in transgenic *Arabidopsis* plants. Cytokinin enhancement of *SrEnod2 5'-gus-SrEnod2 3'* gene expression was found to be abolished in the *Arabidopsis* cytokinin-resistant mutant *cyr1*, but was regulated normally in the auxin resistant mutant, *axr2*. These results indicate the existence of a conserved cytokinin signal transduction pathway between legumes and *Arabidopsis*, and open the way for its genetic dissection.

INTRODUCTION

Since the discovery of cytokinins (Miller et al., 1955), a considerable amount of research has been dedicated to understanding the physiological, developmental and molecular mechanisms of cytokinin action in plants. Cytokinins appear to play a crucial role in a large number of developmental processes, including regulation of cell division, nutrient mobilization, chloroplast development, and apical dominance (Binns, 1994). The complexity of cytokinin effects has hindered the elucidation of the mechanism of cytokinin action. Many genes have been described which are regulated by cytokinin (Chen et al., 1993; Hobbie et al., 1994), but the majority of these genes are also co-regulated by other factors, such as other hormones, light, nutrient status, and environmental stresses (Chen et al, 1993). One exception may be the *SrEnod2* gene from the tropical legume *Sesbania rostrata*. This gene was shown to be specifically regulated in response to physiologically relevant concentrations of cytokinin, without requiring other hormones, physical or chemical stresses as co-regulating factors (Dehio and de Bruijn, 1992). The steady state level of *SrEnod2* mRNA is increased within 2 hours of treatment of non-nodulated roots with cytokinin, and this relatively short response time suggests that the mRNA increase could be a primary effect of cytokinin treatment (Dehio and de Bruijn, 1992). Study of the regulation of this gene by cytokinins may reveal general mechanisms of cytokinin action.

The *SrEnod2* gene codes for a proline-rich protein, which is expressed during nodule formation, specifically in the nodule parenchyma (Van de Wiel et al., 1990), and is highly conserved among legume plants (Franssen et al., 1987; Dickstein et al., 1988; Govers et al., 1990; Van de Wiel et al., 1990; Szczyglowski and Legocki, 1990; Dehio and de Bruijn, 1992). The nodule parenchyma is typically comprised of a few cytoplasmically dense cell layers that surround the central cells of the nodule, which harbor the nitrogen fixing bacteroids (Van de Wiel et al., 1990). Based on the location of expression of the *Enod2* gene in pea and soybean, it has been proposed that the Enod2 protein may function

as part of an oxygen diffusion barrier (Tjepkema and Yocum, 1974; Witty et al., 1986; Van de Wiel et al., 1990). Such an oxygen diffusion barrier might protect the bacterial nitrogen fixing enzyme nitrogenase, which is highly oxygen sensitive, in the infected core cells of the nodule. However, the function of the Enod2 protein still remains to be determined.

The results of nuclear run-on assays using nuclei from cytokinin-treated roots of *S. rostrata* suggested that *SrEnod2* mRNA accumulation in response to cytokinin is due to posttranscriptional regulation (Silver et al., 1996). The accumulation of *SrEnod2* mRNA appears to occur primarily in the cytoplasm, and is inhibited by the translational inhibitor cycloheximide, inhibitors of protein phosphatases, and kinases (Silver et al., 1996). In addition, *SrEnod2* mRNA levels decay rapidly upon treatment with cycloheximide, suggesting that ongoing protein synthesis is required for the accumulation, as well as maintenance, of *SrEnod2* mRNA levels (Silver et al., 1996). In order to better understand the mechanism of this regulatory process, it is necessary to delimit the sequence elements of the *SrEnod2* locus which are required for the observed cytokinin response and to isolate proteins involved in the regulation of cytokinin-modulated mRNA stability.

In this report, I have developed a model system using *Arabidopsis thaliana* to study the mechanism of *SrEnod2* mRNA accumulation mediated by cytokinin. I chose *Arabidopsis*, in part, because of the existence of mutants affected in hormone response that would be useful for investigating the regulation of this gene, in particular, the *cytokinin resistant 1 (cyr1)* mutant, which appears to be specifically resistant to cytokinin based on a root-elongation assay (Deikman and Ulrich, 1995). The *cyr1* mutation results in a ten-fold reduction in sensitivity of roots to BAP, but does not affect sensitivity to IAA and ACC. However, this mutant has increased sensitivity to ABA. The *cyr1* mutation is pleiotropic, causing shoot abnormalities, including limited leaf production, and the development of a single infertile flower. Some aspects of this phenotype are consistent with a defect in cytokinin response, such as a failure of the cotyledons to expand, reduced accumulation of

chlorophyll, and failure to accumulate anthocyanins in response to cytokinins. However, the *cyr1* gene has not been isolated, and the nature of the defect is not precisely known.

Many plant developmental processes require the action of both cytokinin and auxin. The classical example is the initiation of cell division in tobacco pith cells, which will expand upon auxin treatment, but only divide in the presence of cytokinin (Miller et al., 1955, 1956). Root nodule formation has also been shown to involve both auxin and cytokinin action (Allen et al., 1953; Hirsch et al., 1989; Cooper and Long, 1994). Therefore, I also used the *auxin resistant 2 (axr2)* mutant in this study. The *axr2* mutation (Wilson et al., 1990) is a dominant mutation which confers auxin-resistant root growth, as well as root growth resistance to inhibition by ethylene and ABA. The *axr2* mutation is also pleiotropic in that *axr2* mutants are dwarfs, affected in both shoot and root gravitropic growth, and lack root hairs.

In this report, I demonstrate that a chimeric gene consisting of the *SrEnod2* 5' upstream region, including its 5' UTR, fused to the *gus* reporter gene, and terminated by the *SrEnod2* 3' downstream region, is specifically regulated by cytokinin, and is expressed in roots of transgenic *Arabidopsis* plants in an identical fashion as in the legumes *S. rostrata* and *Lotus japonicus*. In addition, expression was found to be localized to newly expanding leaves, trichomes of these leaves, and stipules of *Arabidopsis*. Both tissue-specific and cytokinin responsive regulation was shown to be dependent on the *SrEnod2* 3' downstream region. I generated *cyr1* mutants that harbored the *SrEnod2* 5'-*gus*-*SrEnod2* 3' gene, and found that GUS activity was not induced upon cytokinin treatment in these plants. This data provides genetic evidence for the hypothesis that the *SrEnod2*5'-*gus*-*SrEnod2*3' construct is regulated by a cytokinin signal transduction pathway that is conserved between legumes and *Arabidopsis*.

METHODS

Plant material and growth conditions

Transgenic *Lotus japonicus* GIFU seeds were germinated on B5 media (Sigma)/ 0.8% agar for one week and then grown for two weeks in pots containing a sterilized vermiculite mix (fine vermiculite 2: coarse vermiculite 3: sand 1) in a growth chamber. The growth chamber conditions were 18 h light/ 6 h dark; 23^0 C day/ 18^0 C night. Plants in pots were watered with Hoagland's media once a week. Transgenic *Arabidopsis thaliana* Columbia plants as well as the *Arabidopsis* mutant lines *cyr1* and *axr2* (obtained from the *Arabidopsis* Biological Resource Center at The Ohio State University) were germinated and grown on MS media (Gibco BRL)/ 0.8% agar for 2 weeks in a growth chamber. *Arabidopsis* plants were grown in *Arabidopsis* Mix (Hummert International, Earth City, MO) in a growth chamber for approximately one month after which plants were allowed to dry, and seeds were harvested. Growth chamber conditions were 18 h light/ 6h dark; 21^0 C day/18^0 C night.

For cytokinin treatments of transgenic *L. japonicus* plants, three-week-old plants were washed free of soil and incubated in distilled water containing 1 μM BAP for 4 h, 8 h, 12 h, and 24 h, respectively. Control plants were incubated only in distilled water for the same time period. Hormone treatments of transgenic *Arabidopsis* plants were carried out as follows: Two-week-old transgenic *Arabidopsis* plants grown on solid MS plates, as described above, were transferred to solid MS plates containing 1 μM BAP for the time period indicated in the Results section. Their roots were isolated and frozen in liquid nitrogen. Treatments with 10 μM GA_3 (Abbott Labs, Chicago, IL), 10 μM ABA (Sigma), 0.02 μM NAA (Sigma), and 40 μL/L ethylene were performed for 24 h under the same

condition as the cytokinin treatments. Ethylene incubations were carried out in a desiccating jar.

Chimeric *gus* reporter gene construction

The construction of the *SrEnod2* 3' and *nos* 3' constructs was carried out as follows: A 5.3 kb *EcoR*1 fragment containing 1.9 kb of *SrEnod2* 5' upstream region, 1.0 kb *SrEnod2* coding region, and 2.5 kb *SrEnod2* 3' downstream sequence was subcloned from the genomic clone λCD1 (Dehio, 1989) into pBluescript KS- vector (Clonetech, Palo Alto, CA). *BamH*I and *Sac*I restriction sites were introduced immediately preceeding the translational initiation site and after the translational stop codon, respectively, as described by Kunkel et al. (1985). The oligonucleotide for introducing the *BamH*I site was (5')GAGTAGTGTAGAGA-GGA**TCC**TTCTATCTAT(3'). The location of the original translational start codon is indicated in bold. The oligonucleotide for introducing a *Sac*I site was (5')GTAGTGGTA-GTGGTTGGAGCTC**TTAA**TTTTTTTTGG(3'). The translational stop codon TAA is indicated in bold. Both the 1.9 kb *EcoR*I-*BamH*I *SrEnod2* 5' fragment and the 2.5 kb *Sac*I-*EcoR*1 *SrEnod2* 3' fragment were subcloned into the pBI101.1 vector (Clonetech) flanking the *gus* reporter gene. This construct was designated as *SrEnod2* 3'. The *nos* 3' construct was made by subcloning only the 1.9 kb *EcoR*I-*BamH*I *SrEnod2* 5' fragment into pBI101.1 5' in front of the *gus* reporter gene which is terminated by the nopaline synthase 3' terminator (*nos 3'*).

Plant transformation

The *SrEnod2* 3' *gus* and *nos* 3' constructs were introduced into the genome of *L. japonicus* GIFU seedlings according to the method of Handberg et al. (1992). *A. thaliana*

Columbia seedlings were transformed with the *SrEnod2* 3' *gus* and *nos* 3' constructs using vacuum infiltration, according to the procedure described by Bent et al. (1994).

RNA isolation and northern blot analysis

Total RNA was isolated from roots of transgenic *Arabidopsis* plants according to the method of Verwoerd et al., (1989). Poly(A) RNA was isolated from total RNA by oligo-dT column chromatography as described by the manufacturer's instructions (5 prime-3 prime, Inc., Boulder, Co). RNA gel blot analysis was performed as according to Maniatis et al. (1982). Approximately 3 ug of poly(A) RNA was used for analysis. The coding region of the *E. coli* ß-glucuronidase gene (*uidA; gus*) was used as a DNA probe for RNA gel analysis. A DNA fragment encompassing part of the *eif*4 coding region (Taylor et al., 1993) was used as a loading control on northern blots. [^{32}P]-dATP labelled *gus* and *eif*4 DNA probes were generated by random priming (Boehringer Mannheim, Indianapolis, IN). Hybridization and washing conditions of RNA gel blots were as previously described (Silver et al., 1996). *gus* and *eif*4 mRNA levels were quantified using phosphorimager analysis (Model 400B PhosphorImager, Molecular Dynamics, Sunnyvale, CA). Radioactive blots were exposed to autoradiography film (Dupont, Wilmington, DE) at -75^{0}C.

Quantification of GUS enzymatic activity

Quantification of GUS enzymatic activity was performed according to the procedure described by Jefferson et al. (1987). The fluorometric assay was performed using a fluorescence spectrophotometer (Model F-2000; Hitachi, Tokyo, Japan). GUS acitivity was reported as picomoles of 4-methylumbelliferone produced *per* minute *per* milligram of

protein in total protein extracts. Total protein concentrations in the extracts were measured using the Bradford assay, with BSA as a standard (Bradford, 1976).

GUS histochemical staining

GUS histochemistry was performed according to the procedure described by Jefferson et al. (1987). Stained plant tissues were photographed under bright-field using a Wild Makroskop M420 light microscope (W. Nuhsbaum, Inc., McHenry, Illinois). Microscopical analysis of stained root cross sections were performed as follows: Root tissues were embedded in historesin (Reichert-Jung; Cambridge Instruments, Heidelberg, Germany) as described by De Block and DeBrouwer (1992). 10 uM sections were made using an ultramicrotome. Sections were then photographed under bright-field using an Axiophot microscope (Zeiss; Oberkochen, Germany).

Genetic crosses

Arabidopsis plants heterozygous for the *cyr1* mutation (*Cyr1/cyr1*) were crossed to kanamycin-resistant wild-type plants (*Cyr1/Cyr1*) harboring the *SrEnod2* 3' *gus* construct. Kanamycin-resistant F_1 plants were allowed to self-pollinate, and kanamycin-resistant F_2 seedlings were selected. Kanamycin-resistant *cyr1* homozygotes were identified based on their phenotype (Diekman and Ulrich, 1995). Homozygous *axr2* mutants (*axr2/axr2*) were crossed to kanamycin-resistant wild-type plants (*Axr2/Axr2*) harboring the *SrEnod2* 3' *gus* construct. Kanamycin-resistant F_1 plants were allowed to self-pollinate, and kanamycin-resistant F_2 seedlings were selected. Kanamycin-resistant *axr2* mutants were identified based on their phenotype (Wilson et al., 1990).

RESULTS

Tissue Specificity of *SrEnod2* gene expression in *L. japonicus* : Requirement of the *SrEnod2* 3' region.

We previously demonstrated that the enhancement of *SrEnod2* mRNA accumulation by cytokinin is regulated at a posttranscriptional level (Silver et al., 1996). Based on this study I sought to define the *SrEnod2* sequence(s) that are necessary for *SrEnod2* mRNA accumulation in response to cytokinin, and to determine whether the *SrEnod2* gene is expressed in a tissue-specific manner in roots, as it is in nodules (Van de Wiel et al., 1990; Chen et al., 1996). To this end, we generated a fusion of a 1.9 kb *SrEnod2* 5' region, including its 5' UTR, to the *gus* reporter gene, flanked by 2.5 kb of the *SrEnod2* 3' region, and designated this construct "*SrEnod2* 3'" (Figure 4.1). The 3' region includes sequences immediately downstream of the stop codon of the *SrEnod2* gene. A second construct was prepared in which the *SrEnod2* 3' region was replaced with the 3' terminator of the nopaline synthase gene, and was designated "*nos* 3'." These two constructs were introduced into the genome of *L. japonicus* via *Agrobacterium tumefaciens*-mediated transformation. Since transformation of *S. rostrata* is not yet possible, *L. japonicus* was used because it is a legume which is self-pollinating, diploid, and amenable to molecular genetics (Handberg et al., 1992).

Three independent lines harboring the constructs described in Figure 4.1 were recovered, and GUS histochemistry was performed. GUS activity in plants harboring the *SrEnod2* 3' construct was localized to specific regions in the root system (Figure 4.2A and B), and was not detected in any other tissues (Data not shown). GUS activity was seen mainly along the primary root in the vascular cylinder and at emerging lateral root sites. The only emerging lateral roots which displayed GUS activity were those located on the primary root, and primary or secondary fully developed lateral roots (Data not shown).

GUS activity was not found in lateral roots produced later during development. Even in the primary root, GUS expression was not observed along the full length of the root and was never found in root tips (Data not shown). Thus, the reporter gene expression pattern was found to be restricted to the primary root and the first emerging lateral roots in a gradient-like fashion. *L. japonicus* plants transformed with the *nos* 3' construct failed to show detectable GUS activity in any tissues (Figure 4.2C, and Data not shown). These data indicate that the *SrEnod2* 3' region is required for root-specific expression in *L. japonicus*. To determine which particular root cell types expressed the *SrEnod2* 3' construct, cross sections of stained primary roots were examined. As shown in Figure 4.2I, GUS activity was localized in the pericycle cells and in the pith parenchyma cells of transgenic *L. japonicus* roots.

Next, I wanted to determine whether the *SrEnod2* 3' and the *nos* 3' chimeric genes would respond to cytokinin treatment. Roots from intact two-week-old transgenic plants harboring the *SrEnod2* 3' and *nos* 3' constructs were incubated in a solution containing 1 μM BAP for 4 h, 8 h, 12 h , and 24 h, respectively. Control plants were incubated in solution without BAP for the same time periods. Treatment with BAP was not found to lead to an enhancement of GUS activity in plants harboring the *SrEnod2* 3' construct, as determined by GUS fluorometric assays (Data not shown). Plants transformed with the *nos* 3' construct failed to show GUS activity in the presence or absence of cytokinin (Data not shown). These results are consistent with the observations that in most legumes, other than *S. rostrata* and alfalfa, *Enod2* genes are not regulated by application of cytokinin (Dehio and de Bruijn, 1992; Hirsch and Fang, 1994).

Figure 4.1. Structure of the chimeric *SrEnod2-gus* reporter gene constructs. The *SrEnod2* 3' construct is composed of 1.9 kb of the *SrEnod2* 5' upstream region (sequences from -1900 to +23), fused to the *gus* reporter gene (cross hatched box), and terminated by 2.5 kb of the *SrEnod2* 3' downstream region (sequences from +1017 to +2500). The black box represents the *SrEnod2* 3' UTR (250 bp). The *nos* 3' construct is identical to the *SrEnod2* 3' construct except that the *SrEnod2* 3' downstream region was replaced by the 300 bp nopaline synthase 3' end (*nos* 3').

Constructs

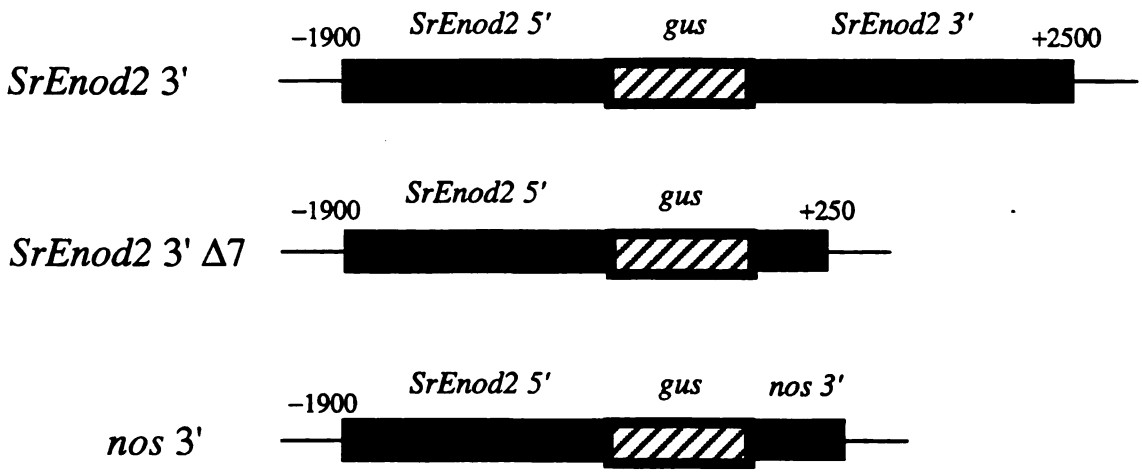
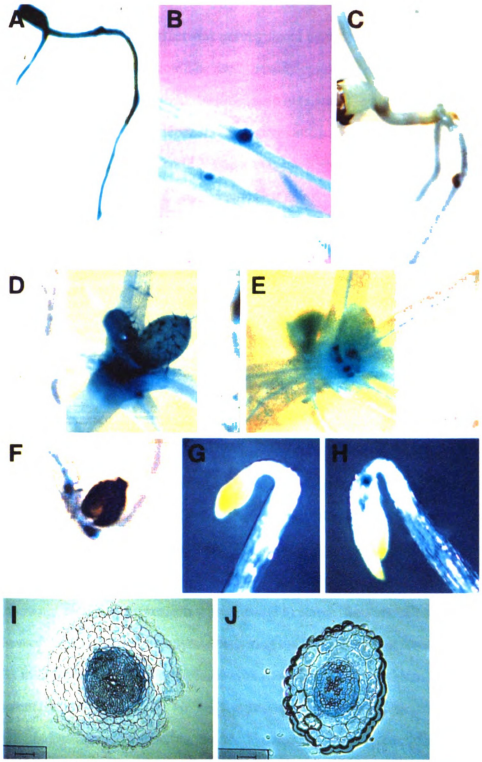

SrEnod2 3'
−1900
SrEnod2 5'
gus
SrEnod2 3'
+2500
SrEnod2 3' Δ7
−1900
SrEnod2 5'
gus
+250
nos 3'
−1900
SrEnod2 5'
gus
nos 3'

Figure 4.2. Histochemical localization of GUS activity in transgenic *L. japonicus* and *Arabidopsis* plants harboring the *SrEnod2* 3' and *nos* 3' constructs. A, GUS staining pattern of 21-day-old *L. japonicus* roots. B, GUS staining of *L. japonicus* emerging lateral roots. C, Absence of GUS staining in *L. japonicus* plants harboring the *nos* 3' construct. D, GUS staining in emerging leaves and stipules of two-week-old *Arabidopsis* seedlings harboring the *SrEnod2* 3' construct. E, GUS staining in stipules of two-week-old *Arabidopsis* seedlings harboring the *nos* 3' construct. F, GUS expression in emerging lateral roots of *Arabidopsis* seedlings harboring the *SrEnod2* 3' construct. G, Absence of GUS expression in the meristematic region of etiolated plants harboring the *nos* 3' construct. H, GUS expression in the meristematic region of etiolated plants harboring the *SrEnod2* 3' construct. I and J, Cross sections of primary roots from 21-day -old *L. japonicus* and two-week-old *Arabidopsis* plants harboring the *SrEnod2* 3' construct, respectively. Sections were generated from the primary root within 2 centimeters from the hypocotyl/root junctions in both plant species. GUS expression can be seen in the vascular cylinder and pericyle cells of both plant species.


A
B
C
D
E
F
G
H
I
J

Expression of the *SrEnod2* 3' *gus* chimeric gene in *Arabidopsis* roots: Tissue specificity and cytokinin induction.

Since the *SrEnod2* 3' construct was not regulated by cytokinin in *L. japonicus*, I investigated whether *Arabidopsis* could be used to study cytokinin regulation of *SrEnod2* expression. To this end, I transformed *Arabidopsis* plants with the *SrEnod2* 3' and *nos* 3' constructs, and recovered seven independent transformants for each construct. *Arabidopsis* plants bearing the *SrEnod2* 3' construct expressed GUS in a tissue-specific manner similar to that observed in *L. japonicus*. That is, expression was confined to the vascular cylinder in the hypocotyl-root junction and to the first few emerging lateral roots along the primary root, and weak expression was observed in the upper part of the primary root (Figure 4.2F and Data not shown). Interestingly, GUS expression in emerging lateral roots was found to be confined to the primary root, and first and second fully developed lateral roots while lateral roots that developed later did not show GUS activity, as had been observed in transgenic *L. japonicus* plants (Figure 4.2F, 4.3B and Data not shown). In contrast to *L. japonicus*, GUS activity was also detected in certain parts of the shoot of *Arabidopsis* plants. GUS activity was visible in emerging leaves, stipules and trichomes (Figure 4.2D) and in the meristematic region of etiolated transgenic plants harboring the *SrEnod2* 3' construct (Figure 4.2H). GUS activity was not found in the meristematic region of transgenic plants harboring the *nos3'* construct (Figure 4.2G). GUS expression decreased as the leaves expanded, and was completely absent in fully developed leaves (Data not shown). No GUS expression was detected in stems, flowers, siliques, and rosette leaves of mature *Arabidopsis* plants harboring the *SrEnod2* 3' construct (Data not shown). As in *L. japonicus*, GUS expression was localized to cells of the root pericycle and pith parenchyma (Figure 4.2J). No GUS expression was seen in any tissues of transgenic *Arabidopsis* plants harboring the *nos* 3' construct, with the exception of stipules (Figure 4.2E and Data not shown).

To examine whether the *SrEnod2* 3' and *nos* 3' constructs were regulated by cytokinin in *Arabidopsis*, two-week-old transgenic plants were placed onto solid media containing 1 μM BAP for 12 h. Controls plants were incubated for the same time period on solid media lacking BAP. The plants were then stained for GUS activity. The intensity of GUS staining in roots of plants bearing the *SrEnod2* 3' construct was found to be substantially increased in response to cytokinin treatment (compare Figure 4.3A and B). However, cytokinin treatment did not result in detectable GUS staining in transgenic plants harboring the *nos* 3' construct (Data not shown). In plants transformed with the *SrEnod2* 3' construct, cytokinin treatment extended GUS expression from the hypocotyl-root junction down the primary root (Figure 4.3B). An increase in GUS activity was detectable by histochemical staining after 4 h of cytokinin treatment of plants harboring the *SrEnod2* 3' construct (Data not shown). However, the time required for staining was found to be twice that of the tissue shown in Figure 4.3B, indicating that expression was significantly lower at 4 h than at 12 h.

To quantify the observed changes in expression of the *SrEnod2* 3' construct in transgenic *Arabidopsis* plants in response to cytokinin treatment, proteins were extracted from the roots of plants incubated with or without 1 μM BAP for 12, 24, and 30 h, and GUS activity was measured. GUS activity was found to increase approximately 2.8-fold in plants treated with cytokinin for 12 h, and at 30 h the GUS activity level was found to be approximately 3.5-fold greater than that in control plants (Figure 4.4). Again, no GUS activity could be detected in extracts of roots harboring the *nos* 3' construct (Data not shown).

The increase in GUS activity in response to cytokinin appeared greater when intact roots were stained for GUS activity compared to the results obtained with the fluorometric assay (Figure 4.3A and B). This apparent discrepancy may be the result of the fact that the *SrEnod2* 3' construct is only expressed in a small portion of the total root tissues, since

Figure 4.3. Histochemical staining of GUS activity in *Arabidopsis* seedlings harboring the *SrEnod2* 3' and *nos* 3' constructs treated with cytokinin. A, *Arabidopsis* seedlings harboring the *SrEnod2* 3' construct incubated on media without BAP for 12 h. B, *Arabidopsis* seedlings harboring the *SrEnod2* 3' construct treated with 1 μM BAP for 12 h. The shoots from seedlings in A and B were removed prior to photography in order to visualize GUS staining in the upper part of the root.

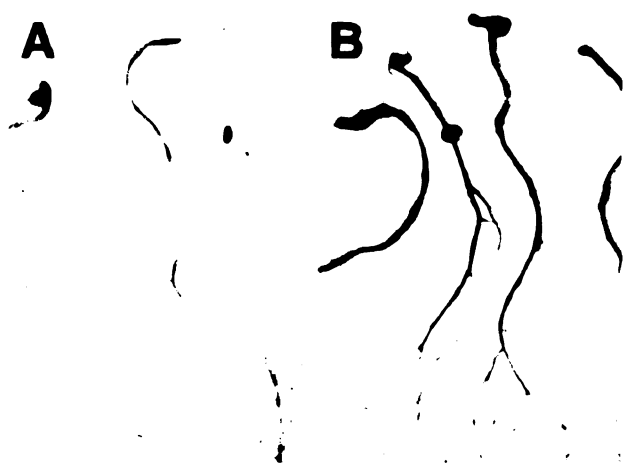
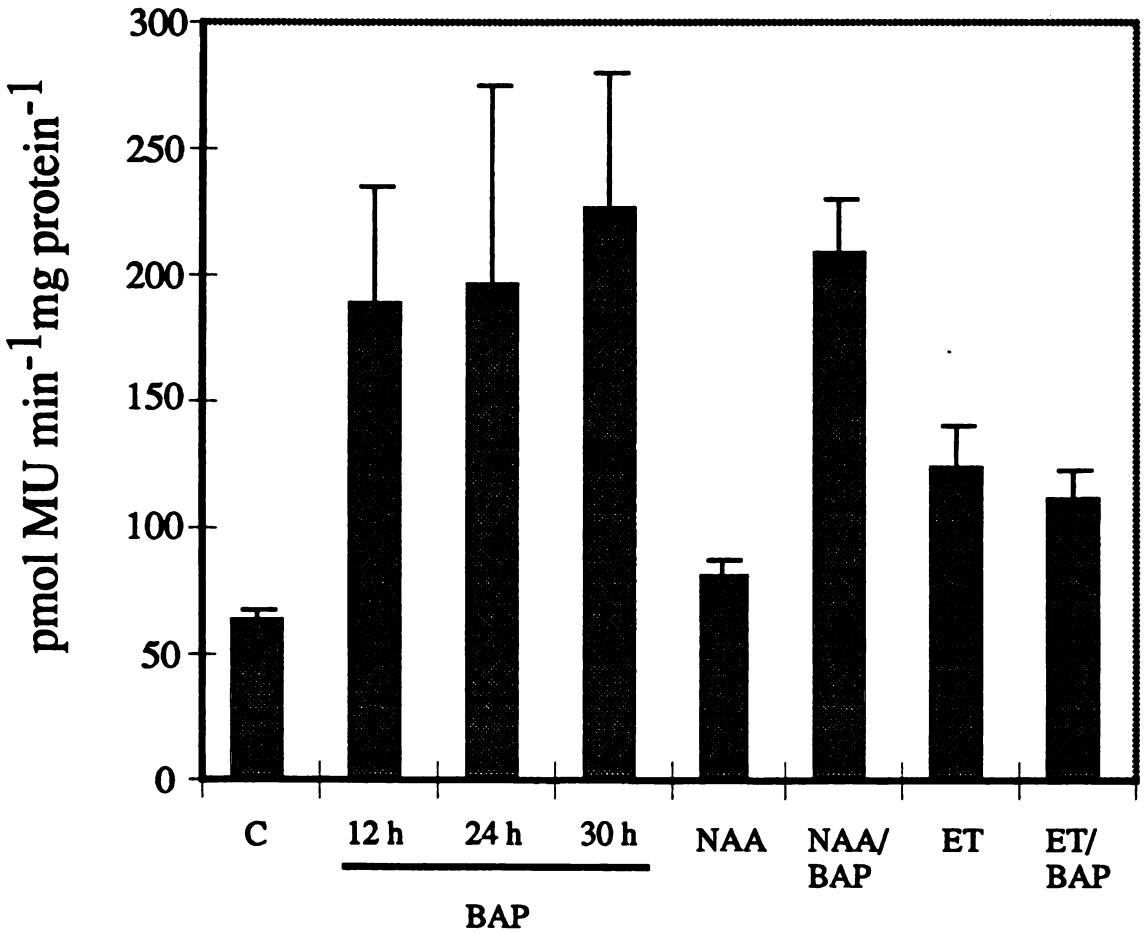

A
B

Figure 4.4. GUS activity of *Arabidopsis* plants harboring the *SrEnod2* 3' construct after hormone treatments. Two-week-old seedlings were treated with 1 μM BAP for the time periods indicated. Control seedlings (C) were incubated for 30 h on media without hormones. Separate seedlings were treated for 24 h with 0.02 μM NAA, and/or 40 μL/L ethylene. Roots were then isolated for GUS measurements. The mean GUS activity from 3 independent experiments is shown and expressed as pmol MU/min/mg protein. Each experiment included 5 seedlings. The error bars represent the SD.


pmol MU min-1mg protein-1
300
250
200
150
100
50
0
C
12 h
24 h
30 h
NAA
NAA/ BAP
ET
ET/ BAP
BAP

lateral roots fail to stain. Therefore, the use of the entire root system for GUS activity measurements, may have diluted the amount of GUS protein in the total root protein extract.

In order to rule out the possibility that cytokinin was acting non-specifically in causing an increase in GUS activity, poly(A) RNA was isolated from roots of transgenic *Arabidopsis* plants harboring the *SrEnod2* 3' and *nos* 3' constructs, incubated with or without BAP for 12 h. *gus* mRNA levels were determined by northern blot hybridization. Treatment with BAP was not found to affect the accumulation of *gus* mRNA in plants transformed with the *nos* 3' construct (Data not shown). However, in plants transformed with the *SrEnod2* 3' construct, treatment with BAP resulted in *gus* mRNA levels more than 4-fold higher than in the control (Data not shown). These data indicate that cytokinin treatment results in accumulation of the *gus* mRNA, and that the *SrEnod2* 3' region is required for this response.

Expression of the *SrEnod2* 3' *gus* construct in *Arabidopsis* is enhanced specifically by cytokinin, and inhibited by ethylene.

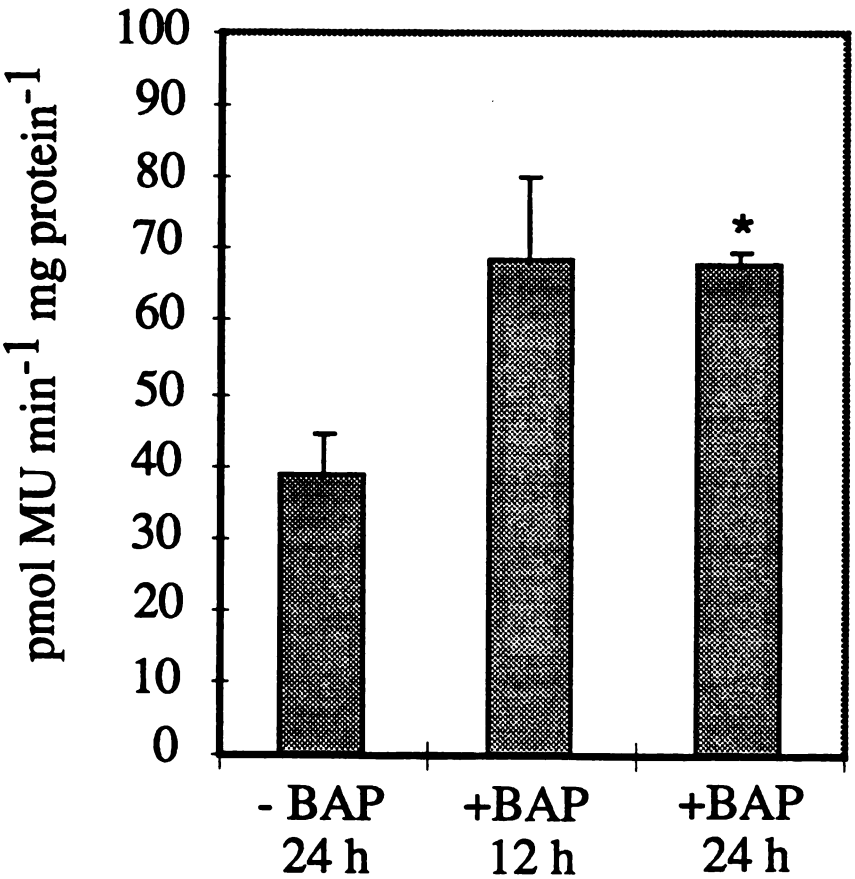
To determine whether the *SrEnod2* 3' *gus* construct was regulated solely by cytokinin or whether its expression is also influenced by other hormones, two-week-old transgenic *Arabidopsis* plants harboring this construct were incubated for 24 h in the presence of BAP, the auxin NAA, and ethylene, alone or in combination. Control plants were incubated under the same conditions without hormones. NAA was found not to affect expression of the *SrEnod2* 3' *gus* construct, and not to affect the cytokinin enhancement of GUS activity (Figure 4.4). In contrast, ethylene appeared to have a significant effect on expression of the *SrEnod2* 3' *gus* construct, since in ethylene treated plants, GUS activity was approximately 2-fold greater than in untreated controls, but ethylene inhibited further induction of GUS activity by BAP (Figure 4.4). Similar effects

of ethylene on *SrEnod2* mRNA levels have been observed in *S. rostrata* plants (D.L. Silver and F.J. de Bruijn, unpublished data). In addition, GA_3 and ABA were tested and did not have any effect on GUS activity (Data not shown).

The *SrEnod2* 3' UTR specifies cytokinin inducibility and tissue-specificity

To define the region of the *SrEnod2* 3' locus which is responsible for tissue-specificity and cytokinin inducibility, a deletion of the *SrEnod2* 3' sequence was constructed, so that only 250 bp of the 3' region, comprising the *SrEnod2* 3' UTR, remained. The corresponding *gus* fusion was designated as "*SrEnod2* 3'Δ7" (Figure 4.1). This construct was introduced into *Arabidopsis*, and six independent transgenic lines were obtained. Two-week-old transgenic plants harboring the *SrEnod2* 3'Δ7 construct were incubated for 12 h and 24 h on solid media with or without 1 μM BAP, and GUS activity was measured using the fluorometric assay. Tissue-specific expression of the reporter gene fusion was found to be identical to that in plants expressing the full *SrEnod2* 3' construct (Data not shown). The level of GUS activity was enhanced about two-fold after 12 h and 24 h of BAP treatment (Figure 4.5), although the overall level of expression of this construct was lower than that observed with the *SrEnod2* 3' construct containing the full 2.5 kb *SrEnod2* 3' region. This decrease in total level of expression was also observed by histochemical staining (Data not shown). These results indicate that sequences outside of the 3' UTR are required for optimal cytokinin inducibility of *SrEnod2* expression. However, these data do delimit a cytokinin response element within the 250 bp 3' UTR of the *SrEnod2* gene.

Figure 4.5. GUS activity of *Arabidopsis* plants harboring the *SrEnod2* 3'UTR construct after cytokinin treatment. Two-week-old *Arabidopsis* plants harboring the *SrEnod2* 3'UTR construct were treated for 12 h and 24 h with 1 μM BAP. Roots were isolated for GUS activity measurements. Control seedlings were incubated for 24 h on media without BAP. The mean GUS activity from 3 independent experiments is shown. Each experiment included 5 seedlings. Error bars represent the SD. The *asterik* indicates a $p < 0.05$.

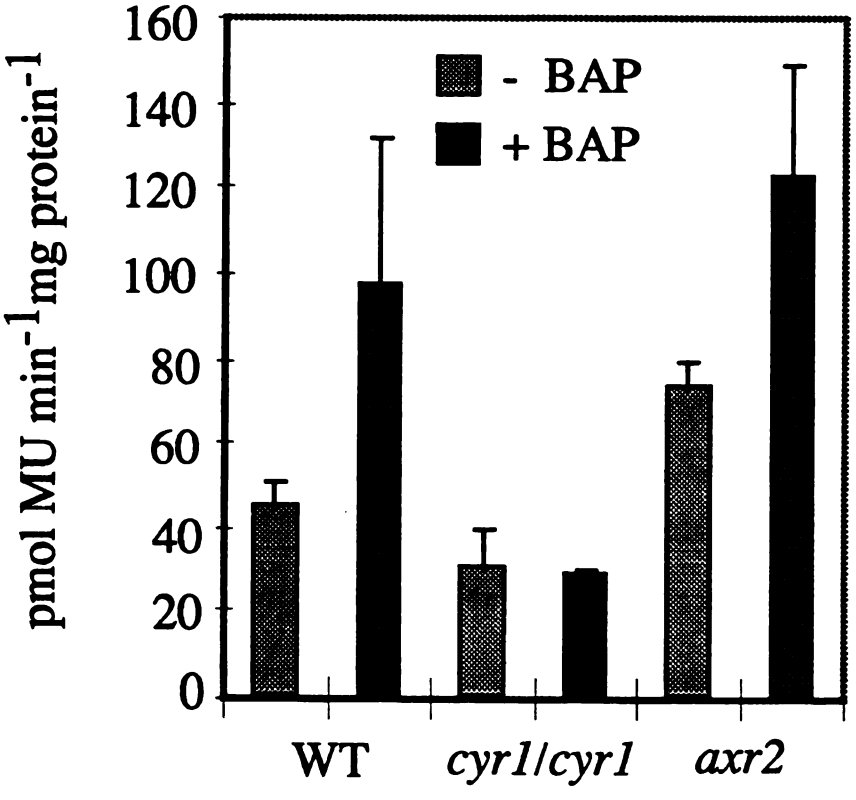

pmol MU min-1 mg protein-1
100
90
80
70
60
50
40
30
20
10
0
*
- BAP
24 h
+BAP
12 h
+BAP
24 h

Expression of the *SrEnod2* 3' *gus* construct in the cytokinin-resistant mutant *cyr1* and auxin-resistant mutant *axr2*

To further examine cytokinin regulation of the *SrEnod2* 3' *gus* construct, I examined the expression of this reporter gene fusion in *Arabidopsis* mutants defective in cytokinin or auxin response. Transgenic *Arabidopsis cyr1* mutant plants harboring the *SrEnod2* 3' construct were generated (see Materials and Methods) and stained for GUS activity. *cyr1/cyr1* plants harboring the construct failed to show GUS staining in roots (Data not shown). Furthermore, the application of BAP did not cause an increase in GUS activity in *cyr1* mutants bearing the *SrEnod2* 3' construct (Figure 4.6). These plants did have GUS staining in stipules, as did plants harboring the *nos 3'* construct (Figure 4.2), but not in expanding leaves or trichomes (Data not shown).

Two-week-old auxin-resistant *axr2 Arabidopsis* plants harboring the *SrEnod2* 3' *gus* construct were also placed onto solid media with or without 1 μM BAP for 24 h. In *axr2* mutants, there appeared to be a slight increase in the level of expression of the *SrEnod2* 3' construct in the absence of BAP, but this increase was not statistically significant (Figure 4.6). GUS activity increased in response to BAP in the *axr2* mutant plants qualitatively similar to wild type (Figure 4.6). The tissue specific pattern of GUS staining in *axr2* plants was the same as in wild-type plants (Data not shown), indicating that the *axr2* mutation does not affect the localized expression pattern of the *SrEnod2* 3' *gus* construct in *Arabidopsis*.

Figure 4.6. Regulation of the *SrEnod2* 3' construct in the cytokinin-resistant mutant *cyr*1, and the dominant auxin-resistant mutant *axr*2. Two-week-old wild-type, *cyr*1/*cyr*1, and *axr*2 seedlings harboring the *SrEnod2* 3' construct were treated for 24 h with 1μM BAP. Control plants were not treated with BAP. Roots were then isolated for GUS activity measurements. The mean GUS activity from 3 independent experiments is shown. Each experiment included 5 seedlings. Error bars represent the SD.


160
140
120
100
80
60
40
20
0
pmol MU min-1mg protein-1
- BAP
+ BAP
WT
cyr1/cyr1
axr2

DISCUSSION

Conserved tissue-specificity and cytokinin regulation of the early nodulin gene *SrEnod2* in *Arabidopsis*

Here it is demonstrated that a chimeric reporter gene construct composed of the 5' and 3' flanking regions of the *SrEnod2* locus of *S. rostrata* and the *gus* reporter gene is expressed in a tissue-specific manner in both the legume plant *L. japonicus* and in *Arabidopsis*. The expression pattern in the roots of these divergent species was found to be identical, namely in the vascular parenchyma, and in emerging lateral roots. However, while transgenic *L. japonicus* plants harboring this construct displayed no detectable expression in shoots, transgenic *Arabidopsis* plants harboring this construct showed distinct GUS expression in emerging leaves, stipules and trichomes (Figure 4.2D). In transgenic *Arabidopsis* plants, the expression of this construct was enhanced by treatment with cytokinin to approximately the same degree as the accumulation of the endogenous *SrEnod2* mRNA of *S. rostrata* (Dehio and de Bruijn, 1992). Expression of the *SrEnod2* 3'-*gus*-*SrEnod2* 5' construct was found not to be enhanced by cytokinin in the legume *L. japonicus*. It is possible that regulation by a nodule-specific developmental program supercedes cytokinin regulation in *L. japonicus*, and that cytokinins are not involved in nodule development of *L. japonicus* as they are in *S. rostrata*. Indeed, we have found that expression of the endogenous *LjEnod2* gene in *L. japonicus* was not enhanced by cytokinin (D.L. Silver, R. Chen, F. J. de Bruijn, unpublished data). However, it is also possible that changes in sensitivity to endogenous cytokinin are involved in regulation of *Enod2* genes in *L. japonicus*, but that the cytokinin response is saturated in developed roots of this species.

Our previous study on the cytokinin enhancement of *SrEnod2* mRNA accumulation in *S. rostrata* suggested a posttranscriptional mechanism. I now show that the tissue-

specific and cytokinin responsive expression of the chimeric *gus* fusion requires the *SrEnod2* 3' region. A chimeric construct which contains the nopaline 3' terminator instead of the *SrEnod2* 3' region, failed to be expressed in roots of either *L. japonicus* or *Arabidopsis* transgenic plants (Figure 4.2C and Figure 4.3C), and could not be enhanced by cytokinin in *Arabidopsis* plants (Figure 4.3D). Furthermore, the 250 bp UTR from the *SrEnod2* gene was found to be sufficient for the cytokinin response in *Arabidopsis* plants (Figure 4.5). The requirement of the *SrEnod2* 3' UTR for nodule parenchyma-specific expression (Chen et al., 1996), root-specific expression in *L. japonicu* and *Arabidopsis*, and cytokinin enhancement of expression in *Arabidopsis*, further suggests a posttranscriptional mechanism. 3' UTRs contain signals needed for transcript processing (for a review see Proudfoot, 1991), and have been shown to contain mRNA stabilizing (Klausner et al., 1993; Weiss and Liebhaber, 1995), or de-stabilizing elements (Newman et al., 1993; Zhang and Mehdy, 1994; Riedl and Jacobs-Lorena, 1996; DeMaria and Brewer, 1996). In our studies we cannot fully rule out the involvement of the *SrEnod2* 5' region in tissue specific- or cytokinin-regulated expression patterns, although Chen et al. (1996) have presented strong evidence that nodule-parenchyma-specific expression of the *SrEnod2* gene is mediated by its 3'UTR. It remains possible that both the *SrEnod2* 5' region and its 3' UTR are essential for posttranscriptional regulation. In fact, there is increasing evidence in the literature supporting an interaction of 5' UTRs and 3' UTRs of mRNAs in gene regulation (Munroe and Jacobson, 1990; Muhlrad et al., 1994; Tarun and Sachs, 1995). These interactions have been shown to be important for posttranscriptional events such as translation and mRNA stability (for review see Beelman and Parker, 1995). One scenario for the mechanism of regulation of the *SrEnod2* gene would be that (a) protein(s) interacting with the 3'UTR, stabilizes the mRNA, as well as confer(s) tissue specificity. A posttranscriptional mechanism for regulating *SrEnod2* expression may afford the cell with a rapid means of regulating *SrEnod2* message levels (Green, 1993). Work is in progress to further delimit the 3'UTR sequences which are involved in this level of regulation.

Evidence that *SrEnod2* is regulated by cytokinin *in vivo*

To learn more about the role of cytokinin in regulation of the *SrEnod2* gene, I examined expression of the *SrEnod2* 3' *gus* construct in transgenic *Arabidopsis* plants that contain a mutation that affects sensitivity to cytokinin; *cyr1* (Deikman and Ulrich, 1995). The *cyr1* mutation, which appears to have pleiotropic effects, was isolated using a root elongation assay which examined root sensitivity to cytokinin. Roots of *cyr1* mutant plants were found to have a 10-fold reduction in cytokinin sensitivity, as compared to wild-type plants (Deikman and Ulrich, 1995). The expression of the *SrEnod2* 3' construct was found not to be affected by cytokinin treatment in *cyr1* plants (Figure 4.6). This interesting observation is consistent with the cytokinin-insensitive nature of the mutant. Furthermore, this construct was not expressed in any tissues of *cyr1* transgenic plants except for stipules. These data suggest that tissue-specific regulation of the *SrEnod2* 3' construct in roots, expanding leaves, and trichomes in *Arabidopsis* requires a functional cytokinin-sensing pathway.

The failure of the *SrEnod2* 3' construct to respond to cytokinin in *cyr1* mutant plants provides the first molecular evidence supporting the proposed cytokinin insensitivity of this mutant, and underscores the value of this mutant for studying cytokinin responses. In addition, these data suggest that the *cyr1* gene is part of a conserved cytokinin signal transduction pathway, found both in *Arabidopsis* and in legume plants. To our knowledge these data are the first to show a link between cytokinin induction of gene expression and the existence of an *in vivo* cytokinin signal transduction pathway.

Another *Arabidopsis* mutant (*amp1*) has been characterized which produces elevated levels of cytokinin (Chaudhury et al., 1993). *amp*1 exhibits a severe shoot phenotype, in that it produces an overabundance of rosette leaves, and has loss of apical dominance, phenotypes which would be expected for a cytokinin overproducer

(Chaudhury et al., 1993). However, *amp1* produces normal roots, suggesting that AMP1 acts only in the shoot. Expression of the *SrEnod2* 3' *gus* fusion in *amp1* roots appeared to be unaffected by the mutation, consistent with the idea that the *amp1* mutation has its primary effects on the shoot (D.L. Silver and F. J. de Bruijn, unpublished data).

SrEnod2 3' *gus* expression in an auxin-resistant *axr2* mutant of *Arabidopsis*

A number of cytokinin regulated genes are also regulated by auxin, such as the gene represented by pLS216 from *Nicotiana plumbaginifolia* (Dominov et al., 1992), the *cim1* gene of tobacco (Crowell, 1994), and others (Crowell et al., 1990; Hemerly et al., 1993; Gough et al., 1995). It has been shown that the expression of the *SrEnod2* gene in *S. rostrata* is not regulated by the application of auxins (Dehio and de Bruijn, 1992). Similarly, our studies indicate that exogenous auxins do not stimulate the expression of the *SrEnod2* 3' *gus* construct in *Arabidopsis,* and it does not affect the cytokinin-enhanced expression of this gene in transgenic plants (Figure 4.4). However, it is possible that endogenous levels of auxin are important for cytokinin enhancement of gene expression. For example, auxin dependence of cytokinin induction of gene expression has been suggested for the regulation of the *cdc2a* gene in *Arabidopsis*. Expression of a *cdc*2a *gus* fusion has been shown to be induced by cytokinin alone only in the upper part of the primary root in intact *Arabidopsis* plants (Hemerly et al., 1993), while auxin is required for cytokinin induction of expression of the *cdc*2a *gus* fusion in excised roots of the same plants. Based on these data, Hemerly et al. (1993) suggest that auxin transport from the shoot is required for cytokinin induction of the *cdc*2a gene.

To determine whether a functional auxin signal transduction pathway was necessary for the cytokinin induction of the *SrEnod2* 3' *gus* chimeric gene, I examined expression of this gene in the auxin resistant mutant *axr2*. Cytokinin-enhanced expression of the

SrEnod2 3' construct was not affected by the *axr2* mutation (Figure 4.6). Taken together, the lack of effect of either exogenous auxin or the *axr2* mutation on expression of the *SrEnod2* 3' *gus* construct suggests that cytokinins act independently of auxin in the regulation of *SrEnod2* gene expression.

Role of ethylene in regulation of the *SrEnod2* gene

Treatment of transgenic *SrEnod2* 3' *Arabidopsis* plants with ethylene alone caused a slight increase in GUS activity compared to control plants, but application of ethylene and cytokinin together appeared to prevent full cytokinin enhancement of gene expression (Figure 4.4). Similarly, we found that *SrEnod2* mRNA accumulated approximately two-fold over controls in ethylene-treated *S. rostrata* plants, and that further *SrEnod2* mRNA accumulation was inhibited in the presence of both ethylene and cytokinin (D.L. Silver and F. J. de Bruijn, unpublished data). Many physiological responses to cytokinin in roots are known to be mediated through ethylene, and application of cytokinins has been shown to be able to increase the production of ethylene in the root (Fuchs and Lieberman, 1968; Radin and Loomis, 1969; Bertell and Eliasson, 1992). The inhibition of root growth by cytokinin in roots of *Arabidopsis* seedlings has been shown to be mediated through ethylene (Cary et al., 1995). The mechanism by which ethylene acts to inhibit cytokinin-induced expression of the *SrEnod2* 3' *gus* construct in *Arabidopsis* roots remains to be determined. One possibility is that BAP acts to enhance expression of the *SrEnod2* 3' construct by stimulating the production of ethylene, but that the relatively high level of ethylene used in our study (40 μl/l) was inhibitory for gene expression. It will be necessary to determine the dose dependence of the response to both ethylene and cytokinin to resolve this question. A genetic approach to better understand the role that ethylene may play in regulating the *SrEnod2* 3' *gus* construct would be to analyze GUS expression in ethylene insensitive mutants (Guzman and Ecker, 1990; Chang et al., 1993; Hua et al.,

1995; Roman et al., 1995), or ethylene overproducing plants (Guzman and Ecker, 1990). These experiments are being initiated presently.

Gradient-like expression pattern of the *SrEnod2* 3' *gus* construct

The expression of the *SrEnod2* 3' construct in only a limited number of emerging lateral roots is interesting. In *S. rostrata* the exact cellular location of *SrEnod2* expression in roots is unknown. However, RNA gel blot analysis indicates that *SrEnod2* is expressed most highly in the primary root of *S. rostrata* plants (D.L. Silver and F.J. de Bruijn, unpublished data). It is tempting to speculate that this pattern of expression is due to the level of cytokinin, which is being transported to the shoot from the root, the proposed site of cytokinin biosynthesis (Torrey 1976; Van Staden and Davey, 1979).

The identical tissue-specific expression patterns and levels of the *SrEnod2* 3' *gus* construct in both *L. japonicus* and *Arabidopsis* plants, namely highest at the hypocotyl-root junction and decreasing toward the root tip, may be indicative of a signal gradient. It is possible that a gradient of cytokinin concentration forms as it is transported from the root tip to the shoot, and in combination with tissue-specific signals, is responsible for the observed pattern of expression. Alternatively, a signal may be transported from the shoot to the root, which allows only root tissues near the shoot junction to perceive the cytokinin signal. An obvious candidate for such a signal would be auxin, which is transported from the shoot. However, the evidence presented in this report is not consistent with auxin as the signal, since exogenous auxin had no significant effect on GUS expression, and the *axr2* mutation did not affect cytokinin inducibility of the *SrEnod2* 3' *gus* fusion. There is precedence for the role of a shoot to root signal molecule in nodulation. It has been found that during rhizobial infection, a regulatory signal is transported from the shoot to the root through the stele, which may be involved in the autoregulation of nodule initiation and development (reviewed by Caetano-Anolles and Gresshoff, 1991; Hirsch and Fang, 1994).

A third possibility is that a negative factor produced in the root apical meristem generates an opposing gradient to expression of the *SrEnod2* 3' construct.

Expression of *SrEnod2* 3' in both *L. japonicus* and *Arabidopsis* was found to be located in the pericycle and vascular pith cells of roots. This cell-specific expression pattern of *SrEnod2* 3' *gus* fusion may also be related to cytokinin transport. Evidence in the literature indicates that cytokinins are synthesized in the root apical meristem and transported to the shoot via the vascular system (Torrey 1976; Van Staden and Davey, 1979). It may be that inactive, conjugated cytokinins are transported from the root meristem to more distal regions of the root and eventually into the shoot where they become active through deconjugation in tissues which require cytokinin action. An intriguing question arises as to the relationship between the cell- and tissue-specific expression pattern seen for the *SrEnod2* 3' *gus* fusion in the root and that seen in nodules. It is known that pericycle cells and outer cortical cells are the site of nodule primordia initiation in determinant-type nodules (Hirsch, 1992). Further investigation is needed to discover the identity of the endogenous signal(s) involved in forming this gradient-like pattern of gene expression.

The role of cytokinin in root nodule formation

An important question remains as to the relationship between cytokinin enhancement of expression of the *SrEnod2* gene in roots, and its expression in nodules. Specifically, it is not known whether *SrEnod2* gene expression is under cytokinin control during nodule development. However, we have found that the *SrEnod2* 3' UTR is required both for the tissue-specific expression pattern seen in nodules (Chen et al., 1996) and cytokinin inducibility in roots. The cytokinin inducibility of the *SrEnod2* gene in *S. rostrata* roots, and of the *SrEnod2* 3' chimeric *gus* fusion in *Arabidopsis*, suggests that legume plants have evolved a mechanism to regulate *SrEnod2* gene expression by making

use of a general cytokinin signal transduction pathway, in addition to tissue-specific factors.

The evidence for the involvement of cytokinin in nodule development is circumstantial. Thimann (1936) first proposed a role for auxin in nodule development as well as the idea that nodule development may be related to lateral root development. Libbenga et al. (1973) in an *in vitro* approach using pea root cortical explants, found that cell divisions took place in the pericycle, the place of lateral root development, if auxin was added. Cortical cell division, the location of nodule initiation, was shown to occur upon the addition of both auxin and cytokinin. It should be noted that nodule development is primarily determined by a plant genetic program and not by the bacteria, as evidenced by the discovery of spontaneous nodulating alfalfa plants (Truchet et al., 1989). In addition, alfalfa plants can be induced to form nodule-like structures upon treatment with auxin transport inhibitors (Allen et al., 1953; Hirsch et al., 1989), or Nod factors (Truchet et al., 1991; Mergaert et al., 1993; Stokkermans et al., 1994). However, it has also been shown that rhizobia secrete cytokinins into the culture medium (Morris, 1986; Sturtevant and Taller, 1989; Taller and Sturtevant, 1991; Upadhyaya et al., 1991) The significance of this source of cytokinin on nodule development remains to be proven, and no cytokinin biosynthetic genes or mutants have been identified in rhizobium to date. Probably the most conclusive study suggesting a role of cytokinins in nodule development comes from the work by Cooper and Long (1994). They expressed the *Agrobacterium* cytokinin biosynthetic gene, *tzs*, in a *Rhizobium meliloti* strain mutant for the *nod* structural genes. This *tzs*-expressing *Rhizobium* was capable of inducing nodule-like structures on alfalfa which expressed the *Enod2* gene, supporting the idea that localized cytokinin production may be important for nodule development. This work does not prove that cytokinin secreted from wild-type rhizobia is required for nodule development, but rather that a localized production of cytokinin may play a role in nodule development, be it from *Rhizobium* or the plant.

The *SrEnod2* gene as a marker for a cytokinin signal transduction pathway

Expression of the *SrEnod2* 3' construct in wild type and *cyr1* mutant transgenic *Arabidopsis* indicates that the cytokinin signal transduction pathway that regulates the expression of this gene is conserved between legume and non-legume plants. Thus, this chimeric gene may be a useful tool to probe the general mechanism of cytokinin signal transduction in plants. The evidence presented here implies that a conserved protein(s) interacts with the *SrEnod2* 3' mRNA region in *Arabidopsis* and in *S. rostrata.* Therefore, endogenous *Arabidopsis* mRNAs must share conserved binding sites with *SrEnod2* 3' mRNA sequences and/or secondary mRNA structures. In order to identify the corresponding *trans*-acting factor(s), we have initiated a genetic screen using the transgenic *Arabidopsis* plants harboring the *SrEnod2* 3'construct, and are searching for RNA binding proteins *in vitro*. These studies may shed light on the molecular basis of cytokinin regulation of plant gene expression in general.

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Chapter 5

Genetic Evidence For the Existence of *SrEnod2* 3'-Interacting Factors

ABSTRACT

It has previously been shown that expression of a *gus* reporter gene under the control of the *SrEnod2* 5' upstream and *SrEnod2* 3' downstream regions (*SrEnod2* 5'-*gus*-*SrEnod2* 3' construct) can be cytokinin-enhanced in *Arabidopsis,* and that sequences necessary for this cytokinin-mediated enhancement of expression are located in the 3' nontranslated region of the *SrEnod2* gene. This indicates that conserved regulatory proteins exist between *Arabidopsis* and legume plants. Here, it is reported that overexpression of the nontranslated *SrEnod2* 3' downstream region via the CaMV 35S promoter (35S *SrEnod2* 3' construct) in *Arabidopsis* gives rise to a novel developmental abnormality, namely the production of more than one shoot apical meristem. This developmental abnormality indicates that the factor(s) which regulate the *SrEnod2* 5'-*gus*-*SrEnod2* 3' construct in *Arabidopsis* plays an important role in development. To provide genetic evidence for the existence of these factors, an *in vivo* titration experiment was performed in *Arabidopsis.* The expression of the *SrEnod2* 5'-*gus*-*SrEnod2* 3' construct in plants harboring the 35S *SrEnod2* 3' construct was not found to be cytokinin enhanced. Therefore, it can be postulated that the factor(s) which interact with the *SrEnod2* 3' region are required for both cytokinin-mediated expression and for regulating meristem number.

INTRODUCTION

Without a doubt cytokinins play an important role in plant development, but unfortunately little is known about their action at the molecular level. As in the studies of other plant hormones, a genetic approach to mutant isolation in *Arabidopsis* has been undertaken towards the understanding of cytokinin signal transduction. Surprisingly, very few mutants have been isolated which appear to be specific to cytokinin. It may be that there exists a large redundancy in genes coding for cytokinin receptors and other interacting proteins in the cytokinin signal transduction pathway, for example.

Studies of signal transduction pathways in plants as well as in animals has utilized marker genes to gain a foothold into determining the components of signal transduction pathways. In conjuction with mutants in a specific pathway, marker genes can serve as a probe to help order the genes comprising a pathway. It is proposed in this thesis that the *SrEnod2* gene may serve as a molecular probe to dissect a cytokinin signal transduction pathway. In this thesis, evidence is presented showing that the cytokinin-enhanced *SrEnod2* mRNA accumulation occurs posttranscriptionally, requires protein synthesis, protein phosphatases and kinases. Furthermore, it is shown that expression of the chimeric *gus* reporter gene *SrEnod2* 5'-*gus*-*SrEnod2* 3' is enhanced specifically by cytokinin, and in a tissue-specific manner in roots of *Arabidopsis* plants. It is also tissue-specifically expressed in *L. japonicus*, but not cytokinin regulated. Cytokinin regulation was shown to require the *SrEnod2 3'* UTR. In addition, it was demonstrated in Chapter 4 that the *SrEnod2* 5'-*gus*-*SrEnod2* 3'construct is not cytokinin-enhanced in the cytokinin-resistant *Arabidopsis* mutant *cyr1*, thus providing genetic evidence that the *SrEnod2* 5'-*gus*-*SrEnod2* 3'construct is regulated by an *in vivo* cytokinin signal transduction pathway.

Here, genetic evidence is presented which may indicate the existence of *trans*-acting factors which are required for the functioning of the *SrEnod2 3'* downstream region in

Arabidopsis, and that overexpression of the *SrEnod2 3'* downstream region in *Arabidopsis* causes a novel developmental phenotype.

METHODS

Plant material and growth conditions

Transgenic *Arabidopsis thaliana* Columbia and *Arabidopsis amp1* plants were germinated and grown on MS media (Gibco BRL)/ 0.8% agar for 2 weeks in a growth chamber. *Arabidopsis* plants were grown in *Arabidopsis* Mix (Hummert International, Earth City, MO) in a growth chamber for approximately one month after which plants were allowed to dry, and seeds harvested. Growth chamber conditions were 18 h light/ 6h dark; 21^0 C day/18^0 C night.

Cytokinin treatments of transgenic *Arabidopsis* plants were as follows: Two-week-old transgenic *Arabidopsis* plants grown on solid MS plates, as described above, were then transferred to solid MS plates containing 1 μM BAP for 24 h. Roots and green tissues were then isolated and frozen in liquid nitrogen.

Chimeric *gus* reporter gene construction

The 35S *SrEnod2* 3' construct was prepared as follows: The CaMV 35S promoter was isolated as a *HindIII-XbaI* fragment from pBI121 (Clonetech). The 35S promoter (*HindIII-XbaI*) was then subcloned into the *SacI-EcoRI* digested pBIB vector to make pBIB35S. The 2.5 kb *SrEnod2* 3' region was removed from the *SrEnod2* 3' construct (see Chapter 4 of this thesis) as a *SacI-EcoRI* fragment and subcloned into the pBIB35S digested with SacI-EcoRI. The *SrEnod2* 3'Δ7 deletion was made using the ExoIII digestion protocol as described by the manufacturer (Erase A Base, Boeringer Mannheim). The 3'Δ7 deletion was subconed into pBIB35S as a *SacI-EcoRI* fragment, making the construct 35S *SrEnod2* 3'Δ7.

Plant Transformation

Transformation of *Arabidopsis thaliana* Columbia plants with the 35S *SrEnod2* 3' 35S *SrEnod2* 3'Δ7, and *SrEnod2* 5'-*codA-SrEnod2* 3' contructs were perfomed using vacuum infiltration according to the procedure described by Bent et al. (1994).

RNA isolation and northern blot analysis

Total RNA was isolated from roots of transgenic *Arabidopsis* plants according to the method of Verwoerd et al., (1989). Poly(A) RNA was then isolated from total RNA by oligo-dT column chromatography as described by the manufacturer's instructions (5 prime- 3 prime, Inc., Boulder, Co). Northern blot analysis was performed as according to Maniatis et al. (1982). Approximately 3 ug of poly(A) RNA was used for analysis. The 2.5 kb *SrEnod2* 3' region, and the 250bp *SrEnod2* 3' UTR was used as a DNA probe for northern analysis. [^{32}P]-dATP labelled *SrEnod2* 3' DNA probes were generated by random priming (Boehringer Mannheim). Hybridization and washing conditions of Northern blots were as previously described (Silver et al., 1996). Radioactive blots were exposed to autoradiography film (Dupont) at -75^{0} C.

Quantification of GUS enzymatic activity

Quantification of GUS enzymatic acitivity was performed according to the procedure described by Jefferson et al. (1987). The fluorometric assay was performed using a fluorescence spectrophotometer (Model F-2000; Hitachi, Tokyo, Japan). GUS acitivity was reported as picomoles of 4-methylumbelliferone produced per minute per milligram of protein in total protein extract. Total protein concentrations in the extracts were measured using the Bradford assay with BSA as a standard (Bradford, 1976).

GUS histochemical staining

GUS histochemistry was performed according to the procedure described by Jefferson et al. (1987). Stained plant tissues were photographed under bright-field using an Axiophot microscope (Zeiss; Oberkochen, Germany).

Genetic crosses

T_2 generation *Arabidopsis* plants harboring the 35S *SrEnod2* 3' construct (hygromycin resistant) were crossed to plants harboring the *SrEnod2* 5'-*gus*-*SrEnod2* 3' construct. Kanamycin/hygromycin resistant F_1 plants were allowed to self-pollinate, and F_2 Kanamycin/hygromycin resistant seedlings were selected, and analyzed for GUS activity. Plants harboring the 35S *SrEnod2* 3' construct were crossed to the *amp1* mutants (Chaudhury et al., 1995) harboring the *SrEnod2* 5'-*gus*-*SrEnod2* 3' construct. Kanamycin/hygromycin resistant F_1 plants were allowed to self-pollinate, and F_2 Kanamycin/hygromycin resistant seedlings were selected, and will be used for further analysis.

RESULTS

Overexpression of the *SrEnod2* 3' downstream region in *Arabidopsis* causes a novel developmental phenotype.

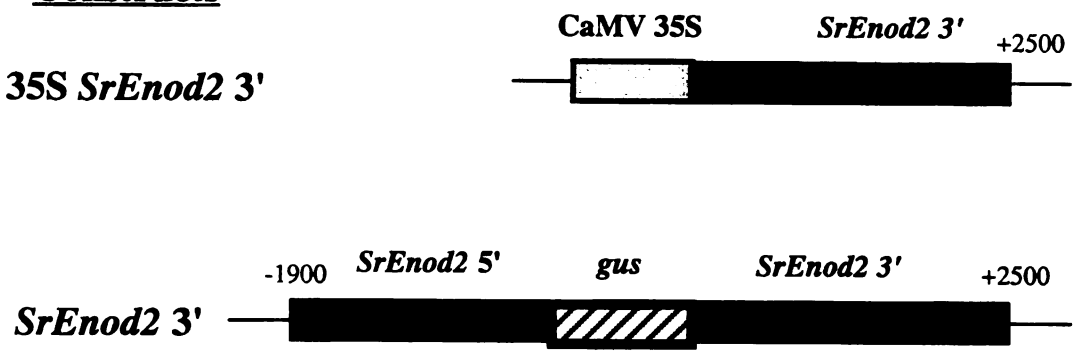
It has been shown that the *SrEnod2* 3' UTR is required for the cytokinin-enhanced expression of the *SrEnod2* 5'-*gus*-*SrEnod2* 3'construct in *Arabidopsis* (Chapter 4). Overall, tissue-specificity and cytokinin-enhancement of gene expression appear to be conserved between *S. rostrata*, *L. japonicus* (tissue-specificity only), and *Arabidopsis*. It is tempting to speculate that factors which regulate the expression of the *SrEnod2* 5'-*gus*-*SrEnod2* 3'construct in *Arabidopsis* are homologous to factors which regulate the *SrEnod2* gene in the legumes *S. rostrata*, and *L. japonicus*. To demonstrate the existence of such factors, an *in vivo* titration experiment was designed as diagrammed in Figure 5.1. The aim was to overexpress the *SrEnod2* 3' region under the control of a constitutive promoter and genetically cross this construct into a homozygous line expressing the *SrEnod2* 5'-*gus*-*SrEnod2* 3'construct in *Arabidopsis* (Figure 5.1). The expectation is that if expression of the *SrEnod2* 3' region can titrate out *SrEnod2* 3'-interacting factors, then GUS expression from the *SrEnod2* 5'-*gus*-*SrEnod2* 3' construct will be signifcantly reduced (Figure 5.2).

To overexpress the *SrEnod2* 3' downstream region in *Arabidopsis*, the 2.5kb *SrEnod2 3'* region was fused to the CaMV 35S promoter and introduced into *Arabidopsis* via *Agrobacterium tumefaciens*. Seven independent lines were isolated harboring the 35S *SrEnod2* 3' construct. Surprisingly, plants expressing these 35S *SrEnod2* 3' constructs exhibited a novel developmental phenotype. As shown in Figure 5.3, plants harboring the 35S *SrEnod2* 3' construct are initially arrested in the production of leaves during the first 10 d after germination while wild-type plants have produced four leaves. After 10 d post-germination, leaf primordia begin to form from more than one

Figure 5.1. ***In vivo*** **titration scheme.** A, Diagram of the 35S *SrEnod2* 3' and *SrEnod2* 3' construct used to determine whether proteins interact with the *SrEnod2* 3'UTR. The 2.5 kb of the *SrEnod2* 3' region was overexpressed under the control of the CaMV 35S promoter. The *SrEnod2* 3' construct contains 1.9 kb of *SrEnod2* 5' region ligated to the *gus* reporter gene (hatched box) fused to the 2.5 kb *SrEnod2* 3' region. The black box represents the *SrEnod2* 3'UTR ending at the poly(A) addition site. B, ***in vivo*** titration experiment was performed by a genetic cross between *Arabidopsis* plants harboring the 35S *SrEnod2* 3' construct and plants harboring the *SrEnod2* 3' construct. F_2 progeny were analyzed for GUS activity.

A

Constructs



B

35S *SrEnod2* 3' X *SrEnod2* 3'

↓

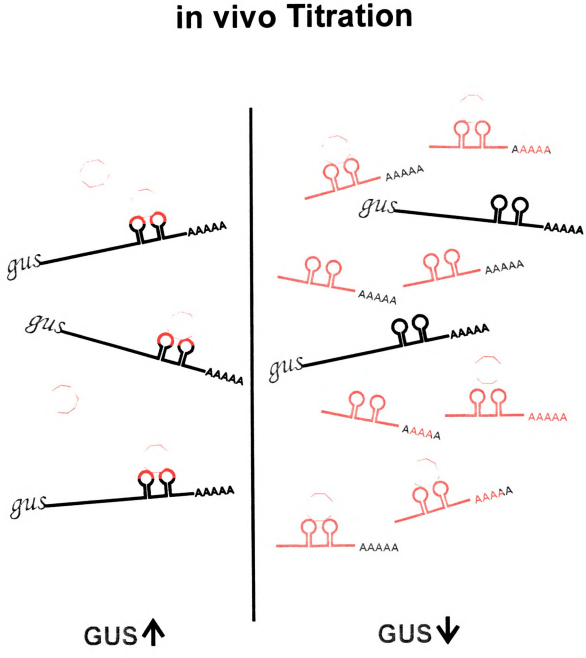
F_1

↓

F_2

GUS activity quantification

Figure 5.2. Hypothetical mechanism for *in vivo* titration. The panel on the left represents a cell in which a protein(s) interacts with the *gus-SrEnod2* 3'UTR mRNA resulting in GUS activity. The panel on the right represents a cell overexpressing the *SrEnod2* 3'UTR which titrates out the *SrEnod2* 3'UTR-binding protein(s) resulting in a decrease in the levels of *gus-SrEnod2* 3'UTR mRNA, and thus a decrease in GUS activity.


in vivo Titration
gus
AAAAA
GUS↑
GUS↓

meristem (Figure 5.3). These plants were named Siamese, for the presence of two meristems (Figure 5.3 and 5.4). Infrequently, three or four meristems were formed. A second phenotype was less frequently observed among the seven lines, which were named Flag. Flag plants produced a single, large leaf at the stage in which wild-type plants produced two primary leaves. Further leaf production was arrested until about day 10, then two possible fates of the meristem occured. One fate is that two meristems form, giving rise to the Siamese phenotype. The second fate is that a normal, single meristem forms, giving rise to a wild-type phenotype, except for production of a the single, large primary leaf. Both Siamese and Flag plants produce normal leaves, trichomes, roots, stems, flowers, and a normal seed set. However, at a low frequency, a more severe phenotype was seen in multiple independent lines. This phenotype exhibited a biforcated or triforcated root system as well as shoot abnormalities (Figure 5.3). To determine if the Siamese/Flag phenotype was due to overexpression of the *SrEnod2* 3' UTR, and / or sequences downstream of the poly(A) addition site, a deletion of the *SrEnod2* 3' region containing 170 bp of the 3' UTR was fused to the CaMV 35S promoter and was designated as 35S *SrEnod2* 3'Δ6 (Figure 5.5). This construct contains 110 bp of an internal fragment of the *SrEnod2* 3' region as a result of the deletion procedure. Sixteen independent lines were generated harboring the 35S *SrEnod2* 3'Δ6 construct. Eight T_2 independent lines harboring the *SrEnod2* 3'Δ6 construct were examined, and all exhibited the Siamese/Flag phenotype. Thus, the Siamese/Flag phenotype is likely due to expression of the *SrEnod2* 3'UTR and not sequences downstream. Three other control constructs were produced to determine whether the Siamese/Flag phenotype was specifically due to the overexpression of the *SrEnod2* 3' UTR or can be caused by the overexpression of any UTR. To overexpress a different 3'UTR, the nopaline synthase 3' terminator (*nos 3'*) was fused to the 35S promoter. Two other constructs contained promoterless nos 3' and *SrEnod2* 3' regions (Figure 5.5). These constructs have recently been transformed into *Arabidopsis* and await further analysis.

Figure 5.3. Phenotypes of seedlings harboring the 35S *SrEnod2* 3' construct. A, Two-week-old non-transformed wild-type plant. B, One-week old Siamese plant lacking visible leaves. C, Two-week-old Siamese plant. Arrows indicate the two independent whorls of leaves. D, Two-week-old Flag plant. Arrow indicates a single primary leaf. E, Biforcated root system of a two-week-old Siamese plant. F, Root system of a non-transformed wild-type plant. G, Siamese plants in soil. H, Non-transformed wild-type plants in soil.

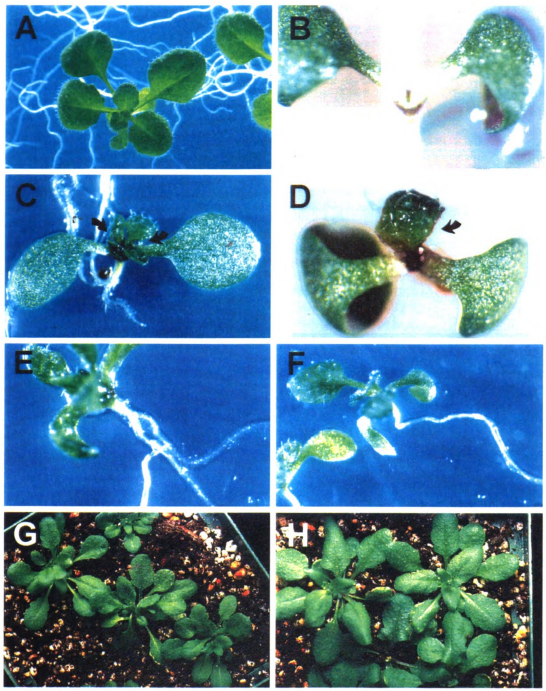

A
B
C
D
E
F
G
H

Figure 5.4. Scanning electron microscopy of 10-day-old plants harboring the 35S *SrEnod2* 3' construct exhibiting Siamese and Flag phenotypes. A, The first two leaf primordia emerging from the apex of a wild-type plant are visible, as indicated by asteriks. (450X magnification). B, A ten-day-old Siamese plant exhibiting multiple structures in the apical region (120X). C, A higher magnifcation (370X) of the apical region of the plant shown in panel B. Multiple structures (leaf primordia and or meristems) are indicated by asteriks. D, The apical region of a Flag plant which has developed two meristems.(220X magnification). Meristems are indicated by arrows; The *asterik* indicates leaf primoridia.

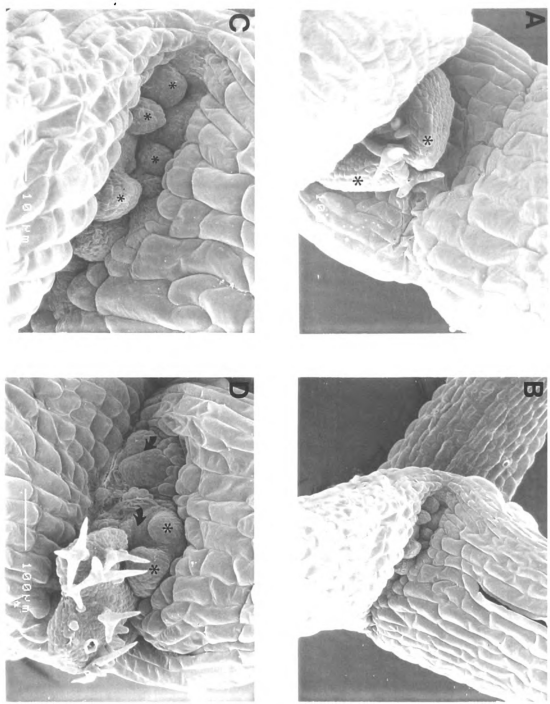
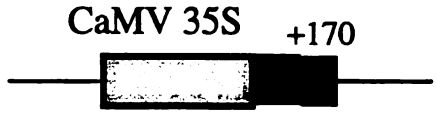

A
B
C
D

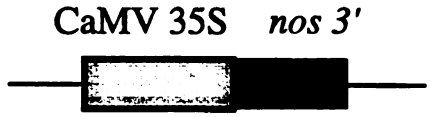
Figure 5.5. Diagram of constructs used as controls. The 35S *SrEnod2* 3'Δ6 construct contains 170 bp of the *SrEnod2* 3'UTR along with 110 bp of an internal region from downstream sequence and was used to determine whether the Siamese/Flag phenotype was due to the overexpression of the *SrEnod2* 3'UTR or sequences downstream. The 35S *nos* 3' construct contains the nopaline synthase 3' terminator (*nos* 3') under the control of the CaMV 35S promoter. Promoterless *SrEnod2* 3' and *nos* 3' sequences were transformed into *Arabidopsis* as additional controls.

Constructs

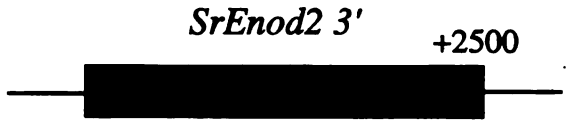
35S *SrEnod2* 3' Δ6



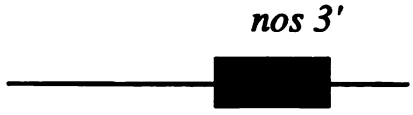
35S *nos* 3'



pBIB *SrEnod2* 3'



pBIB



Inheritence of the Siamese and Flag phenotype across three generations was in a non-Mendelian fashion (data not shown). Plants exhibiting a wild-type phenotype gave rise to Siamese/Flag plants, and Siamese/Flag plants gave rise to plants with wild-type phenotype. Therefore, it was of interest to determine whether the expression level of the *SrEnod2 3'* region correlates with phenotype. Total RNA from two-week-old wild-type and Siamese plants were isolated for nothern blot anlaysis. A roughly 300 nt RNA was detected when northern blots were probed with either a full length *SrEnod2* 3' region or only the *SrEnod2* 3' UTR (Figure 5.6), indicating that the 2.5 kb 3' end may be processed at the normally used poly(A) addition site (Dehio and de Bruijn, 1992). This idea was supported by finding this same size transcript in the poly(A)$^+$ RNA fraction (Figure 5.7). Overall, the expression levels of the 35S *SrEnod2* 3' construct in multiple independent lines did not correlate with phenotype (Figure 5.6).

Genetic Evidence for the existence of *SrEnod2* 3'-interacting factors

Genetic crosses were made between twelve independent lines harboring the 35S *SrEnod2* 3' construct and an inbred line (T_4 generation) harboring a single copy of the *SrEnod2* 5'-*gus*-*SrEnod2* 3'construct (Figure 5.1). Figure 5.8 shows the results of GUS staining of F_1 plants from this cross. In general, F_1 plants showed a dramatic decrease in expression in apical and root tissues. Plants harboring the *SrEnod2* 5'-*gus*-*SrEnod2* 3'construct typically show GUS staining in trichomes of newly expanding leaves and infrequently in the expanding leaf (Figure 5.8). However, F_1 progeny showed reduced or absence of GUS staining in trichomes of newly expanding leaves. In addition, tissue-specific staining in roots not treated with BAP was significantly reduced or absent. F_2 plants were then analyzed for GUS activity in response to cytokinin. Two-week-old

Figure 5.6. Northern blot analysis of *Arabidopsis* plants harboring the 35S *SrEnod2* 3' construct. Lines WT7-6 and WT1-9 did not segregate for the Siamese/Flag phenotype. The other lines shown segregated for the Siamese/Flag phenotype. C = non-transformed wild-type plant. Total RNA was isolated from entire two-week-old seedlings and probed with 2.5 kb *SrEnod2* 3' DNA. A ubiquitin DNA probe (*ubi-1*) was used as a loading control. Lines were named based as described in the following example: WT1-9; WT refers to the phenotype of the parent line (F_2) from which the F_3 line was derived. 9 refers to the parental F_1 vacuum infiltrated plant. 1 denotes the individual line derived from plant 9. WT = wild-type phenotype, Sia = Siamese phenotype. Six additional lines were analysed and yielded similar results.

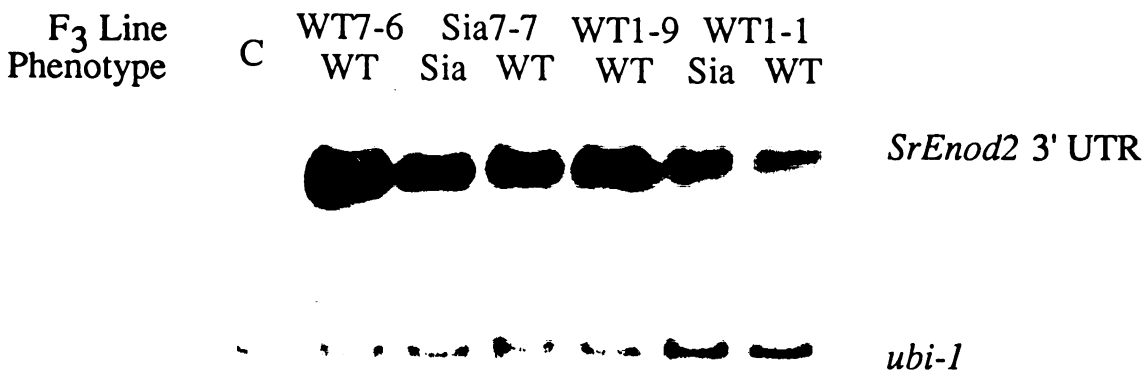
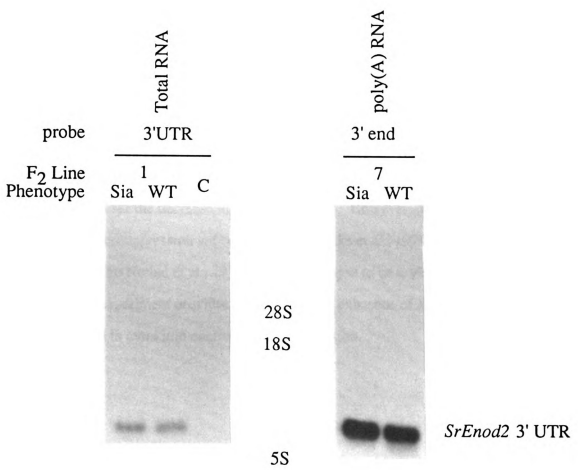

F3 Line
Phenotype
C
WT7-6
WT
Sia7-7
Sia
WT1-9
WT
WT1-1
Sia
WT
SrEnod2 3' UTR
ubi-1

Figure 5.7. Accumulation of the *SrEnod2* 3'UTR mRNA. Total RNA from Line 1 segregating for the Siamese (Sia) and wild-type (wild type) phenotype was probed with a 250 bp DNA probe encompasing the *SrEnod2* 3'UTR (3'UTR). C = non-transformed wild-type plant. Poly(A)$^+$ RNA from Line 7 segregating for the Siamese/WT pheotype was probed with 2.5 kb of the *SrEnod2* 3' downstream region (3'end). The location of the 28S, 18S and 5S ribosomal RNAs as size markers are indicated.


Total RNA
poly(A) RNA
probe
3'UTR
3' end
F2 Line
1
7
Phenotype
Sia
WT
C
Sia
WT
28S
18S
5S
SrEnod2 3' UTR

Siamese and wild-type phenotype F_2 plants and plants harboring the *SrEnod2* 5'-*gus*-*SrEnod2* 3' construct were placed onto solid media with or without 1 μM BAP for 24 h. Protein was extracted from roots and GUS activity was quantified. Figure 5.9 shows that the levels of GUS staining in roots was absent in both Siamese and wild-type phenotype F_2 plants in five out of six lines tested (only line WT7-3 showed staining). In addition, GUS staining was not enhanced in five out of 6 F_2 lines treated with BAP (again, only line WT7-3 showed staining). Also, GUS staining of trichomes was reduced or absent in three out of six F_2 lines (lines: Sia1-9, Sia1-10, Sia7-7). *gus* reporter gene expression, as measured by GUS acitivty in roots of both Siamese and wild-type phenotype F_2 plants, was not cytokinin enhanced except for line WT7-3 (Figure 5.9). Indeed, this decrease in GUS activity may define a form of cosuppression, in which the titration of *trans*-acting factors accounts for the decrease in GUS expression. However, other proposed mechanisms of cosuppression are conceivable (Matzke et al., 1994; Van Blokland et al., 1994; de Carvalho Neibel et al., 1995), and would need to be investigated. Thus, this *in vivo* tiratration experiment provides evidence for the existence of *SrEnod2 3'*-interacting factors involved in cytokinin control of gene expression.

Figure 5.8. GUS expression in F_1 progeny from crosses between plants harboring both of the *SrEnod2* 3' and 35S *SrEnod2* 3' constructs. A and B, GUS staining in two-week-old seedlings harboring the *SrEnod2* 3' construct not treated and treated with 1 μM BAP for 24 h, respectively. C and D, GUS staining of F_1 progeny.

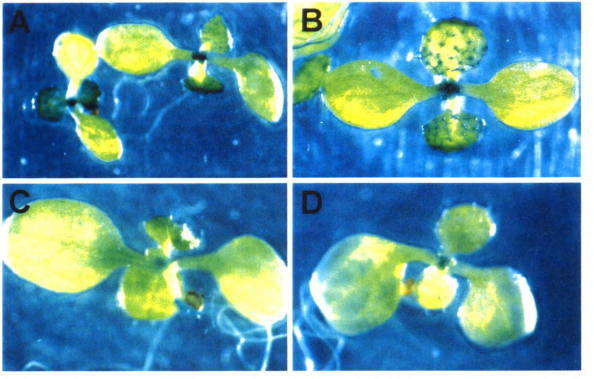
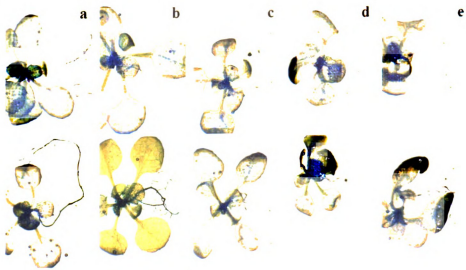
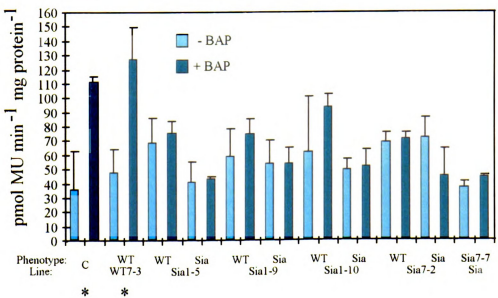

A
B
C
D

Figure 5.9. GUS expression in F_2 progeny from crosses between plants harboring both of the *SrEnod2* 3' and 35S *SrEnod2* 3' constructs. A, Top panels show plants not treated with 1 μM BAP, and bottom panels show plants of the same line treated with BAP for 24 h. Panels a,b,c,d,e show a control plant and representitive F_2 progeny of lines WT7-3 WT, Sia1-5 WT, Sia1-5 Sia, Sia7-2 Sia, respectively. GUS staining was seen only in plants in panels a and b. B, GUS activity measurements of six F_2 lines. C = plants harboring only the *SrEnod2* 3' construct; *asteriks* denotes samples which showed BAP-enhanced GUS staining. Refer to Figure 5.6 for explanation of nomenclature for lines.

A


a
b
c
d
e

B


pmol MU min -1 mg protein-1
160
150
140
130
120
110
100
90
80
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60
50
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30
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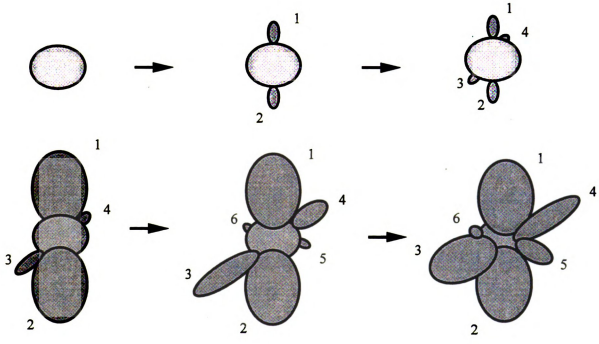
- BAP
+ BAP
Phenotype:
Line:
C
WT
WT7-3
WT
Sia
Sia1-5
WT
Sia
Sia1-9
WT
Sia
Sia1-10
WT
Sia
Sia7-2
Sia7-7
Sia
*
*

Figure 5.10. Diagram illustrating normal *Arabidopsis* apical meristem development. Central circle represents the shoot apical meristem. The first two leaf primordia are formed opposite each other at nearly the same time. Subsequent leaf primordia are formed in succession giving rise to a spiral phyllotaxy. Numbers denote the chronological order in which leaf primordia form.

Arabidopsis Apical Meristem Development



DISCUSSION

Future characterization of the plants expressing the 35S *SrEnod2* 3' construct.

Many examples for the involvement of 3'UTRs in the regulation of *Drosophila* development have been reported (for review see Johnston, 1995). For example, the anterior determinant bicoid, a homeodomain protein, binds to the 3'UTR of its posterior counterpart caudal, a homeodomain transcription factor, and negatively regulates its translation in a posterior to anterior gradient (Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996). In embryos lacking bicoid expression, caudal protein will accumulate evenly throughout the embryo, giving rise to deletion of head and thoracic segmentation (Mlodzik, et al., 1990). Another important example comes from the study of *C. elegans* development. Expression of the *fem-3* gene in the hermaphrodite germ line allows the switch to spermatogenesis and negative regulation of *fem-3* expression is required for the switch to oogenesis (Barton et al., 1987). Negative regulation of *fem-3* expression was shown to localize to the 3'UTR of *fem-3* (Ahringer and Kimble, 1991). In an *in vivo* tritration experiment, overexpression of the 3'UTR of *fem-3* caused 21% masculinization of the hermaphrodite germ line.

In the *SrEnod2* 3' *in vivo* titration experiment, the overexpression of the *SrEnod2* *3'*UTR in *Arabidopsis* resulted in an unexpected novel developmental phenotype, the Siamese/Flag phenotype. It has been been shown that the *SrEnod2* 5'-*gus-SrEnod2* 3' construct is expressed at a lower level in the meristematic region of *Arabidopsis* (see chapter 4), as compared to expression in the root system. These alterations in the determination of meristem number of Siamese plants may reflect the level of expression of *SrEnod2* 3'-interacting proteins in these tissues as compared to the root system, where a root phenotype is only rarely observed. It may be that this lower level of expression of the

SrEnod2 5'-*gus*-*SrEnod2* 3' construct reflects a low level of expression of *SrEnod2* 3'-interacting proteins in these tissues. Therefore, expression of these *SrEnod2 3'*-interacting proteins in the meristem may be low enough that they can be titrated away from their normal *in vivo* targets, whereas the higher expression of the *SrEnod2 3'*-interacting proteins in roots may not allow for a significant decrease in their effective concentrations by titration. Analysis of control plants harboring the promoterless *nos 3'* and *SrEnod2 3'* regions are in progress. To better understand the Siamese phenotype, a detailed developmental anlaysis will need to be performed using scanning electron microscopy. It is not known at what stage of meristem development is effected to give rise to the Siamese/Flag phenotype. The only evidence which supports a postembryonic effect is the existence of Flag plants. Figure 5.10 shows a diagram of normal *Arabidopsis* meristem development. The first two leaf primordia are formed opposite each other at approximately the same time within the first two days after germination. Following the development of these primary leaves, single primordia develop in a clockwise or counter-clockwise fashion, until a mature whorl of leaves is formed (Medford et al., 1992). The presence of a single primary leaf in Flag plants suggests that the meristem was functional at an early stage after germination, but soon changed fate. A close examination of the meristem in the embryo, and during germination may determine whether this developmental abnormality occurs embryonically or postembryonically.

The existence of multiple idependent lines makes it likely that the Siamese phenotype is indeed due to expression of the 35S *SrEnod2 3'* construct, and not do to random insertional mutagenesis. A question remains as to the possible involvement of components of a cytokinin signal transduction pathway in the Siamese phenotype. To gain some insight into this question, the cytokinin-overproducing mutant *amp1* (Chaudhury et al., 1995) harboring the *SrEnod2* 5'-*gus*-*SrEnod2* 3' construct was crossed to plants harboring the 35S SrEnod2 3' construct. The *amp1* mutation gives rise to an increase in leaf production, as well as a loss of apical dominance (Chaudhury et al., 1995). F_2 plants

of being analysed for phenotype and GUS activity. Another question is what is the connection between this meristem phenotype and *SrEnod2* expression in roots and nodules? The answer to this question will lie in the isolation of *trans*-acting factors and the study of their function. One can only speculate that the protein(s) which may be titrated away from its normal *in vivo* target is a negative regulator of a gene(s) involved in meristem development (perhaps through translational regulation).

It is important to note that overexpression of only the *SrEnod2* 3'UTR gives rise to the Siamese/Flag phenotype, and downregulation of the *gus* reporter construct. This phenotype and measurement of GUS activity may be usefull assays for the further delimitation of the *SrEnod2 3'*UTR for sequences important for its function. In conjunction with these assays, deletions or linker-scanning mutations of the *SrEnod2 3'*UTR-*gus* fusions may allow for the identification of sequences important for cytokinin regulation of the *SrEnod2* gene. It will be interesting to determine whether overexpression of sequences giving rise to the Siamese/Flag phenotype are the same as those which are important for cytokinin regulation.

The utility of this *in vivo* titration experiment may extend to answering questions regarding the existence of *SrEnod2* 3'-binding proteins in nodules, as well as the function of the SrEnod2 protein in nodules. The latter idea is based on the assumption that titration of *SrEnod2* 3'-binding proteins may down regulate the *SrEnod2* gene, thereby giving a cosuppression effect. Multiple independent lines of *Lotus corniculatus* plants harboring the 35S *SrEnod2* 3' construct have been generated and are now in the process of being analyzed for alterations in endogenous *LcEnod2* gene expression.

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Chapter 6

FUTURE PERSPECTIVES

A central problem to the study of the molecular action of cytokinins has been the lack of a gene(s) which is specifically regulated by cytokinin to be used to dissect cytokinin signal transduction pathways. The work presented in this thesis was based on that by Dehio and de Bruijn (1992) showing that expression of the *SrEnod2* gene can be specifically enhanced by application of cytokinin. Their work led me to further determine the important parameters of this regulation by cytokinin and encouraged me to try to develop a system in *Arabidopsis* utilizing the *SrEnod2* gene as a marker for cytokinin signal transduction. Surprisingly, expression of the *SrEnod2* gene is specifically enhanced by cytokinin in *Arabidopsis*, although it is not in legumes other than *S. rostrata* and alfalfa. In addition, genetic evidence was presented in Chapter 5 which indicates the existence of *SrEnod2* 3'-interacting proteins in *Arabidpsis*. This thesis opens the way for the genetic dissecton of a cytokinin signal transduction pathway in *Arabidopsis*, and specifically the isolation of *SrEnod2* 3'-interacting factors.

A selection scheme was devised based on the cytokinin-specific enhancement of expression of the *SrEnod2* 5'-*gus-SrEnod2* 3' construct in *Arabidopsis*. This scheme utilizes the *E. coli* cytosine deaminase gene (*codA*). The *codA* gene functions to deaminate cytosine as part of the pyrimidine salvage pathway in *E. coli*, whereas higher eukaryotes lack the ability to deaminate cytosine (Danielsen et al., 1992). It has been shown that *Arabidopsis* plants expressing the *codA* gene under the control of the 35S promoter grown in the presence of 5-fluorocytosine were severely inhibited in growth (Perra et al., 1993). This inhibition is due to the deamination of 5-fluorocytosine to 5-fluorouricil, an inhibitor of thymidilate synthase. *Arabidopsis* plants which harbor a single copy of the *SrEnod25'-gus-SrEnod2* 3' construct were transformed with the *SrEnod25'-codA-SrEnod23'* construct. Work is in progress to create inbred lines which harbor both constructs. A schematic diagram of this selection scheme is shown in Figure 6.1. Plants which harbor both GUS and *codA* genes under the control of the *SrEnod2* 5' and 3' regions will be

mutagenized with EMS, and M_2 plants selected for growth on plates containing 5-fluorocytosine. To eliminate mutations in the *SrEnod25'-codA-SrEnod2* 3' T-DNA, which would give rise to 5-fluorocytosine resistant plants, plants which survive this selection will be assayed for GUS expression. Future work will focus on the characterization of mutants from this screen and the isolation of their corresponding genes. Any gene which can be isolated from such mutants, will be used as a probe to isolate possible legume homologs, and used to study function in the legume plant. Alternative approachs to consider for the isolation of *SrEnod2* 3' RNA binding proteins are by screening a nodule-specific cDNA expression library using a translational repression system in *E. coli* (Jain and Belasco, 1996) or by the three-hybrid system in yeast (SenGupta, 1996).

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Figure 6.1. Genetic selection scheme. *Arabidopsis* plants harboring both the *SrEnod2* 5'-*gus*-*SrEnod2* 3' and *SrEnod2* 5'-*codA*-*SrEnod2* 3' construct will be mutagenized with EMS and F_2 plants selected for growth on 5-Fluorocytosine. 5-Fluorocytosine-resistant plants will then be analysed for lack of GUS activity. The construction of the *SrEnod2* 5'-*codA*-*SrEnod2* 3' was as follows: The *codA* coding region was subcloned as a ClaI-XbaI fragment (1285 bp) from the pNE3 vector (Stougaard, 1993) into pBluescript SK+ (Stratagene). The *codA* coding region in SK+ was subcloned as a SacI-HindII fragment into a BamHI (made blunt ended)-SacI digested *SrEnod2* 5'-*gus*-*SrEnod2* 3' construct (with *gus* gene removed). The construction of the *SrEnod2* 5'-*gus*-*SrEnod2* 3'construct was as described in Chapter 4.

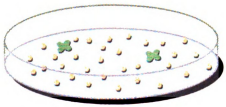
Selection Scheme

SrEnod2 5'-gus-SrEnod2 3'
SrEnod2 5'-codA-SrEnod2 3'

EMS mutagenize

M_1

M_2



Select
5-fluorocytosine
resistance

Test for lack of
GUS activity