This is to certify that the

dissertation entitled

Cytokinin and Nitrate
Regulation of Nitrate Reductases

in Agrostemma githago

presented by

Ronda White

has been accepted towards fulfillment
of the requirements for

Ph. D. degree in Biochemistry/Genetics

Major professor

Date 4/19/96

CYTOKININ AND NITRATE REGULATION OF NITRATE REDUCTASE IN AGROSTEMMA GITHAGO

By

Ronda White

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degrees of

DOCTOR OF PHILOSOPHY

Department of Biochemistry
and
Genetics Program

1996

ABSTRACT

CYTOKININ AND NITRATE REGULATION OF NITRATE REDUCTASE IN AGROSTEMMA GITHAGO

By

Ronda White

Cytokinins are a class of phytohormones that regulate various aspects of plant growth and development. To understand the mechanism of cytokinin regulation, research has focussed on cytokinin regulation of gene expression. We studied the regulation of nitrate reductase (NR) by cytokinin in *Agrostemma githago* because it is a fast and specific response, and we are studying an enzyme where much biochemical and molecular biological information is known.

Three partial NR cDNA clones were isolated and showed high sequence homology. All three genes were regulated by nitrate; two genes were regulated by cytokinin. Changes in mRNA levels were transient and preceded changes in NR activity. Cytokinin increased NR activity in the cotyledons but increased NR mRNA levels in roots and cotyledons. Nitrate increased NR activity and NR mRNA levels in roots and cotyledons. Increases in NR mRNA levels were observed sooner in the cotyledons than in the roots. Agnr2 mRNA levels were much less abundant than Agnr1 or Agnr3 mRNA levels, and were increased only by nitrate, primarily in the roots.

There were low levels of NR activity at the zero time point that transiently increased with incubation of embryos on water. At the zero time point when there were low levels of NR activity, there were very high levels of

NR mRNAs. The mRNA levels greatly decreased within 2 h of incubation on water.

Nuclear runoff transcription analysis suggested nitrate regulated Agnr1 and Agnr3 at least partially at the transcriptional level, and cytokinin regulated the genes at the post-transcriptional level. When NR activity was additive, NR mRNA levels were not additive. And there were high levels of mRNAs present at the zero time point when there was low NR activity. Taken together, the results suggest multiple levels of regulation by nitrate and cytokinin. Two genomic clones, corresponding to the genes regulated by cytokinin and nitrate, were isolated. These results may lead to further analysis of the mechanism(s) of cytokinin regulation of NR, and to further understanding of how cytokinin regulate plant growth and development.

To Joseph, Laura, and Baby

Without you, I would not be where I am

ACKNOWLEDGEMENTS

I would like to thank Hans Kende, in whose laboratory I spent many years on this project. I would also to like thank my committee members for their assistance: Pam Green, Lee Kroos, Lee McIntosh, and Shelagh Ferguson-Miller.

I would also like to thank all past and present members of the Kende Lab who have taught and supported me in my work. Special thanks go to Wilfried Peters, who was the only other person working on this project in the lab. I enjoyed our discussions and his eternal optimism. Thank you to Sergei Mekhodov, who shared discussions on the RT-PCR analysis. And thank you to David Silver and Alexander Pinaev who helped in the nuclei isolations.

And thank you to Joseph White, for everything.

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CHAPTER 1

Introduction

Cytokinins are a class of phytohormones that regulate processes in plant growth and development which are usually measurable only after days or weeks. Since the first identification of the cytokinin kinetin in 1955 (Miller *et al.*, 1955), much of cytokinin biology has focussed on the characterization of naturally occurring cytokinins and their metabolites and the response of plants to applied cytokinins. The advances in biochemical analysis, molecular biology, and genetics have brought further analysis and understanding of the role of cytokinins in plant growth and development. However, there are still many unanswered questions concerning the synthesis, metabolism, and activities of cytokinins. The identification of genes whose expression is affected by cytokinins will lead to answers about the mechanism of cytokinin action. This work examines the regulation of nitrate reductase by cytokinins and nitrate in *Agrostemma githago* embryos.

CYTOKININS

In the early 1950's, Skoog and colleagues were studying the stimulation of growth by auxin in cultured tobacco pith cells. In response to auxin, these cells enlarged but did not divide. When segments of vascular tissue were placed on the pith or in the medium next to the pith, cell division occurred in

the pith tissue. Cell division was also stimulated by adding coconut milk or yeast extract to the medium. This work lead to the isolation and identification of the cytokinin kinetin from autoclaved herring-sperm DNA (The cytokinin resulted from a chemical rearrangement of the DNA during autoclaving; for a review, see Skoog, 1994). The first naturally occurring cytokinin identified was zeatin, isolated from corn endosperm independently by both Letham and Miller in 1964.

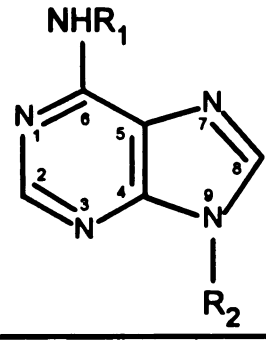
Cytokinins are defined as substituted adenine compounds that promote cell division in defined tissue systems (Skoog *et al.*, 1965; Salisbury and Ross, 1992). The structures of some cytokinins are shown in Figure 1.1. Matsubara (1990) reviewed the activity of more than 200 natural and synthetic cytokinins in bioassays. Cytokinin activity varied greatly depending on the side groups present. In general, the N^6 side chain primarily defined cytokinin activity. Side chains at positions N^9 and, to a lesser extent, N^7 also influenced cytokinin activity.

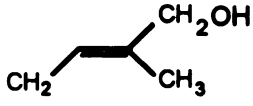
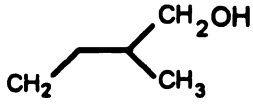
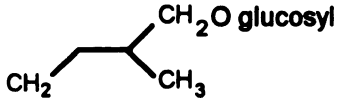
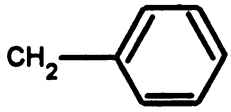
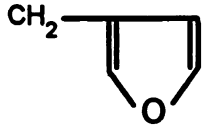
LOCALIZATION

Cytokinins are specifically found at the base adjacent to the 3′ end of the anticodon of tRNAs recognizing codons beginning with uridine in essentially all organisms and are known to be synthesized during post-transcriptional processing (Murai, 1994; Taller, 1994). This bound cytokinin is not believed to contribute significantly to free cytokinins in plants responsible for hormonal effects (Letham and Palni, 1983; McGaw, 1988; Binns, 1994). In prokaryotes, the cytokinins in tRNAs have been found to influence aspects of translation.

Figure 1.1. Structures of cytokinins.

The structures of the first discovered cytokinin, kinetin, and the first naturally occurring cytokinin identified, *trans*-zeatin, are shown along with other cytokinins. Benzyladenine is a synthetic cytokinin, as is kinetin. Glucosylation may also be found at the *N*-3 and *N*-7 positions.



R_1	R_2	Trivial name
	H	isopentenyl adenine
	ribosyl	isopentenyl adenosine
	ribotide	isopentenyl AMP
	H	*trans*-zeatin
	ribosyl	*t*-zeatin riboside
	glucosyl	*t*-zeatin-9-glucoside
	ribotide	*t*-zeatin ribotide
	H	dihydrozeatin
	ribosyl	dihydrozeatin riboside
	ribotide	dihydrozeatin ribotide
	glucosyl	dihydrozeatin-9-glucoside
	H	dihydrozeatin O-glucoside
	ribosyl	dihydrozeatin riboside O-glucoside
	H	benzyladenine
	ribosyl	benzyladenosine
	H	kinetin

Experiments with mutants have shown that cytokinin nucleotides in tRNA are not required for cell growth or protein synthesis, but appear to have a regulatory role (Taller, 1994 and references within). Little is known about the role of cytokinins in tRNAs in plants, but presumably their function is analogous to that in prokaryotes.

Free cytokinins are found in plants and microorganisms that form associations with plants and are present at <1-200 nmoles per 100 g fresh weight (McGaw *et al.*, 1984). Cytokinins are probably found in all plant tissues and cells. Cytokinins and their metabolites are differentially distributed between species, plant organs, and developmental stages (for a review, see Jameson, 1994). While experimental evidence has supported a compartmentalization of some cytokinins, only one study has directly shown that cytokinin-*O*-glucosides are localized within the vacuole (Fusseder and Ziegler, 1988). The vacuole may serve as a storage compartment for *O*-glucosides and other cytokinins, protecting them from degradation and irreversible inactivation.

BIOSYNTHESIS

Indirect synthesis. Since some tRNAs contain cytokinins, biosynthesis of free cytokinins could occur *via* the hydrolysis of the tRNA. However, there is evidence that hydrolysis of cytokinins in tRNA does not contribute significantly to the pool of free cytokinins. Most of the biologically active naturally occurring cytokinins do not occur as constituents of tRNA (McGaw, 1988). In plant tissues, the free and tRNA-bound cytokinins are usually structurally distinct, *i.e.*, free zeatin occurs mainly as the *trans* isomer while

zeatin present in tRNA occurs mainly as the *cis* isomer. Cytokinin-requiring plant tissue cultures contain cytokinins in their tRNAs but still require exogenous cytokinins for growth (Chen and Hall, 1969). The rate of tRNA turnover is not rapid enough to account for the levels of endogenous cytokinins in most tissues (Letham and Palni, 1983).

The recent partial purification and characterization of a *cis-trans* zeatin isomerase from bean endosperm (Bassil *et al.*, 1993) suggests the possibility that tRNA cytokinins could be precursors of biologically active free cytokinins, but this has not been shown. However, tRNA hydrolysis may contribute to the free cytokinin pool to some degree in specific tissues or developmental stages, but the likely primary route for free cytokinin biosynthesis in plants is *via* the *de novo* biosynthetic pathway (McGaw, 1988; Binns, 1994).

De novo synthesis. Except in *Agrobacterium tumefaciens*-induced crown gall tumors, the biosynthetic pathway of cytokinins has not been unequivocally elucidated. The currently favored model shown in Figure 1.2 predicts the formation of isopentenyl adenosine-5'-phosphate (isopentenyl AMP) from AMP and Δ-2-isopentenyl pyrophosphate, catalyzed by isopentenyl transferase (IPT). Isopentenyl AMP can be modified in a series of reactions to yield other cytokinins, their ribotides, ribosides, and free bases.

Since cytokinins are present at low levels in plants, cytokinin biosynthetic enzymes are also expected to be present at low levels. The low levels and instability of IPT, as well as the susceptibility of the substrates and products to phosphatases, has made purification of the enzyme difficult. IPT has been

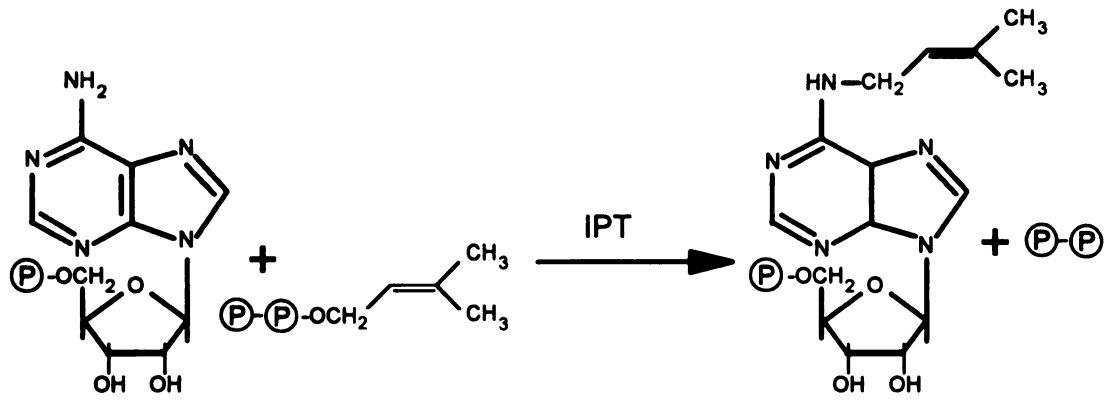


Figure 1.2. Cytokinin biosynthesis.

Cytokinins are synthesized *de novo* by transfer of an isopentenyl group from Δ-2-isopentenyl pyrophosphate (iPePP) to 5'-AMP by isopentenyl transferase (IPT). Isopentenyl AMP (iPeAMP) is the first cytokinin synthesized, and is then converted to zeatin and other cytokinins.

partially purified from tobacco cell lines and slime mold (for a review, see Chen and Ertl, 1994), suggesting the *de novo* synthetic pathway is also present in higher plants. Unfortunately, the enzyme has not been purified in quantities sufficient for antibody generation or microsequencing. Although a variety of bacteria contain *ipt* or *ipt*-like genes, plant DNA sequences homologous to the bacterial *ipt* have not been reported. Comparisons of bacterial *ipt* genes reveal conserved sequences that may lead to the isolation of a plant gene (Crespi *et al.*, 1992; Chen and Ertl, 1994).

Site of biosynthesis and translocation. While cytokinins have been found in most plant tissues, the site of biosynthesis is difficult to determine with certainty. The extremely low levels of endogenous cytokinins in plant tissues and the central role of the putative adenine-derived precursors in cellular metabolism have posed technical problems in identifying sites of cytokinin biosynthesis. The primary site of cytokinin biosynthesis is likely the root tips, but synthesis can also occur in the shoots (Letham, 1994). Germinating seeds may also synthesize cytokinins. Cytokinins are translocated primarily from the roots to the shoots through the xylem (Kende, 1965; Letham, 1994). Ribosides, nucleotides, and *O*-glucosides appear to be important in root-to-shoot translocation (Letham, 1994). Evidence also supports some movement of cytokinins through the phloem. Cytokinins may also be translocated from the embryonic axis to the cotyledons early in seed germination (for a review, see Letham, 1994).

CYTOKININ METABOLISM

An important way to regulate the availability of active cytokinins in plant tissues is through metabolic interconversions. The first metabolic step is the stereospecific hydroxylation of isopentenyl AMP to *trans*-zeatin derivatives. Further cytokinin metabolism can be grouped into purine ring and side chain modifications (Jameson, 1994). Changes in the purine ring include base-riboside-nucleotide conversions, N^3-, N^7-, and N^9-glucosylations, and N^9-alanylation. Changes in the N^6-side chain include side chain reduction, cleavage, and conjugation (including *O*-glucosylation, *O*-xylosylation, and *O*-acetylation; for a review, see Jameson, 1994). The elucidation of the metabolic pathways has been based largely on the use of radiolabelled cytokinins. The cytokinin nucleotides are the prominent metabolites formed immediately after cytokinin uptake. Ultimately, applied cytokinins are either broken down by side chain cleavage to the corresponding adenine derivatives or are conjugated to glucose, xylose, alanine, or acetate (Jameson, 1994).

Which molecules have biological activity, which are used for temporary storage, and which are used to remove cytokinin from the active pool? Zeatin, its riboside and nucleotide are all active in bioassays, but because of the interconversions it is not known which molecules are active *per se*. The N-glucosides are very stable and have low biological activity, apparently withdrawing cytokinin from the active pool. The N^9-alanylation may be a storage form of the cytokinin. Reduced side chains (dihydro derivatives) are not substrates of cytokinin oxidase, which cleaves side chains, and are, therefore,

more stable. Reduction of the side chain appears to precede conjugation of the side chain with glucose, xylose, or acetate. Cytokinins with conjugated side chains may be involved with transport and storage. Side chain cleavage leads to irreversible loss of biological activity (for a review, see Jameson, 1994).

There are reports of crude activities for many of the cytokinin metabolic enzymes (for reviews, see Armstrong, 1994; Mok and Martin, 1994), but few have been purified sufficiently for antibody production or microsequencing. The most advanced molecular analysis of a cytokinin metabolic enzyme is the purification and characterization of zeatin O-xylosyl transferase from *Phaseolus vulgaris* embryos (Turner *et al.*, 1987). A monoclonal antibody has been used to show that the transferase is found in both the cytoplasm and the nucleus (Martin *et al.*, 1993a) and to isolate a cDNA clone containing an insert proposed to code for the transferase (Martin *et al.*, 1993b). As more metabolic genes are identified, transgenic methodologies could be used to alter cytokinin metabolism and determine the role of metabolism in cytokinin activities.

CYTOKININ ACTIVITIES

Since the discovery of cytokinins in the 1950's, there have been many studies to elucidate the effects of cytokinins on plant growth and development. Most effects have been observed by application of cytokinins or analysis of endogenous cytokinins. However, results observed with exogenous cytokinins do not necessarily indicate the involvement of endogenous cytokinins. Many studies of endogenous cytokinins have supported the involvement of cytokinins in developmental processes, but the analyses were often flawed by inadequate

analytical techniques, reliance on bioassays, or determination of only a few cytokinin metabolites (Mok, 1994). Still, much has been learned about cytokinin effects on plant growth and development.

It is clear that plant development is determined by many internal and external factors, and cytokinins are just one of these factors. Other hormones may interact cooperatively, independently, or antagonistically with cytokinins. Environmental stimuli such as light and temperature affect plant growth and development. It is often difficult to undertstand the interaction between the various signals. In summary, cytokinins play a role throughout development, from seed germination to leaf senescence. Some of the effects include the following: promotion of cell division; enhanced seed germination under non-permissive conditions; bud formation in tissue culture at high cytokinin-to-auxin ratios; release of lateral buds from apical dominance; stimulation of leaf expansion; delay of leaf senescence; increase in chloroplast development; and regulation of the cell cycle. More detailed reviews on cytokinin effects have been written recently (Jacqmard *et al.*, 1994; Mok, 1994; Musgrave, 1994; Reski, 1994).

GENE REGULATION BY CYTOKININS

The diversity of cytokinin effects suggests that changes in gene expression may mediate the response of plant cells to cytokinins. Studies have shown that cytokinins affect steady-state mRNA levels, protein levels, and the activity of numerous enzymes. Table 1 lists many of these enzymes, showing regulation of various enzymes that might affect plant growth and development.

[A]NR=nitrate reductase; LHCP=light-harvesting chlorophyll *a/b* binding protein; PEPC=phospho*enol*pyruvate carboxylase; CA=carbonic anhydrase; SSU=small subunit of ribulose-1,5-bisphosphate; HPR=hydroxypyruvate reductase; CO=cytokinin oxidase; A=activity of the enzyme.

B

1. Borriss 1967.
2. Kende *et al.* 1971.
3. Kulaeva *et al.* 1976.
4. Hänisch ten Cate and Breteler 1982.
5. Knypl 1973.
6. Guadinová 1990.
7. Rao *et al.* 1984.
8. Suty *et al.* 1993.
9. Roth-Bejerano and Lips 1970.
10. Banowetz 1992.
11. Gzik and Günther 1984.
12. Lu *et al.* 1990.
13. Flores and Tobin 1986.
14. Flores and Tobin 1988.
15. Teyssendier de la Serve *et al.* 1985.
16. Abdelghani *et al.* 1991.
17. Longo *et al.* 1990.
18. Sugiharto *et al.* 1992.
19. Suzuki *et al.* 1994.
20. Thomas *et al.* 1992.
21. Axelos *et al.* 1987.
22. Lerbs *et al.* 1984.
23. Chen and Leisner 1985.
24. Chen *et al.* 1993.
25. Deikman and Hammer 1995.
26. Simmons *et al.* 1992.
27. Mohnen *et al.* 1985.
28. Crowell 1994.
29. Sano and Youssefian 1994.
30. Dehio and de Bruijn 1992.
31. Teramoto *et al.* 1995.
32. Cotton *et al.* 1990.
33. Watillon *et al.* 1991.
34. Crowell *et al.* 1990.
35. Teramoto *et al.* 1993.
36. Chen *et al.* 1987.
37. Dominov *et al.* 1992.
38. Chatfield and Armstrong 1986.

[C]The treatment time that gave a significant difference in the results, or the only time tested. A: <4 hours; B: 4-24 hours; C: > 24 hours.

Table 1.1. Examples of enzymes regulated by cytokinins.

Plant	Enzyme[A]	Regulation	Ref.[B]	Time[C]
A. githago	NR	↑ NRA	1-3	A,B
Bean		↑ NRA	4	B
Cucumber		↑ NRA	5,6	B
Maize		↑ NRA	7	A
Tobacco		↑ NRA	8,9	C
Wheat		↑ NRA	6,10	B,C
ß. vulgaris		↑ NRA	11	A
C. album		↑ NRA	11	A
Barley		↑ NRA, ↑ mRNA	6,12	A,C
L. gibba	LHCP	↑ mRNA	13,14	B
Tobacco		↑ mRNA	15,16	C
Watermelon		↑ mRNA, ↑ LHCP	17	C
Maize	PEPC, CA	↑ mRNA	18,19	A
M. crystallinum	PEPC	↑ mRNA	20	C
L. gibba	SSU	↑ mRNA	13	B
Tobacco		↑ mRNA,	16,21	
Pumpkin		↑ SSU, ↑ SSUA	22	C
Pumpkin	HPR	↑ HPRA	23,24	B
A. thaliana	4 anthocyanin biosynthetic genes	↑ mRNA	25	A
Rice	ß1,3;1,4-glucanase	↑ mRNA	26	C
Tobacco	ß1,3-glucanase	↓ mRNA	27	B
Soybean	pollen allergen	↑ mRNA	28	A
Wheat	*wpk4*	↑ mRNA	29	A
S. rostrata	*Enod2*	↑ mRNA	30	A
Cucumber	*CR9, CR20*	↓ mRNA	31	A
	phytochrome	↓ mRNA	32	A
Apple	unknown	↑ mRNA	33	C
	10-kD PSII	↓ mRNA	33	C
Soybean	20 cDNAs (2 ribosomal proteins)	↑ mRNAs	34	A
Cucumber	unknowns	↑↓ transl.	35,36	A,B
Pumpkin	unknowns	mRNAs	23	C
N. plumbaginifolia	pLS216	↑ mRNA	37	B
Bean	cytokinin oxidase	↑ COA	38	A

Most of these experiments have lead to the identification of genes whose expression is enhanced by cytokinins. Other experiments have shown that cytokinins also suppress gene expression (ref. 23, 31, 32, 35, 36 from Table 1).

Most cytokinin-responsive genes also respond to other signals, such as light, stress, other plant hormones, sucrose, nutrient status, or other factors. It is often difficult to separate the effects of independent stimuli, or to understand how multiple stimuli interact. Many of the cytokinin-responsive genes are regulated over longer periods of time (Table 1), and are, therefore, not likely to represent the primary molecular responses to cytokinins but rather the results of a series of signalling activities. Studies of rapid responses to cytokinins are likely to lead to the identification of the early events in the cytokinin signal transduction pathway.

The mechanism(s) by which cytokinins regulate plant gene expression remains unclear but information is starting to accumulate. Inhibitors of transcription or translation have been used to show the requirement of either or both of these processes in enhancement of gene expression (Kende and Shen, 1972; ref. 3, 12, 22, 23, 34, 38 from Table 1). Results of nuclear runoff experiments have indicated both transcriptional (ref. 12, 19, 25, 37 from Table 1) and post-transcriptional (ref. 14, 19, 25, 30 from Table 1) regulation of cytokinin-responsive genes.

A role for transcription in cytokinin regulation of specific plant genes has been confirmed by the isolation and characterization of gene promoters that are

regulated by cytokinins. Studies have shown that the mannopine synthase (*mas*) promoter (Langridge *et al.*, 1989) and the gene-5 promoter (Boerjan *et al.*, 1992) from *Agrobacterium tumefaciens* plasmids are regulated by cytokinin and auxin in transformed tobacco plants. Therefore, genes carried by prokaryotic plasmids have acquired *cis* elements that function following transfer and integration into plant cells. A promoter from *Oryza sativa* is induced by both cytokinin and auxin in transformed tobacco plants, indicating that a monocotyledonous promotor is active and recognized by the hormonal signal transduction mechanism in a dicotyledonous plant (Claes *et al.*, 1991).

Protein kinases and phosphatases are also involved in the regulation of gene expression by cytokinins. Dominov *et al.* (1992) showed that the enhancement of *pLS216* mRNA levels by cytokinin is blocked by a protein kinase inhibitor (staurosporine) and stimulated by a protein phosphatase inhibitor (okadaic acid). In contrast, Crowell (1994) showed that the enhancement of *cim1* mRNA levels is stimulated by staurosporine and blocked by okadaic acid.

These observations indicate that different cytokinin signal transduction pathways may regulate the expression of different plant genes. Future work will likely reveal many of the molecular events that mediate the effects of cytokinin on gene expression and, thus, on the growth and development of plants. Similarities and differences to animal and other plant hormone signal transduction systems will emerge as more information is obtained.

NITRATE REDUCTASE

Inorganic nitrogen in the biosphere must be converted to a biologically useful form either by the fixation of N_2 or the assimilation of nitrate. For plants that do not establish N_2-fixing symbioses, the important nitrogen sources are nitrate and ammonium. Because ammonium is readily oxidized to nitrate by soil bacteria, nitrate is usually the main nitrogen source available to most higher plants. Nitrate assimilation is estimated to produce in excess of 20,000 megatons of organic nitrogen per year compared with 200 megatons from nitrogen fixation (Guerrero *et al.*, 1981). Nitrate assimilation occurs in higher plants, algae, fungi, and some bacteria. These organisms then supply reduced nitrogen to other forms of life.

Agriculture has used nitrogen fertilizers to enhance crop yield. However, there is wide concern over the use of these fertilizers. Fertilizer production uses fossil fuel and is costly; the highly soluble nitrate can accumulate in surface and ground waters, resulting in health concerns since in humans nitrate is reduced by bacteria to toxic compounds; and nitrous oxides are generated *via* denitrification in soils, leading to air pollution (Campbell and Kinghorn, 1990). Enhancing the efficiency of nitrate assimilation has become one of the objectives of research in plant nutrition. To achieve this, understanding the mechanisms and regulation of nitrate uptake and assimilation is of great importance.

There are at least two reasons why plants must regulate nitrate reduction. First, the process is very energy consuming, with as much as 25%

of the energy generated by photosynthesis consumed in driving nitrate assimilation (Solomonson and Barber, 1990). Second, nitrite, the primary product of nitrate reduction, as well as ammonium, are cytotoxic.

The pathway of nitrate assimilation is shown in Figure 1.3. After uptake, nitrate is reduced to nitrite by nitrate reductase (NR) in the cytoplasm, then nitrite is transported into the chloroplast and reduced to ammonium by nitrite reductase. Ammonium is incorporated into glutamine and other organic compounds. The rate-limiting and regulated step is the reduction of nitrate to nitrite by NR. This enzyme is considered to be a limiting factor for growth, development, and protein production in plants (Solomonson and Barber, 1990).

FUNCTIONAL PROPERTIES

Because NR is a large and unstable enzyme of low abundance, purification of higher plant NR has been difficult (for reviews, see Beevers and Hageman, 1983; Campbell and Smarrelli, 1986; Crawford and Davis, 1988; Solomonson and Barber, 1990). Even though Evans and Nason (1953) discovered NR more than forty years ago, major advances in understanding the enzyme came within the last decade with better purification procedures (Notton *et al.*, 1977; Campbell and Smarrelli, 1978) and the production of monoclonal antibodies (Snapp *et al.*, 1984; Notton *et al.*, 1985; Hyde *et al.*, 1989). The first isolation of a partial cDNA clone encoding a NR in higher plants was from squash (Crawford *et al.*, 1986). Since then, more than eleven cDNAs encoding NRs have been isolated (for a review, see Rouzé and Caboche, 1992). Many higher plants have two NR genes, including *Arabidopsis thaliana*, which is often

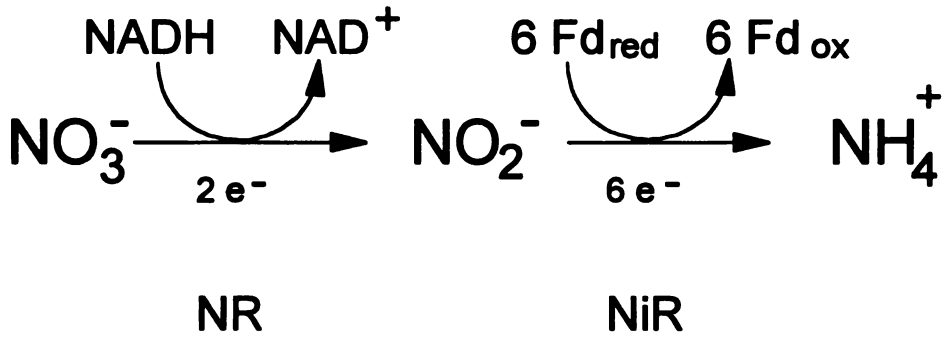


Figure 1.3. Nitrate assimilation pathway.

The assimilation of nitrate occurs *via* a two-step reduction. Nitrate reductase (NR) catalyzes the reduction of nitrate to nitrite, and nitrite reductase (NiR) the reduction of nitrite to ammonium.

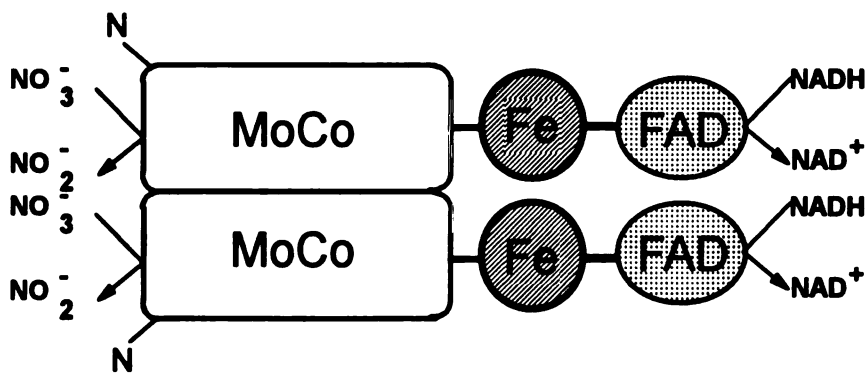


Figure 1.4. Structural diagram of nitrate reductase.

A schematic diagram of the nitrate reductase homodimer with the functional domains indicated. MoCo, molybdenum cofactor domain; Fe, heme domain; FAD, flavin domain; N, N terminus of nitrate reductase.

used in molecular genetics because of its small genome size.

The sizes of the subunits of the homodimeric NR from ten higher plants range from 100-120 kD (Campbell and Smarrelli, 1986; Solomonson and Barber, 1990). NR is a multicenter redox enzyme where each subunit contains specific binding domains for FAD, heme, and a molybdenum-pterin cofactor in a 1:1:1 stoichiometry. Three forms of NR have been found in eukaryotes. Most common in higher plants is a NADH-specific NR. A NAD(P)H-bispecific NR may occur as a second isoform, or may occur as the only form. NADPH-specific NR has not been found in higher plants, but is present in fungi and mosses. A structural model for NR is shown in Figure 1.4. Electrons are transferred sequentially from NADH through the prosthetic groups to nitrate, reducing nitrate to nitrite.

REGULATION of NR

NR activity is inducible by nitrate in all organisms and is one of only a few substrate-inducible enzymes in plants. In general, there is little or no NR mRNA or protein in the absence of nitrate. The addition of nitrate increases the level of mRNA, protein, and enzyme activity to varying degrees, depending on plant species, plant tissue, and plant age. While nitrate is the primary signal that enhances NR expression, other factors also influence NR gene expression, *i.e.*, light, circadian rhythms, CO_2, reduced nitrogen compounds, sucrose, cytokinins, and a plastidic factor. NR has been shown to be regulated in various ways by these factors, including transcriptionally, post-transcriptionally, and post-translationally (for reviews, see Crawford, 1993; Hoff *et al.*, 1994;

Rouzé and Caboche, 1992; Crawford *et al.*, 1992). In plants with more than one NR gene, differential regulation has been found.

LOCALIZATION of NR

NR from higher plants is generally believed to be localized in the cytosol, although a minor fraction may be associated with membranes (Ward *et al.*, 1988). Little is known about the spatial localization of NR in plants. Some studies examined tissue specificity by measuring NR protein, activity, or mRNA in roots versus leaves or in the scutellum, roots, and leaves in corn (Redinbaugh and Campbell, 1981; Sorger *et al.*, 1986). The only studies in higher plants examining cellular localization of NR are with corn. Using immunogold labelling, NR in corn leaves was found to be localized exclusively in the cytoplasm of leaf mesophyll cells (Vaughn and Campbell, 1988), in the cytoplasm of root epidermal and cortical cells, and in the parenchyma and pericycle within the vascular cylinder (Federova *et al.*, 1994). NR was shown to be localized in epidermal cells of corn roots by measuring NR protein content in epidermal, cortical, and stelar cells (Rufty *et al.*, 1986). No work has addressed the localization of NR mRNA in plants.

AGROSTEMMA GITHAGO

Agrostemma githago was widely distributed in many temperate regions throughout the world. It is believed to have originated in the eastern Mediterranean area and was introduced into Britain around 500 B.C. (Firbank, 1988; Svensson and Wigren, 1983). The plant was a prevalent weed and

found as a contaminant of cereal grains for at least 4000 years. Improved seed cleaning techniques of this century have essentially eliminated the plant in nature. Plants are now maintained in reserves and are becoming more popular as garden plants.

A. githago, known by the common name corn cockle, is a tetraploid dicot (n = 24) in the family of the *Caryophyllacea*. Borriss was the first to study physiological processes in *A. githago*, namely after-ripening and seed germination (Borriss, 1967; Borriss, 1977). *A. githago* seeds are ripe about 33 days after fertilization (Svensson and Wigren, 1983), but are in a dormant stage and cannot germinate. During an after-ripening period of several months to a year at 15°C to 20°C (Borriss, 1977; Firbank, 1988), the germination rate increases up to 100%. Dormancy can be preserved for several years by maintaining the seeds at -15°C to -20°C (Borriss, 1977). The viability of the seeds may be maintained for several years in dry storage (Borriss, 1977; Firbank, 1988).

Borriss (1977) examined the effect of plant hormones on enzyme activities during early germination of *A. githago*. Plant hormones applied to isolated embryos regulated specific enzymes dependent on the development stage of the seeds, *i.e*, dormant versus after-ripened. One enzyme examined was NR (Borriss, 1967; Borriss, 1977). Applied auxin and gibberellin had no effect on NR activity, while cytokinin increased and abscisic acid decreased NR activity of dark grown embryos. Electrophoresis of *in vivo* labelled proteins identified differences in patterns of newly synthesized proteins between

imbibed, after-ripened young and aged embryos (Bernhardt *et al.*, 1993). Differences were also shown between cotyledons and axes. Based on Borriss' results (1967), Kende and colleagues studied the regulation of NR by cytokinins in *A. githago*. In particular, the question was asked whether cytokinins affected NR directly or through the substrate nitrate, which also increases NR activity. Kende *et al.* (1971) showed that cytokinin and nitrate enhanced NR activity independently, and that this was a result of *de novo* protein synthesis (Kende and Shen, 1972).

Changes in gene expression may mediate the various responses of plant cells to cytokinins. Identification of genes regulated by cytokinins as well as the mechanism of that regulation will further increase the knowledge of cytokinin action in plants. The regulation of NR by cytokinins in *A. githago* was chosen for further study for several reasons. The induction of NR activity in *A. githago* embryos occurs in less than one hour (Kende *et al.*, 1971), allowing examination of an **early response** to cytokinins. This is the only plant where it has been shown that induction by cytokinins is **specific** and not dependent on exogenous or endogenous nitrate or the presence of light (Kende *et al.*, 1971). While cytokinin regulation of unknown genes will surely prove useful, the regulation of NR allows the study of a **known enzyme** for which much biochemical, genetic, and more recently molecular biological information is known.

OBJECTIVES

Cytokinins were initially identified and characterized because of their role in cell division. Since then, cytokinins have been found to be involved with various aspects of plant growth and development. However, little is known about the primary mode of action of cytokinins. Regulation of gene expression by cytokinins has been studied at the molecular level only in recent years, and the mechanism(s) of regulation are just starting to be elucidated.

The goal of this project was to study the regulation of NR by cytokinins and nitrate by addressing the following questions: How many NR genes are there in *A. githago*? Is (are) the same gene(s) regulated by nitrate and by cytokinin? Do cytokinin and nitrate regulate NR differentially? If there is more than one gene, are the genes differentially regulated? Are the genes regulated at the transcriptional or post-transcriptional levels? Is expression of the nitrate- and cytokinin-regulated NR gene(s) localized in the same or in different cells of the embryo?

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CHAPTER 2

The Biochemical Analysis of Nitrate Reductase in *Agrostemma githago* Embryos

ABSTRACT

Nitrate reductase (NR) activity was induced independently by cytokinins and nitrate in *A. githago* embryos, and the inductions were additive at 6 h of treatment. Nitrate-induced activity increased faster and decreased faster than did cytokinin-induced activity. Nitrate-induced activity peaked at 6 to 12 h, cytokinin-induced activity peaked at 12 to 18 h, and both nitrate and cytokinins enhanced enzyme activity 4 to 6 times over that of water-treated embryos. Cytokinin-induced activity had a lag phase that was dependent on the state of the embryos and was localized primarily in the cotyledons, while nitrate-induced activity showed no lag phase and was localized in both roots and cotyledons. The transient increase in activity in the controls was localized in the roots. The decrease in nitrate-induced activity after 12 h was primarily due to loss of activity in the cotyledons. The induction by cytokinin was specific; several cytokinins increased NR activity while adenine did not.

Ambient room light did not affect the induction of NR activity at 6 h. Ammonia had no effect on NR activity, either by itself or in combination with nitrate or cytokinin. Ethylene increased NR activity ~50% above the control, but had no effect on nitrate-induced activity. Contrary to published results,

ethylene inhibited cytokinin-induced NR activity after 6 h of incubation. NR activity varied during the course of imbibition of the seeds. This activity was temperature dependent, as was the increase in activity in isolated embryos incubated in water.

INTRODUCTION

Nitrate reductase (NR) has been studied in a large number of plants for many years. All plants have nitrate-inducible NR activity, and nitrate is the primary regulator of NR. In most plants, NR is also regulated secondarily by factors such as light, circadian rhythms, CO_2, reduced nitrogen compounds, sucrose, cytokinins, and a plastidic factor (for reviews, see Crawford *et al.*, 1992; Hoff *et al.*, 1992; Rouzé and Caboche, 1992; Crawford *et al.*, 1993, Hoff *et al.*, 1994; Crawford, 1995).

Cytokinins regulate NR activity in several plants (see Table 1.1). However, other factors are usually required for induction of enzyme activity, *e.g.* light and/or nitrate, and cytokinins enhance the response to these factors. Some nitrate is stored in plant vacuoles (Granstedt and Huffaker, 1982), and regulators may increase activity by releasing the nitrate into the metabolic pool where it induces NR activity. Gzik and Günther (1984) showed that cytokinins increased NR activity in the dark in the absence of exogenous nitrate in *Beta vulgaris* and *Chenopodium album* leaf discs. Although cytokinin inducible in the dark, these activities were very low in comparison to those in light. Only in *A. githago* embryos has it been shown that cytokinins induce NR activity independently of the presence of light or exogenous or endogenous nitrate (Kende *et al.*, 1971). Thus, the study of cytokinin regulation of NR in *A. githago* embryos allows the study of a specific cytokinin response without other interacting factors such as nitrate and light. It has been shown that the effects of cytokinin and nitrate on the induction of NR activity are additive and require

de novo synthesis of the enzyme (Kende *et al.*, 1971; Kende *et al.*, 1974; Kuznetsov *et al.*, 1979). Schmerder and Borriss (1986) also demonstrated that ethylene increased NR activity in *A. githago* embryos, and that cytokinin regulation only occurred in the presence of ethylene. The purpose of this work was to examine the regulation of NR in *A. githago* embryos in comparison to previous results in *A. githago* and other plants, and to determine optimum conditions for further molecular biological studies. Because of variations in seed lots, the inductive effects of cytokinin and nitrate needed to be reexamined.

MATERIALS AND METHODS

Plant material. Seeds of *A. githago* were collected from plants grown in the greenhouse and size fractionated to select for larger seeds. After harvest, the seeds were kept in dry storage to break dormancy. Dr. K.H. Köhler (Greifswald University, Germany) also provided multiple seed lots used in these experiments.

Incubation procedures. A. githago seeds were surface sterilized in 1% NaOCl, rinsed five times in sterile water, and then placed in sterile Petri plates on two layers of Whatman filter paper moistened with 5 ml sterile water. The length of imbibition varied as indicated in the figures. Unless specified otherwise, seeds were imbibed at room temperature (RT) in the dark. Embryos were then isolated in ambient room light (except for the experiments in Figure 2.5 where embryos were isolated under a green safelight) and placed in new sterile Petri plates on two layers of Whatman filter paper moistened with 5 ml of the test solution. Embryos were incubated in the dark at 30°C unless otherwise indicated. For experiments using ethylene, the Petri plates were placed without lids in a closed glass container, and ethylene was injected into the container when appropriate. At the end of the indicated treatments, embryos were dried off, weighed when necessary, frozen in liquid nitrogen and stored at -80°C until they were assayed. In the experiment shown in Table 2.1, embryos were first divided into roots and cotyledons.

Tissue extraction and NADH-NR assay. Tissue was homogenized on ice with 0.6 ml of extraction buffer (25 mM KPO_4, pH 7.5, 5 mM EDTA, 5 mM

cysteine), then spun in a microcentrifuge for 10 to 15 min at 14,000 rpm. The supernatant was used for assaying. NR activity was assayed *in vitro* according to Wray and Filner (1970). This assay measures spectrophotometrically the production of nitrite by NR. Typically, four embryos or eight roots or cotyledon pairs were homogenized per sample, and 100 μl of supernatant was assayed in duplicate. Each experiment was done with at least triplicate samples.

RESULTS

Inducibility of NR by cytokinins and nitrate. Both benzyladenine (BA) and nitrate showed dosage-dependent responses on the increase in NR activity (data not shown). Therefore, experiments were done with optimum concentrations of 1-5 μM BA or 50 mM nitrate. Figure 2.1 shows that there was a background level of NR activity in embryos incubated in water. Both BA and nitrate increased the enzyme activity, and, at 6 h, gave an additive effect (Figure 2.1). This additive effect was observed over an entire 24 h incubation period but not in all experiments. Kuznetsov *et al.* (1979) observed an additive effect up to 18 h of incubation (peak activities were 18 h for nitrate and 24 h for BA), but the enzyme activities for BA plus nitrate were not as large experimentally as would be expected from BA and nitrate treatments alone after 18 h of treatment. Kuznetsov suggested that this was due to carbohydrate starvation of the embryos since the addition of glucose could increase NR activity at this time. Therefore, depending on the state of the seeds and the embryos, cytokinin and nitrate increased NR activity and showed an additive effect.

Many NR activities are reported on a fresh-weight basis and vary greatly between 75 nmol/h·g FW in cucumber cotyledons (Guadinova, 1990) to 40,000 nmol/h·g FW in rice seedlings (Kleinhof *et al.*, 1988). Maximum inductions observed in *A. githago* fall in this range at 5000 nmol/h·g FW (data not shown).

Figure 2.2 shows a representative time-course experiment. Although the absolute activities varied between experiments, certain results were consistent. NR activity typically increased transiently in embryos incubated in water.

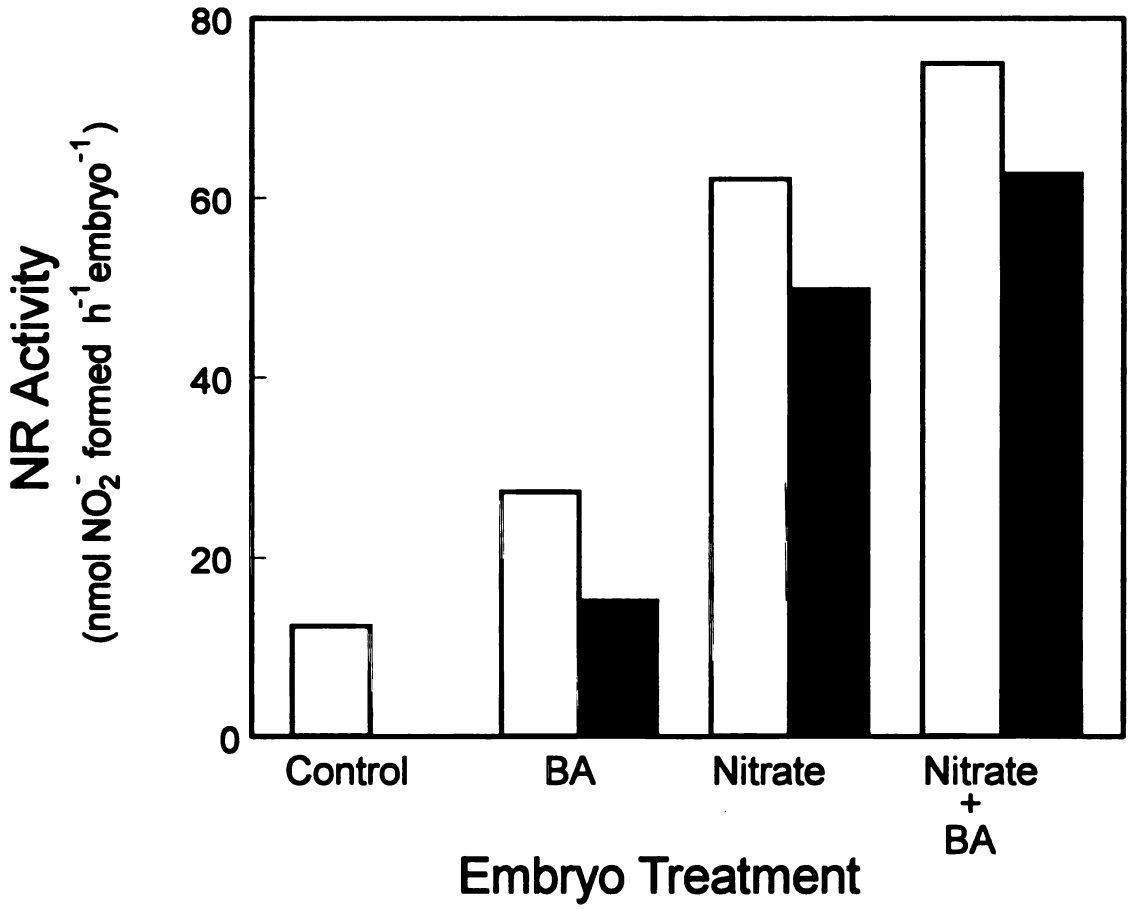

Figure 2.1. Induction and additive effect of cytokinin and nitrate on NR activity.

Seeds were imbibed for 12 h at RT. The embryos were then isolated and incubated at 30°C for 6 h in water (control), 5 µM BA, 50 mM nitrate, or nitrate plus BA. Each point represents the average of triplicate samples, 4 embryos per sample. The darkened bars represent NR activity with the water control subtracted.

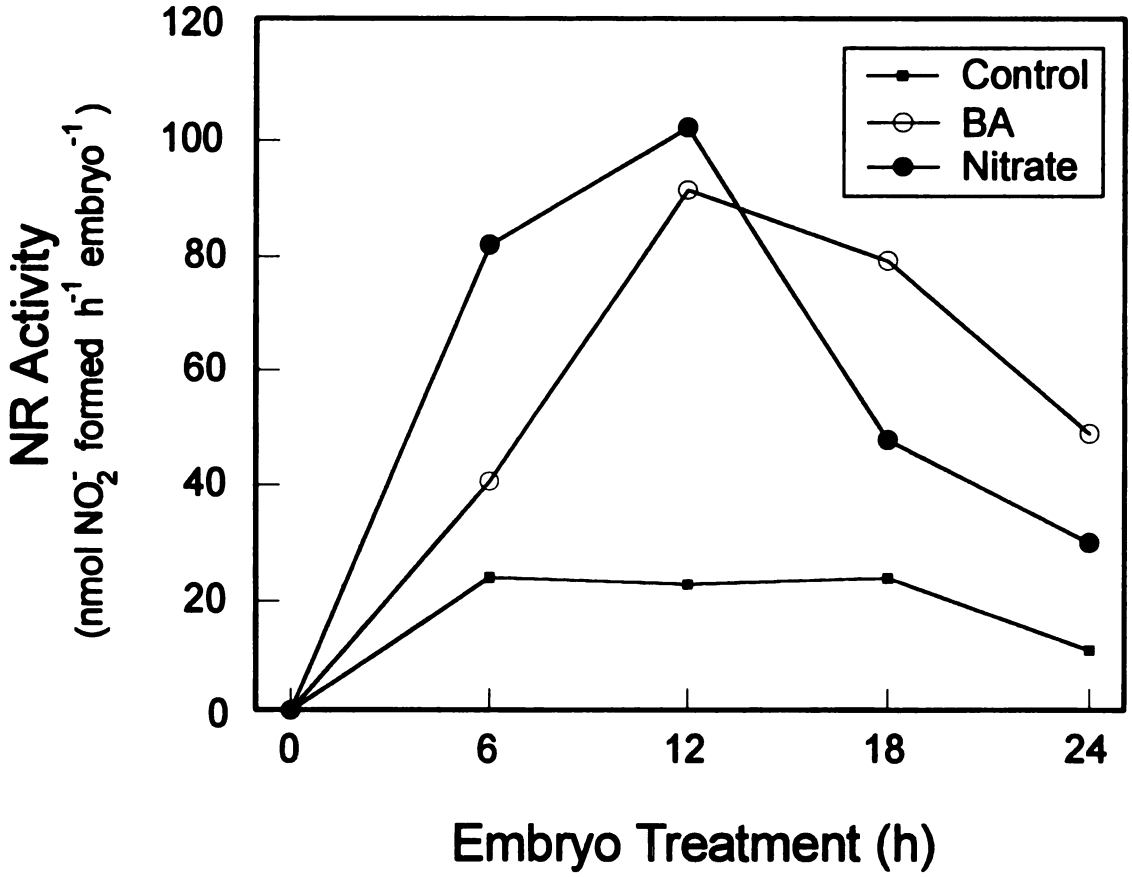

Figure 2.2. Time course for cytokinin and nitrate induction of NR activity.

Seeds were imbibed for 12 h at RT. The embryos were then isolated and incubated at 30°C for the indicated times in water (control), 5 µM BA, or 50 mM nitrate. Each point represents the average of triplicate samples, 4 embryos per sample.

Nitrate-induced activity always increased faster than did BA-induced activity, but BA-induced activity remained elevated longer than nitrate-induced activity. Nitrate-induced activity peaked at 6 to 12 h and BA-induced activity peaked at 12 to 18 h. The activity in water-incubated embryos varied significantly with various seed lots, age of seeds, and time of imbibition (personal observations and Figure 2.9). Overall, both BA and nitrate increased NR activity 4 to 6 times over control.

Closer examination of the early time points showed a lag phase for BA-induced activity that was not observed for nitrate-induced activity (Figure 2.3). The time when a significant increase in BA-induced NR activity could be detected varied with seed lot, age of seeds, and individual experiments from 4-12 h. This lag phase could be due to time for cytokinin uptake or to the state of the embryos. Pre-incubation of the embryos for 4 h in water followed by a 2-h incubation in BA showed no lag phase in the enhancement of NR activity (Figure 2.3). This means that the presence of the lag phase was not due to time required for BA uptake, but rather due to the state of the embryos.

Localization of NR activity. Table 2.1 shows the localization of NR activity. CK-induced activity was found primarily in the cotyledons, while nitrate-induced activity was found throughout the embryo. The transient increase in NR activity in water-incubated embryos was found in the roots. The decrease in nitrate-induced activity in the embryos after 12 h (Figure 2.2) was primarily due to loss of activity in the cotyledons, although there was some loss in the roots also (Table 2.1).

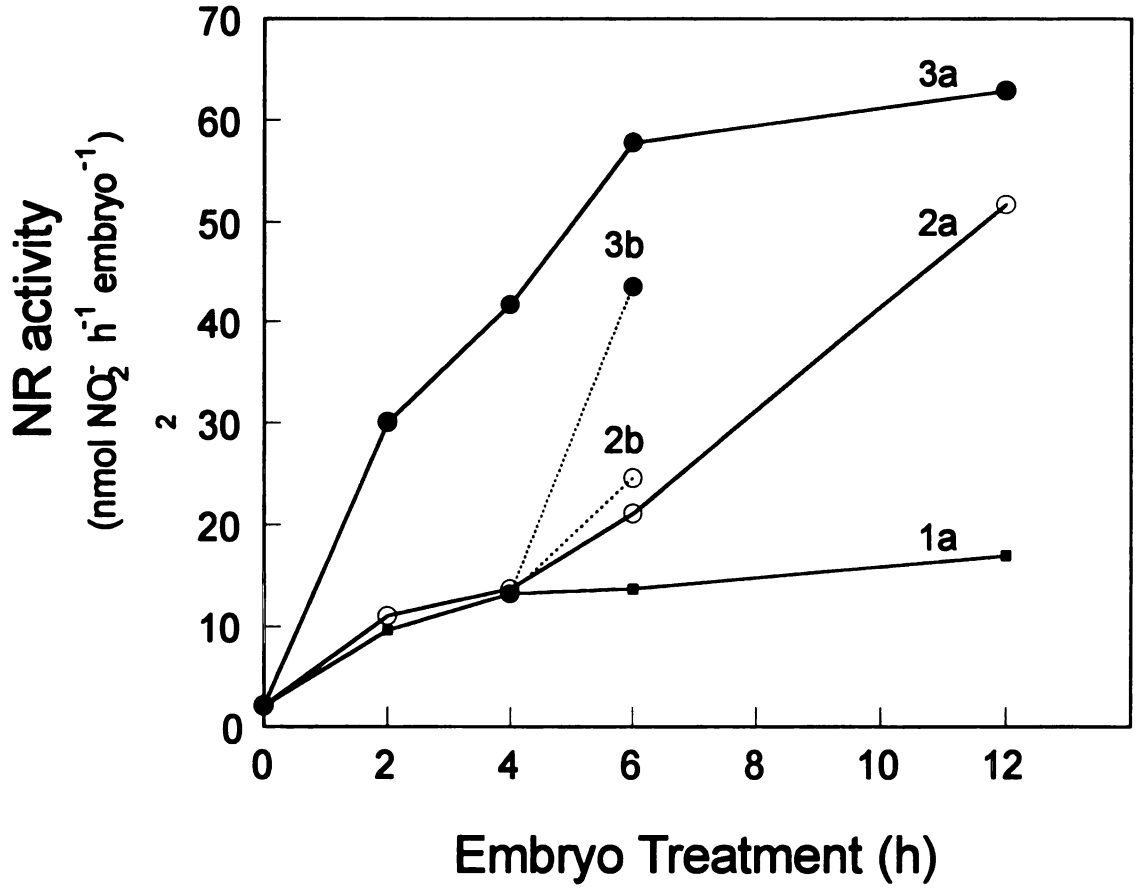

Figure 2.3. The effect of pre-incubation of embryos on cytokinin-induced NR activity.

Seeds were imbibed for 12 h at RT. The embryos were then isolated and incubated at 30°C for the indicated times in water (1a), 5 µM BA (2a), or 50 mM nitrate (3a). Seeds were imbibed for 12 h at RT. The embryos were isolated and incubated at 30°C for 4 h in water, and the embryos were then transferred to BA (2b) or nitrate (3b) for 2 h. The results represent the average of triplicate samples, 4 embryos per sample.

Table 2.1. The localization of cytokinin- and nitrate-induced NR activity in embryos, roots, and cotyledon pairs.

Seeds were imbibed for 12 h at RT, then the embryos were isolated and incubated at 30 °C for 6 or 12 h in water (control), 5 µM BA, or 50 mM nitrate. At the end of the incubation period, some embryos were separated into roots and cotyledon pairs (CP) for measurement of NR activity. Results are ± SEM (*n*=10-12 for 6 h; *n*=6-9 for 12 h). Values represent NR activity, nmoles nitrite/h•embryo, root, or cotyledon pair (CP).

6 hours

	Embryo	Root	CP
Control	16.0 ± 1.4	7.0 ± 0.7	11.8 ± 1.1
BA	30.3 ± 4.7	5.9 ± 0.6	23.1 ± 3.3
Nitrate	64.7 ± 6.4	27.0 ± 2.3	42.0 ± 4.3

12 hours

	Embryo	Root	CP
Control	24.2 ± 3.5	13.9 ± 1.6	9.5 ± 1.5
BA	64.4 ± 11.8	15.2 ± 1.7	41.8 ± 5.3
Nitrate	54.5 ± 7.9	22.0 ± 3.2	22.7 ± 2.7

Specificity of cytokinin-induced NR activity. If NR activity is regulated by cytokinins, the response should be specific. Six cytokinins (BA, BA riboside, zeatin, *i*PA (Figure 2.4), kinetin, and zeatin riboside (data not shown) increased NR activity to varying degrees. Adenine was ineffective (Figure 2.4). Abscisic acid has been shown to be antagonistic to both cytokinin- and nitrate-induced activities in *A. githago* embryos (Kende *et al.*, 1971; Kuznetsov *et al.*, 1979) and barley seedlings (Lu *et al.*, 1992). Abscisic acid (1 μM) inhibited NR activity by ~37% below the control level (data not shown).

Regulation of NR activity.

Light. Light regulates NR at various levels in many plants. While seeds have been imbibed and embryos incubated in the dark in previous experiments (Kende *et al.*, 1971; Kuznetsov, 1979), embryos have usually been isolated in the light. The effect of ambient room light on NR activity was tested. Seeds were imbibed, isolated, and incubated in the light or in the dark. Figure 2.5 shows that light had no effect on background or induced NR activity. Since these experiments were done at different times and under different conditions, the absolute activities cannot be compared between experiments, but within each experiment no effect by light was observed. Thus, in all other experiments, imbibition and incubation were carried out in the dark, but embryos were isolated as quickly as possible in the light.

Ammonia. Ammonia, the product of nitrite reduction, has varying effects on NR activity. In *A. githago* embryos, ammonia has no effect on background or cytokinin- or nitrate-induced NR activity (Figure 2.6). The ratio of nitrate to

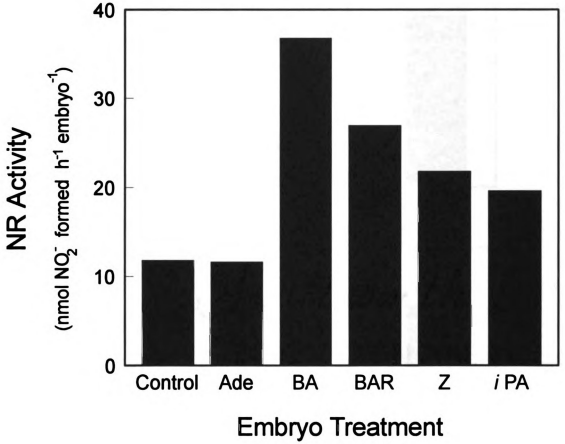

Figure 2.4. The effect of various cytokinins on NR activity.

Seeds were imbibed for 12 h at RT. The embryos were then isolated and incubated at 30°C for 12 h in water (control), 5 µM Ade, or 5 µM of the indicated cytokinins. The results represent the average of triplicate samples, 4 embryos per sample. Ade=adenine; BA=benzyladenine; BAR=benzyladenosine; Z=zeatin; *i*PA=N^6-(Δ^2-isopentenyladenine).

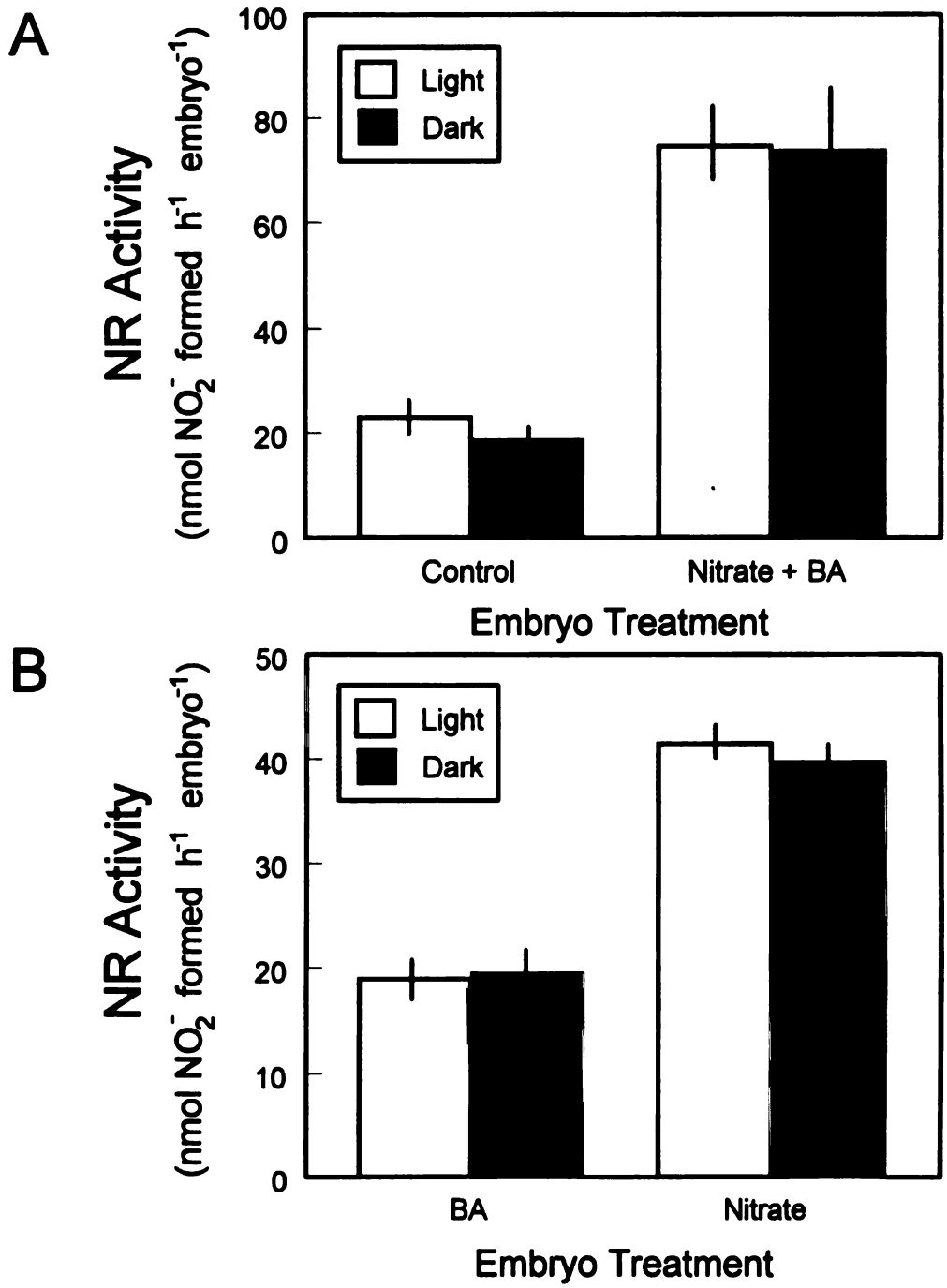

Figure 2.5. The effect of light on induction of NR activity.

Seeds were imbibed for 7 h (A) or 16 h (B) at RT. The embryos were then isolated and incubated at 30°C for 6 h in water (control), 1 µM BA, 50 mM nitrate or BA plus nitrate. Embryos were imbibed, excised, and incubated in room light or in the dark. The embryos for the dark treatment were isolated under green safelights. Bars indicate ± SEM (A:*n*=9; B:*n*= 5 to 7).

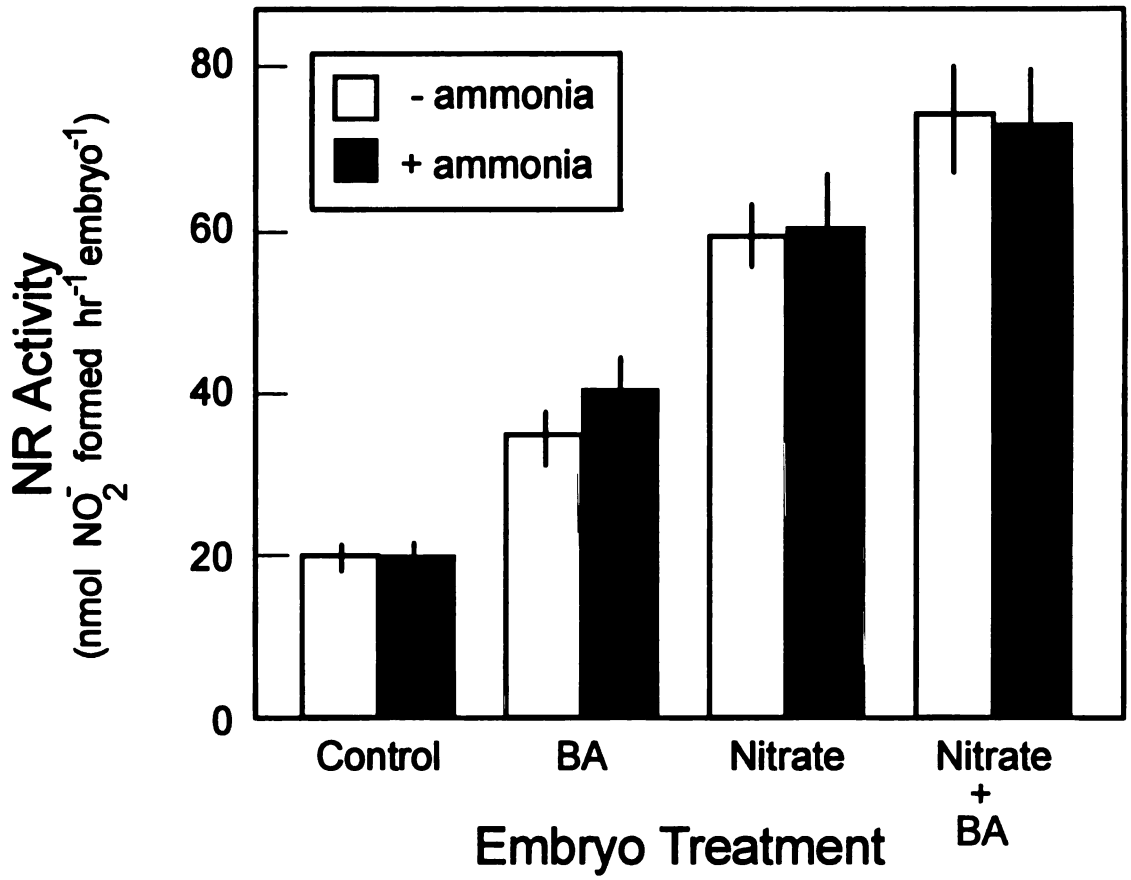

Figure 2.6. The effect of ammonia on cytokinin- and nitrate-induced NR activity.

Seeds were imbibed for 7 h at RT. The embryos were then isolated and incubated at 30°C for 6 h in water (control), 1 µM BA, 50 mM nitrate, or 1 µM BA plus 50 mM nitrate, plus or minus 50 mM ammonium chloride. Bars indicate ± SEM (*n*=8 to 9).

ammonia has been shown to affect nitrate uptake and utilization (Takács and Técsi, 1992); this was also examined in *A. githago* embryos. Ammonia by itself or in equivalent amounts with nitrate had no effect on NR activity (Figure 2.7, 0:0 vs. 0:1 and 1:0 vs. 1:0+0:1). While keeping the total nitrogen concentraton at 50 mM, the ratios of nitrate to ammonia were varied. Only when nitrate was decreased to 12.5 mM (Figure 2.7, lane 1:3) was there any decrease in NR activity.

Ethylene. Schmerder and Borriss (1986) showed that ethylene increased NR activity in *A. githago* embryos, and that ethylene and cytokinin acted synergistically to enhance NR activity. Figure 2.8 shows results from ethylene treatments. Ethylene increased NR activity ~50% over control, but had no effect on nitrate-induced NR activity. Contrary to Schmerder and Borriss' results, ethylene had an inhibitory effect on cytokinin-induced NR activity. Similar results were obtained in four independent experiments.

The effect of imbibition on NR activity. Different seed lots had varying levels of background NR activity, both at the zero time point when embryos were first isolated, and during the incubation of the embryos in water (personal observation). The changes in NR activity during the course of imbibition were measured. The low level of NR activity in imbibed embryos increased with a longer imbibition time. This increase was temperature dependent, with imbibition at 30°C increasing activity more than imbibition at 23°C (Figure 2.9, 1a, 2a). This increase was observed in many experiments, but the time of the initial increase in activity varied between seed lots and with the age of seeds.

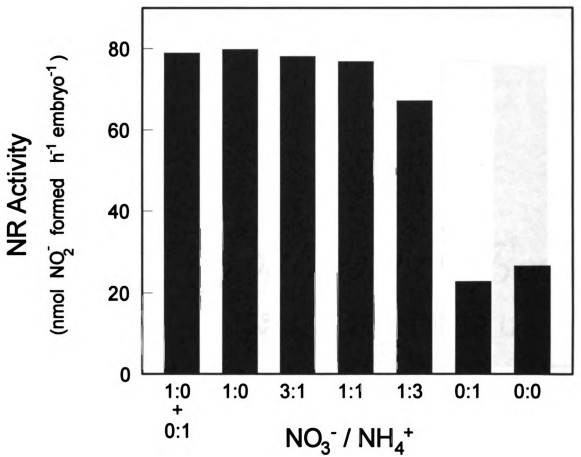

Figure 2.7. The effect of various ratios of nitrate to ammonia on NR activity.

Seeds were imbibed for 12 h at RT. The embryos were then isolated and incubated at 30°C for 12 h in various concentrations of nitrate/ammonia. 1:0+0:1=50 mM nitrate plus 50 mM ammonia; 1:0, 3:1, 1:1, 1:3, and 0:1=ratios of nitrate/ ammonia so that the sum of nitrogen is 50 mM; 0:0=water. The results represent the average of triplicate samples, four embryos per sample. Ammonia was applied in the form of ammonium chloride.

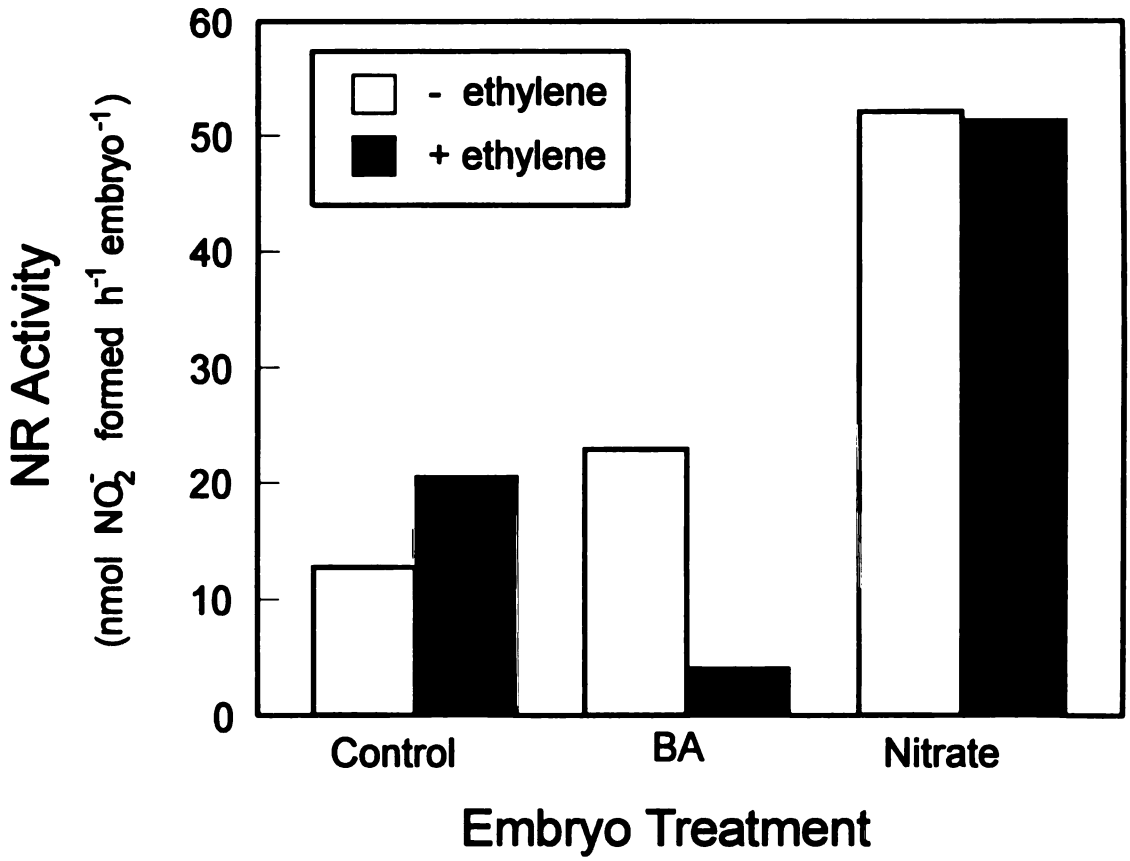

Figure 2.8. The effect of ethylene on cytokinin- and nitrate-induced NR activity.

Seeds were imbibed for 13 h at RT. The embryos were then isolated and incubated at 28°C for 6 h in water (control), 1 μM BA, or 50 mM nitrate, plus or minus 14 μl/l ethylene. The results represent the average of triplicate samples, 4 embryos per sample.

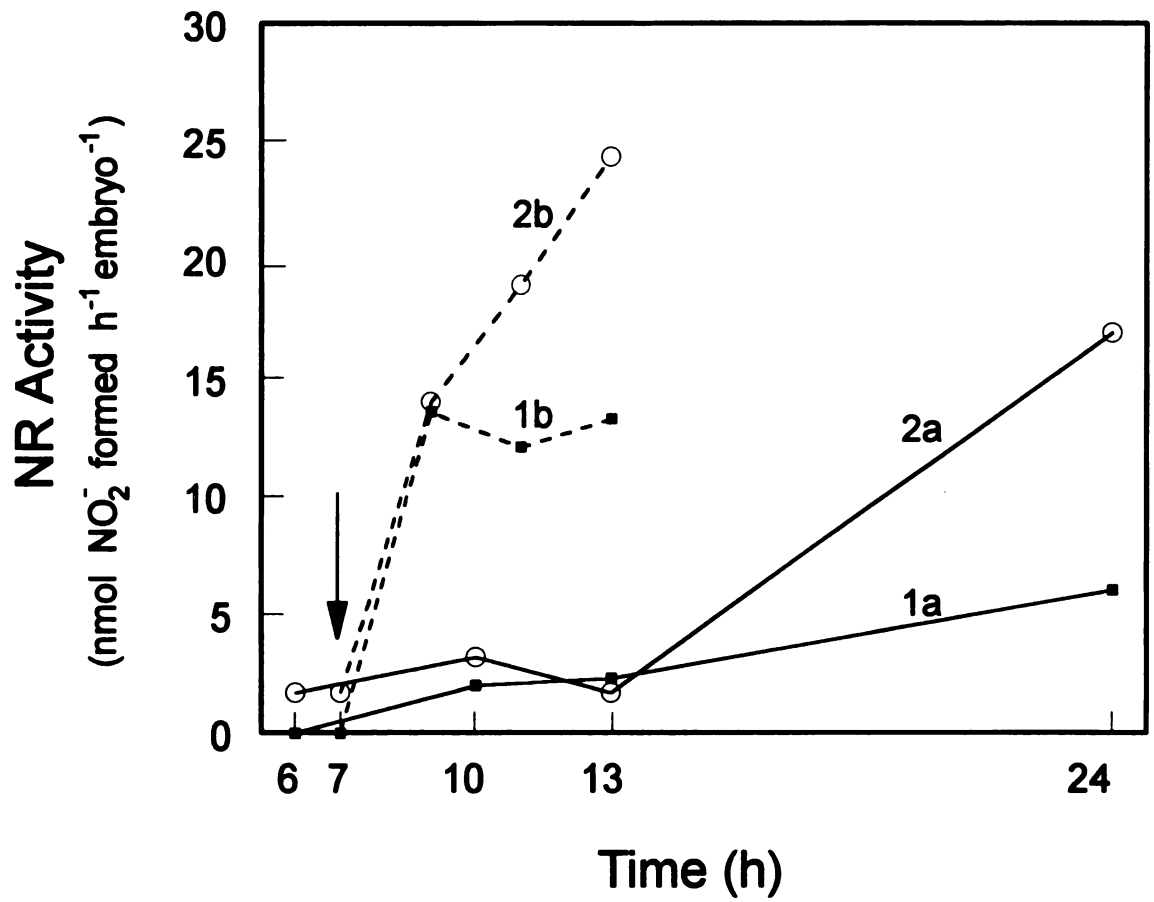

Figure 2.9. Temperature-dependent NR activity in imbibed seeds and isolated embryos.

Seeds were imbibed in water at 23°C (1a) or 30°C (2a) for the indicated times. The embryos were then isolated, frozen and NR activity was measured . Seeds were imbibed for 7 h at 23°C (1b) or 30°C (2b). The embryos were then isolated and incubated in water at the same temperature as during imbibition. The times for 1b and 2b represent cumulative times of imbibition and incubation. The arrow represents the time of embryo isolation for 1b and 2b. The results represent the average of triplicate samples, 4 embryos per sample.

Figures 2.2 and 2.3 show that the low level of NR activity present when embryos were first isolated increased when the embryos were incubated in water. The reason for this increase is unknown. Figure 2.9 shows that this increase in activity was also temperature dependent with a greater increase at a higher temperature (1b, 2b).

DISCUSSION

Studies of cytokinin and nitrate regulation of NR activity in *A. githago* embryos have shown variations in the seeds. Studies (Kende *et al.*, 1971; Kuznetsov *et al.*, 1979; Schmerder and Borriss, 1986) have shown differences in the times of maximum induction, the presence or absence of a lag phase for cytokinin induction, and whether cytokinin or nitrate increased activity to a greater extent. These results, as well as differences observed in these experiments, suggest that the state of the seeds is important in cytokinin and nitrate regulation of NR activity.

Many factors regulate NR activity in plants and some of them were examined in *A. githago* embryos. Light is known to regulate NR in many plants in one or more of the following ways (for a review, see Hoff *et al.*, 1994). In green tissues, light activates one or both of the photosystems of photosynthesis, causing an increase in transport of nitrate from the vacuole to the cytosol where NR induction occurs (Granstedt and Huffaker, 1982). Light may also activate phytochrome, which in turn increases NR mRNA level and enzyme activity. By activation of photosynthesis, light increases the carbohydrate supply and NADH level necessary for nitrate reduction, and thus increases NR activity.

A. githago embryos have typically been imbibed and incubated in the dark, and embryos isolated either under green safelights or in room light. Since large numbers of embryos were needed for molecular biological experiments, the effect of room light on NR activity was examined and found to have no

effect on cytokinin- or nitrate-induced activity. Since individual wavelengths of light and longer incubation times were not tested, regulation by light under certain conditions is still possible. However, in most of these and future experiments, embryos were isolated in room light as quickly as possible, keeping light exposure to under 10 minutes.

While ammonia had no effect on NR activity, ethylene did regulate activity. The effects of ethylene by itself and in combination with nitrate were similar to reported results (Schmerder and Borriss, 1986). However, the enhancement of cytokinin-induced activity by ethylene (Schmerder and Borriss, 1986) was not observed here, rather a significant and reproducible inhibition was observed. Since these experiments were done in only one set of seeds, differences due to seed lots are possible. One might expect that endogenous cytokinin and ethylene levels may affect the regulation by exogenous factors, or perhaps the differences in the times of incubation account for the differing results.

Cytokinins have been shown to be synthesized in the embryonic axis of germinating seeds and transported to the cotyledons where they increase the development of enzymatic activities (*i.e.* amyolytic and proteolytic activities; for a review, see Letham, 1994). The cytokinin-induced NR activity in *A. githago* embryos was localized primarily in the cotyledons, similar to some other cytokinin-regulated proteins.

There are low levels of endogenous cytokinins in *A. githago* embryos that vary during imbibition and germination and that are dependent on the state of

the seeds (Schmerder and Borriss, 1986; Borriss, 1977). Schmerder and Borriss (1986) suggested that changes in NR activity in water-incubated embryos were due to endogenous cytokinins. Higher levels of NR activity in water-incubated embryos were sometimes observed when the seeds were imbibed long enough for the radicles to protrude (personal observation). Since roots are known to synthesize cytokinins (Letham, 1994), perhaps the higher levels of NR activity in water-incubated embryos are due to regulation by endogenous cytokinins. Experiments testing NR activity in imbibing seeds showed an increase in NR activity over time, perhaps also due to changes in endogenous cytokinin levels. Kende *et al.* (1974) showed that while nitrate could induce NR activity in seeds that had been imbibed for up to 65 h, cytokinin could induce activity only up to 24 h of imbibition. This time might correspond with germination, or radicle emergence, that brings about changes in the embryos such that they are no longer susceptible to exogenous cytokinin induction of NR activity.

Since cytokinins are known to be involved with cell growth and differentiation, it may not be surprising to find cytokinins present and regulating NR activity in fast growing tissues such as embryos. If endogenous cytokinin levels vary with germination, perhaps they enhance NR activity so that more nitrogen is reduced for early growth of embryos. Differences in seed lots may partially be due to plant growth conditions and how much cytokinin and nitrate were stored in the seeds during development, or to the ability of the embryo to synthesize cytokinins.

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CHAPTER 3

Molecular Cloning of Three Nitrate Reductase cDNA Clones from *Agrostemma githago* Embryos

ABSTRACT

Degenerate oligonucleotide primers synthesized to conserved regions of nitrate reductases were used to obtain 495-bp clones from *A. githago* using the polymerase chain reaction (PCR). Three groups of PCR-clones were identified. Partial cDNA clones corresponding to each of the PCR-clones were isolated from an *A. githago* cDNA library. A representative clone from each group was characterized in detail. The inserts of Agnr125, Agnr223, and Agnr321 are 2046 bp, 1502 bp, and 1729 bp, respectively. All three clones are partial clones with the 5'-ends beginning in the molybdenum-binding domain and the 3'-ends containing untranslated sequences. In most plants, NR cDNA clones hybridize to 3 to 3.7-kb mRNAs.

Agnr125 and Agnr321 were highly homologous to each other, with 94% nucleotide identity within the coding region and high homology extending into the 3'-untranslated region. Agnr223 had 82% nucleotide identity to either Agnr125 or Agnr321 and did not share homology within the 3'-untranslated region. The three *A. githago* NR clones had 60-77% nucleotide identity to other plant NR clones. The greatest homology was to spinach, with 77% nucleotide homology and 86% amino acid homology. The hinge region lying between the molybdenum and heme binding domains had the least homology.

INTRODUCTION

Plants obtain most of their nitrogen in the form of nitrate. Nitrate is actively transported into the cell and reduced by cytosolic nitrate reductase (NR) to nitrite. Nitrite is toxic to the cell and is translocated into the plastids where it is reduced to ammonium by nitrite reductase (NiR). Ammonium ions enter the amino acid pool primarily via the action of glutamine synthetase.

Because the rate-limiting and regulated step of nitrate assimilation is catalyzed by nitrate reductase (NR), much research has focussed on NR and its regulation (for reviews, see Rouzé and Caboche, 1992; Hoff *et al.*, 1994; Crawford, 1995). NR is a multifunctional homodimer containing molybdenum cofactor, heme, and FAD as prosthetic groups in each of the 100 to 115-kDa subunits. Electrons are transferred from NADH or NADPH through the prosthetic groups to nitrate.

The NR gene, which is nuclear encoded, has now been isolated from many plant species. More than one gene has been found in various plants, including in the relatively small genome of *Arabidopsis thaliana* (Cheng *et al.*, 1988). Based on sequence homology to the members of the cytochrome b5 superfamily and cytochrome b5 reductase (Calza *et al.*, 1987), the boundaries of the heme and flavin domains have been defined. A number of proteins utilize a molybdopterin, but only sulfite oxidase has significant sequence homology to the assimilatory NR molybdenum cofactor binding domain. Protease-sensitive regions separate the functional domains of NR and are less conserved.

NR is regulated by nitrate in all plants. NR may also be regulated by

light, circadian rhythms, CO_2, reduced nitrogen compounds, sucrose, phytohormones, and a plastidic factor (for a review, see Rouzé and Caboche, 1992). While the physiological responses to these factors has been well studied, the molecular mechanisms of these responses are only beginning to be understood.

Cytokinins and nitrate have been shown to increase NR activity independently in *A. githago* embryos (Kende *et al.*, 1971), but only a single NADH-NR protein could be identified (Dilworth and Kende, 1974). Kende and Shen (1972) showed that both nitrate and cytokinins induce the synthesis of new NR protein. The aim of this study was to isolate NR cDNA clones from *A. githago* and to investigate the regulation of NR mRNA(s) by cytokinins and nitrate.

MATERIALS AND METHODS

Plant material, Incubation procedures, Tissue extraction and NADH-NR assay. See Materials and Methods, Chapter 2.

Polymerase chain reaction. PCR was performed on lambda DNA isolated from an *A. githago* cDNA library. The following degenerate primers were used: HK35 (upstream primer), 5'-TGGTGYTGGTGYTTYTGG-3' and HK34 (downstream primer), 5'-TCRAAYTCYTCNGTRCARTC-3'. The 50 μl PCR reaction was run in standard PCR buffer containing 1 μM HK35, 3 μM HK34, and 0.8 μg lambda DNA under the following conditions: 5 min at 94°C and then 35 cycles of 1 min at 94°C, 2 min at 50°C, and 2 min at 72°C. In the last cycle, the extension time was prolonged to 9 min. DNA products were gel purified, proteinase K treated, ligated into the TA cloning vector pCRII (Invitrogen, San Diego, CA), and transformed into InvαF' cells (Invitrogen, San Diego, CA). NR clones were initially identified by hybridization to an internal degenerate oligonucleotide HK30: 5'-AACCARCARTTRTTCATCAT-3'.

Gene-specific oligonucleotides. Gene-specific anti-sense oligonucleotides were synthesized to the hinge 1 region of each of the three PCR clones. These oligonucleotides were used to further screeen the PCR clones and to identify cDNA clones.

HK39 (Agnr1): 5'-GGTTATTGCCACCTGGCACA-3';

HK38 (Agnr2): 5'-ACGCGTGGCTAACTGCGTCC-3';

HK76 (Agnr3): 5'-AGTTATTGCCACCCGGCACT-3'.

RNA extraction. Total RNA was isolated according to Puissant and Houdeline (1990). One hundred embryos weighed 1.1 to 1.2 g, and total RNA yield was 800-1400 μg RNA per g fresh weight. Poly(A)$^+$ RNA was isolated using the PolyATract Isolation System (Promega, Madison, WI) or by selection on an oligo(dT) cellulose (Boehringer Mannheim, Indianapolis, IN) column according to Schleif and Wensink (1981) with yields of 1.0 to 1.4%.

Construction of cDNA library. A. githago seeds were imbibed for 7 h at 23°C, and isolated embryos were incubated in 1 μM BA plus 50 mM nitrate for 4 to 6 h at 30°C. Isolated poly(A)$^+$ RNA was size fractionated on 5 to 30% linear sucrose gradients by centrifugation for 16 h at 28,000 rpm in a SW40 rotor (Beckman, Fullerton, CA; Schleif and Wensink, 1981). Fractions containing 2.5 to 3.5 kb RNA that cross-hybridized with a 1671-bp partial tobacco nia-2 gene (Calza *et al.*, 1987; Vaucheret *et al.*, 1989) were combined. An *A. githago* cDNA library was constructed by Stratagene (La Jolla, CA) in the *Eco*RI site of lambda ZapII. The oligo(dT)-primed library contained 4.2 X 10^6 recombinants with <5% nonrecombinants and inserts greater than 1 kb.

Library screening. The *A. githago* unamplified cDNA library was screened with the random primer-labelled insert of Agpcr1, an *A. githago* 495- bp PCR NR clone. *E. coli* Sure cells (Stratagene) were used to plate 35 to 50,000 pfu per 150 mm plate. Bacterial lawns were lifted in duplicate with nitrocellulose filters (Schleicher and Schuell, Keene, NH). Pre-hybridizations and hybridizations were carried out at 55°C or 60°C in 6XSSC, 50 mM sodium phosphate, 5X Denhardt's solution, 0.5% SDS, and 0.25 mg/ml of denatured

salmon sperm DNA [20X SSC: 3M NaCl, 0.3M sodium citrate, pH 7.0; 50 X Denhardt's solution: 1% Ficoll (w/v), 1% polyvinylpyrrolidine (w/v), 1% bovine serum albumin (w/v)]. pBluescript SK$^-$ clones were isolated from positive lambda ZapII clones using R408 helper phage according to Stratagene.

Radiolabelled probes. Isolated DNA fragments were radiolabelled using a random-primed labelling kit (Boehringer Mannheim). Oligonucleotides were end-labelled according to Maniatis *et al.* (1982). All radioactive materials were supplied by New England Nuclear (Wilmington, DE).

DNA sequencing. The DNA sequences were determined by the dideoxy chain-termination method (Sanger *et al.*, 1977) using a T7 polymerase Sequenase kit (United States Biochemical Corp., Cleveland, OH). Subcloning of restriction enzyme-digested fragments into pBluescript SK$^-$ (Stratagene) and synthetic oligonucleotide primers were used for further sequencing. All DNA sequences were determined completely from both strands.

Subclones. Agnr1-1 was generated by digesting Agpcr1 with *Eco*RI, then digesting the isolated 495-bp fragment with *Hpa*II. The isolated 171 bp *Hpa*II fragment was ligated into the *Sma*I site of pBluescript SK$^-$. Agnr2-1 was generated by digesting a 3'-truncated Agpcr2 clone with *Sma*I/*Eco*RI and ligating the 144-bp DNA fragment into pBluescript SK$^-$. Agnr3-1 was generated by digesting Apcr3 with *Sma*I/*Eco*RI and ligating the 245-bp DNA fragment into pBluescript SK$^-$.

Computer programs. All analyses of the nucleic acid and derived amino acid sequences were performed using the Genetics Computer Group Wisconsin

Package.

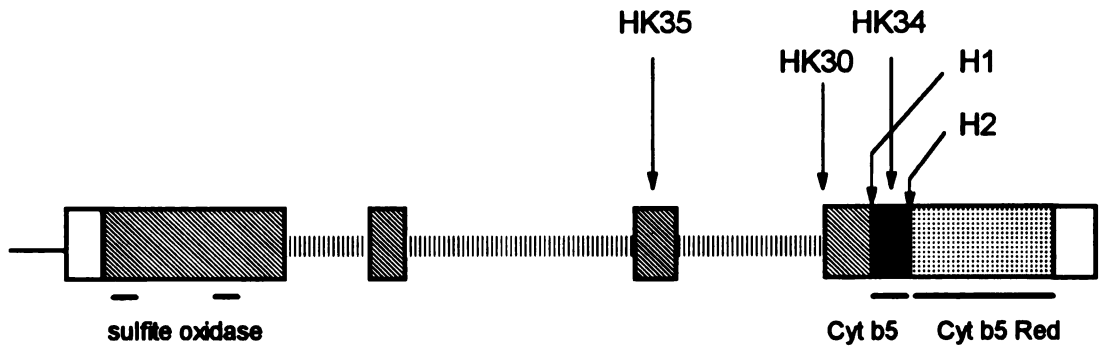
Standard molecular biological methods. Most other methods were performed according to standard molecular biological techniques (Maniatis *et al.*, 1982; Sambrook *et al.*, 1989).

RESULTS

Isolation of three PCR clones. Figure 3.1 shows the structure of the tobacco nia-2 NR gene (Vaucheret *et al.*, 1989). Based on the plant NR sequences available, degenerate oligonucleotides were synthesized to three conserved regions in the coding sequence of NR genes. HK35, located in the molybdenum binding domain, and HK34, located in the heme binding domain (Figure 3.1), were used to amplify by PCR DNA isolated from the *A. githago* cDNA library. There were 3 to 4 PCR products, but only one 495-bp PCR product hybridized to the internal HK30 degenerate oligonucleotide (Figure 3.1). This DNA was ligated into pCRII (Invitrogen), and three different groups of NR clones were identified by DNA sequencing. Eleven clones corresponded to Agpcr1, two clones to Agpcr2, and one clone to Agpcr3.

The three groups of PCR clones were highly homologous to each other. Agpcr1 shared 95% and 85% nucleotide indentity with Agpcr3 and Agpcr2, respectively. The PCR clones contained the hinge 1 region (Figure 3.1) that lies between the molybdenum and heme binding domains and this region had less homology. Three gene-specific oligonucleotides were synthesized to the hinge 1 sequences (HK39, HK38, HK76; Figure 3.2). Even though HK39 and HK76 differ by only three nucleotides, all three of the end-labelled oligonucleotides hybridized only with their corresponding cDNA clones (data not shown).

Isolation of NR cDNA clones from A. githago. Due to the high sequence homology between the three PCR clones, the 495-bp insert of Agpcr1 was used to screen an *A. githago* cDNA library under intermediate stringency conditions



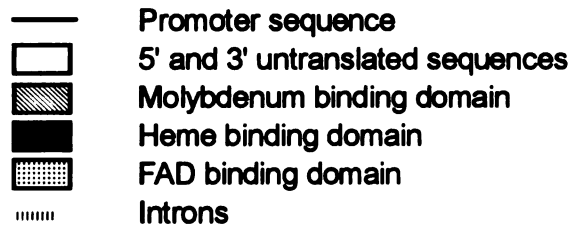


Figure 3.1. The linear structure of nitrate reductase.

The organization of the *Nicotiana tabacum* NR gene, *nia-2* (Vaucheret *et al.*, 1989), is shown. NR has three binding domains as indicated, with two hinge regions (H1, H2) lying between the domains. The locations of the degenerate oligonucleotides used for PCR amplification from *A. githago* DNA (HK35 and HK34) and for screening the PCR clones (HK30) are indicated (sequences shown in Material and Methods). Areas of sequence homology to other genes are identified below nitrate reductase: rat sulfite oxidase (Crawford *et al.*, 1988), bovine liver cytochrome b5 (Ozols and Strittmatter, 1969), and human cytochrome b5 reductase (Yubisui *et al.*, 1984).

Hinge 1

Figure 3.2. The sequences of the hinge 1 region of three *A. githago* NR clones.

The 48 nucleotides of the hinge 1 region (Daniel-Vedele *et al.*,1989) of *A. githago* NR clones are shown. Identical nucleotides between sequences are indicated with asterisks. The sites of the gene-specific 20-mer oligonucleotides are boxed, and their names are indicated on the right.

that would allow cross-hybridization to all three NR clones. Seventy primary phage were isolated from 280,000 recombinant clones. Twenty clones were selected for further purification, and fifteen of these were carried through to isolation. These clones were grouped based on hybridization to the gene-specific oligonucleotides (HK39, HK38, and HK76). Ten clones corresponded to Agnr1, one clone to Agnr2, and four clones to Agnr3.

Analysis of the nucleotide sequences of A. githago NR cDNA clones. The largest clone from each of the three NR groups in *A. githago* was chosen for detailed analysis. To determine the nucleotide sequences of the clones, restriction enzyme digested DNA fragments were subcloned into pBluescript SK$^-$ and sequenced from the ends. Oligonucleotides were synthesized to complete the sequences of all three clones on both strands. Figure 3.3 shows the sequencing strategies for Agnr125 (2046 bp), Agnr223 (1502 bp) and Agnr321 (1729 bp). Since NR cDNAs hybridize to 3 to 3.7-kb mRNAs in other plants, all three isolated clones are partial clones missing the 5'-ends of the sequences.

The nucleotide sequences and comparisons of the three NR clones are shown in Figure 3.4. All three clones begin in the molybdenum-binding domain and extend into the 3'-untranslated region. Table 3.1 shows that there is great homology between the clones, with Agnr125 and Agnr321 sharing 94% nucleotide sequence identity within the coding region. The nucleotide differences between the sequences are all single base-pair changes. Portions of the nine clones corresponding to Agnr1 and the three clones corresponding to Agnr3 were sequenced.

Figure 3.3. The structure and sequencing strategy of three *A. githago* NR partial cDNA clones.

The sequencing strategies of representative clones for each of the three NR genes are shown. The three NR binding domains and hinge regions (H1 and H2) are indicated based on sequence homology to NR sequences from other plants. The stop codon and scale in bp are also indicated. The numbered base pair (+900) corresponds to the analagous position in the spinach NR cDNA sequence (Prosser and Lazarus, 1990) relative to the start of transcription (+1). The horizontal arrows denote the directions and positions of sequencing. Agnr125, Agnr223, and Agnr321 are 2046, 1502, and 1729 bp, respectively.

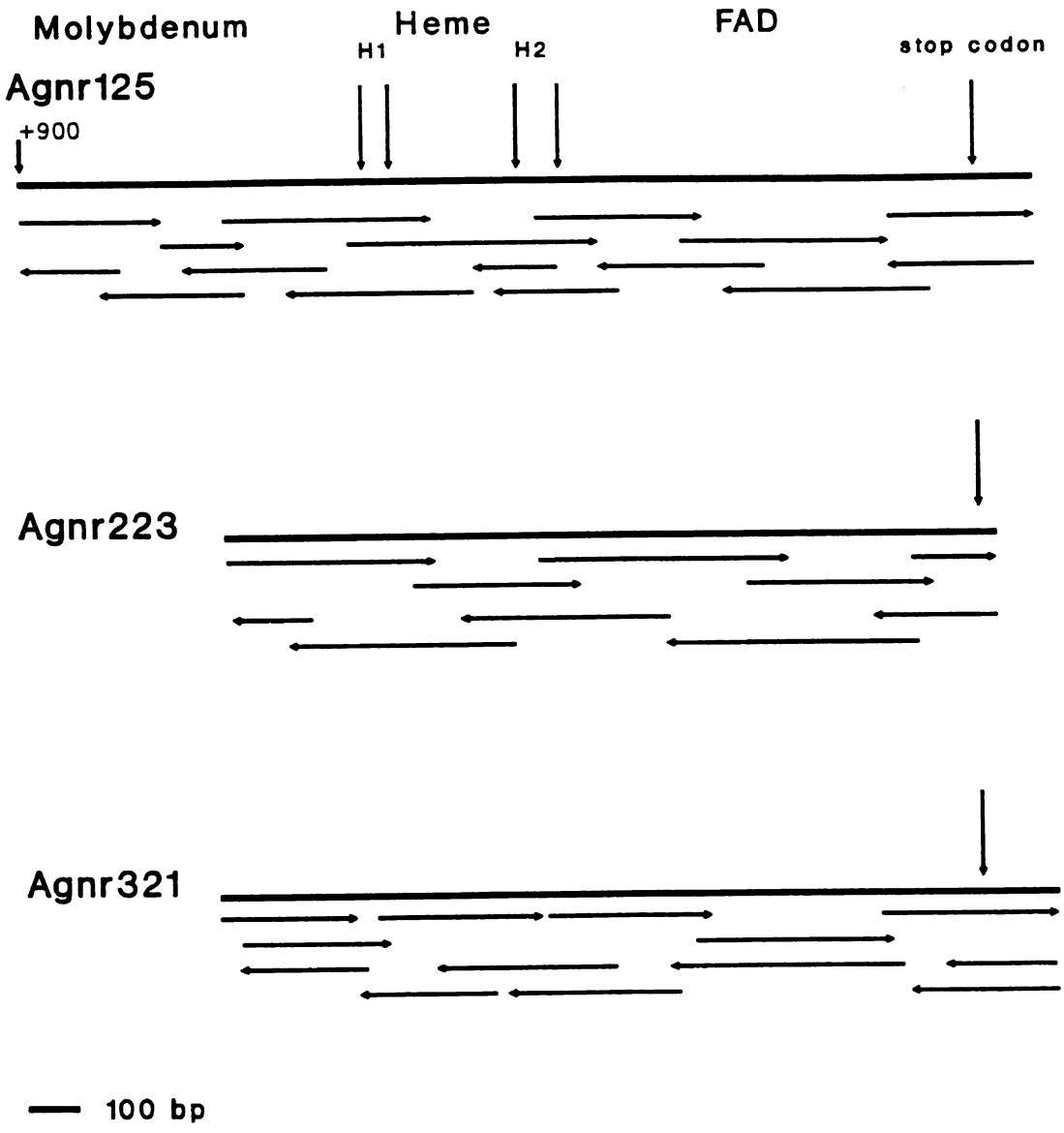


Figure 3.3. The structure and sequencing strategy of three *A. githago* NR partial cDNA clones.

Figure 3.4. Nucleotide sequence comparison between three *A. githago* NR partial cDNA clones.

The sequences of three *A. githago* cDNA clones are compared. Identical nucleotides to Agnr125 are indicated by asterisks. Gap positions, inserted to optimize the alignment, are indicated by dashes. The three binding domains are indicated above the sequences according to Daniel-Vedele *et al.* (1989). The locations of the degenerate oligonucleotides used for PCR amplification (HK35 and HK34) and for screening the PCR clones (HK30) are underlined. The borders of the three subclones surrounding the hinge 1 region are bracketed (Agnr1-1, Agnr2-1, Agnr3-1). The position of the gene-specific oligonucleotides is boxed (HK39, HK38, HK76). Asterisks above the sequences identify the stop codons, and putative polyadenylation signals are double underlined. The conserved positions of the three introns (I1, I2, I3) in NR genes are indicated (Daniel-Vedele *et al.*, 1989).

Figure 3.4. Nucleotide sequence comparison between three A. githago NR partial cDNA clones.

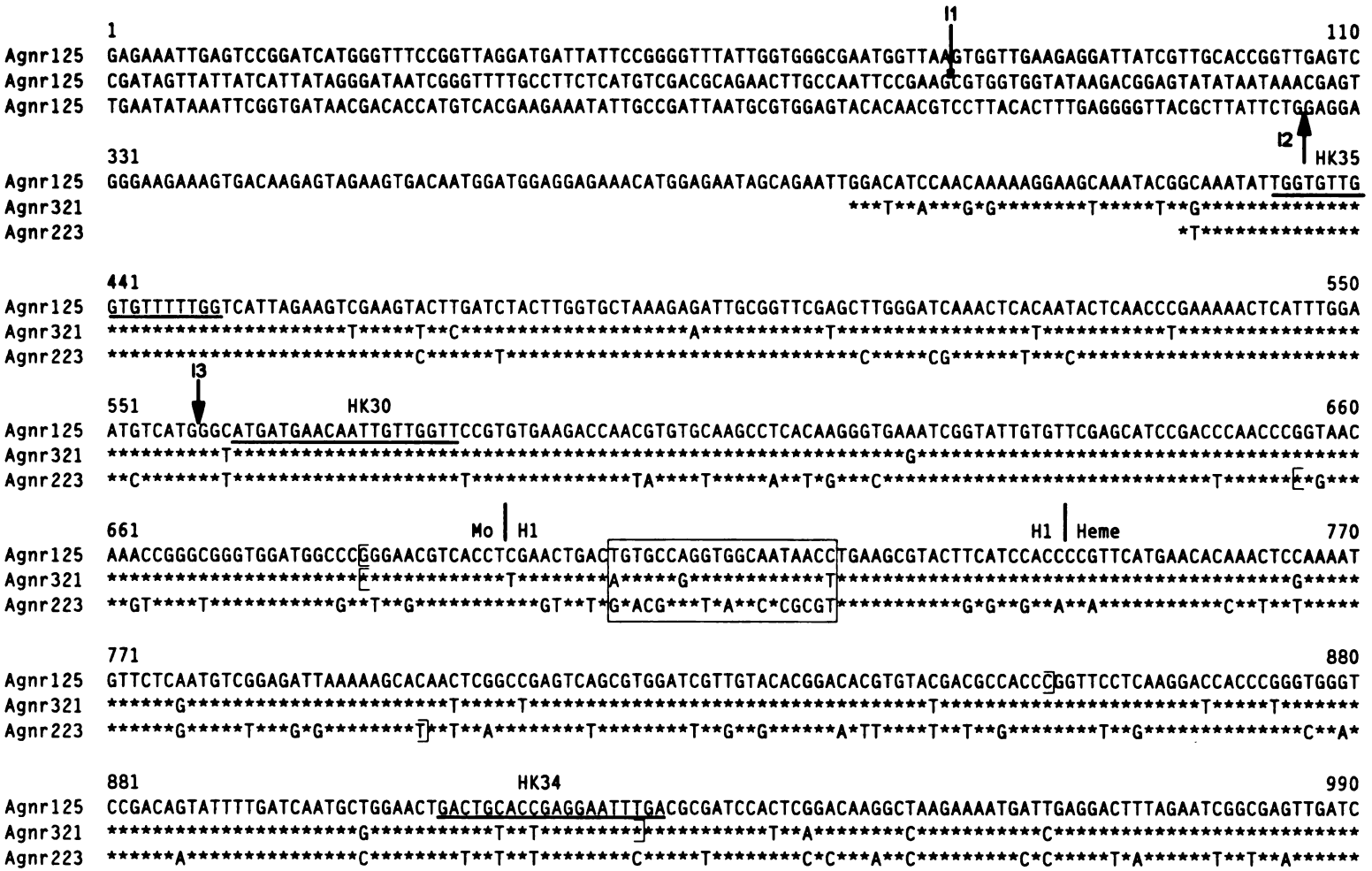


Figure 3.4 cont...

Figure 3.4 cont...

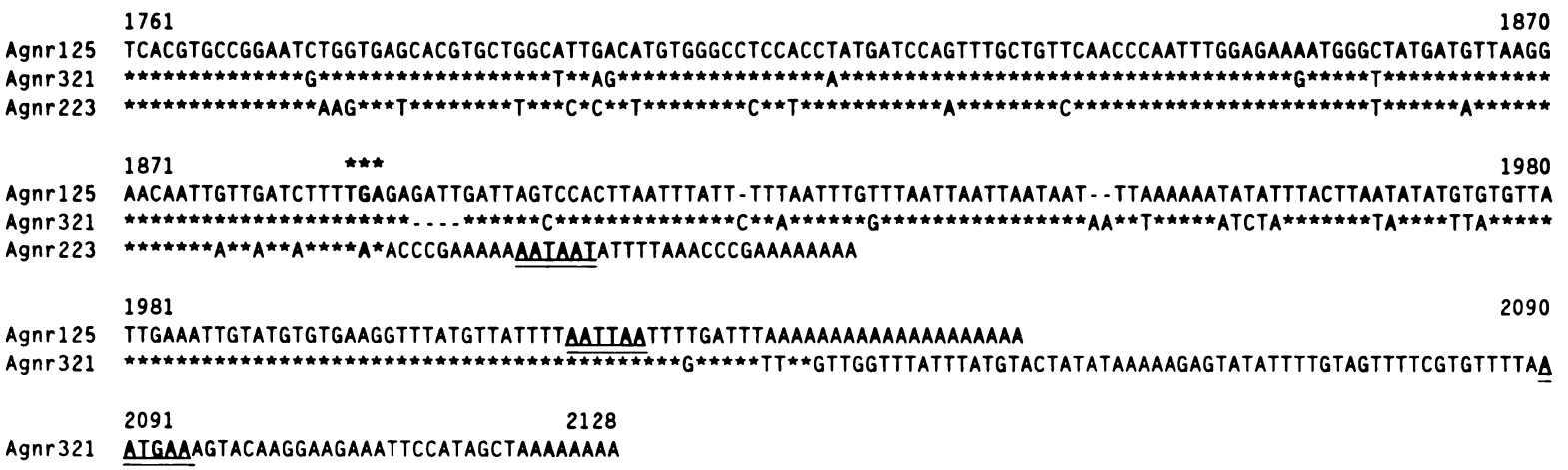


Table 3.1. Nucleotide sequence homology of three *A. githago* nitrate reductase partial cDNA clones.

The nucleotide sequence homology is calculated as a percentage of perfect matches between the cDNA clones over the length of the shorter clone. The greatest homology between any of the *A. githago* clones and other plant sequences is to spinach (Prosser and Lazarus, 1990).

	% Homology			
	Agnr125	Agnr223	Agnr321	Spinach
Agnr125	——	82	94	77
Agnr223		——	82	77
Agnr321			——	76

Analysis of the amino acid sequences of A. githago NR cDNA clones. The translations of the three NR cDNA clones give open reading frames of 630, 488, and 498 amino acids. Full-length NR proteins vary from 881 amino acids in bean (Hoff *et al.*, 1991) to 926 amino acids in spinach (Prosser and Lazarus, 1990). Therefore, 1/3 to 1/2 of the amino-terminal portion of the *A. githago* NRs have not been cloned. Many of the nucleotide differences between the sequences are at the third base of the codon. Amino acid sequence identities between Agnr125 and either Agnr223 or Agnr321 increase to 90% and 98%, respectively.

Comparison of the A. githago NR sequences with NRs from other plants. Figure 3.5 shows the amino acid sequence alignment of twenty partial or full length NRs from higher plants. The three *A. githago* NR clones have 60-77% nucleotide sequence identity to other plant NR sequences, with greatest homology to the spinach sequence (Prosser and Lazarus, 1990). The derived *A. githago* sequences show 86% amino acid identity to the spinach amino acid sequence. While the homologies between plant NRs are less in the hinge 1 and hinge 2 region, the three *A. githago* sequences were quite conserved in the hinge 2 region.

Analysis of the 3'-untranslated regions of A. githago NR sequences. As seen in Figure 3.4, the 3'-untranslated regions of the three *A. githago* NR clones vary in length. All three clones carry putative polyadenylation signals (Joshi, 1987) and polyA tails. However, Agnr223 has a very short 3'-untranslated sequence that may be incomplete. No other Agnr2 clones were

Figure 3.5. Amino acid sequence alignment between NRs from various higher plants.

Three *A. githago* NR sequences (Agnr125, Agnr223, Agnr321) are compared to other higher-plant NRs. Positions where the same amino acid is found as in the Arab2 sequence are shown with asterisks. Gap sequences, inserted to optimize the alignment, are indicated by dashes. Periods indicate the unavailability of sequence information. The binding domains and the N-terminal region are indicated above the sequences according to Daniel-Vedele *et al.* (1989). The conserved positions of the three introns (I1, I2, I3) in NR genes are indicated (Daniel-Vedele *et al.*, 1989). References are as indicated at the end of the sequences. Boxes above the sequences identify amino acids referred to in the discussion.

Figure 3.5.


1
90
Arab2 MAASVD-NRQYARLEPGLNGV---VRSYKPPVPG--------RSDSPKA-HQN--QTTNQ----------TVFLKPAKVHDDDEDV
Arab1 ....
Petunia *****E-***FSH*****S**----***F**----------*****VR-GC*FPLNNEL----------*N*Q--K*PNTTIYLD
Tob1 *****E-***FSHI*AGLS-------**F**----------*****VR-GC*F-PPPNS----------*N*Q--K*PNSTIFLD
Tob2 *****E-***FSH**AGLS-------**F**----------*****VR-GC*F-PSPNS----------*N*Q--K*PNSTIYLD
Tomato *****E-***YTH*****SA*---G*TF**----------*P***VR-GC*FPPSSNH----------ELP*Q--K*QNPPIYLD
Agnr321 ....
Agnr125 ....
Agnr223 ....
Spinach *******----***-H-PAPMSG*----**TPFSNH---------H*****VRNGYTFSNPPSSNGVVKPGEKIKLVDNNSNSNNGSNNNNNRY
Birch *****E-**RFTHH**---A*NGL**TF**VPNS-------H*****DL-G----------RQIPSSPKKQVATG-
Squash ******-****GP*Q*P*S**----***F*NGPN--------H*A***VR-GCNFPNSNVD----------YNNNRPLKSSVKIQEAAAE
Bean1 *****E-H*PFTSHQH--GV----***F*SYPDVPRP----------KKLPLPQ-
Bean2 *****G-N**F*THMN--GV----***--CGQDLKPS----------LPL*F*L-
Barley2 ...**E-P**PFGRLDAPATAPTARA--------PGSNGIRR*A***VR-GCGFPS----------*IS*PRKGPVAEE
Corn ....
Rice *****Q-P**FGH****SAP*RGAAS*NGAKAYPP-ANGIPR*A***VR-GCGFPP.....*VS*PPRKPPS--
Barley1 *****EY***VSAHPWPT*AQPKAAFDLFSSSGG*-----RR**GA----------
Sbean1 ******-******---GTHI*A*---**A--CGPDFNT----------PLPS*F*L-
Sbean2 ******-Q*P*---PGIH*A*---**P--LKPGPDI----------PRPKKLPQAPPPLS
91
N-term Mo
180
Arab2 SSEDENETHNSNAVYYKEMIRKSNAELEPSVLDPRDEYTADSWIERNPSMVRLTGKHPFNSEAPLNRLMHHGFITPVPLHYVRNHGHVPK
Arab1 ....
Petunia C*SS*DDDDDDDKNE*LQ****GKL*V****H*I***G***N*****N**I**********P**A**********P***
Tob1 Y*SS*DDDDDDEKNE*LQ**K*G*S******H*S***G***N*****F*LI**********P**********P***
Tob2 Y*SS*DDDDDDEKNE*LQ**K*G*S******H*T***G***N*****F**I**********P**********P***
Tomato Y*SS*D*DDDDEKNE*VQ**K*G*T*****IH*T***G***N*****F*LI***R*******P**S**********P***
Agnr321 ....
Agnr125 ....
Agnr223 ....
Spinach D*DS*EDDDENEMNVWN***K*G*S*****SV*S***G***Q********I**********P**T*******L**********P**N
Birch --*DSSSEDENEND-***L*Q*G*G*****I******A***N*V***AT**********T*********A*********P***
Squash EM*DSCSEDENENE-FRDL*V*G*R*****I**H***G***N*****A**********P**********V***
Bean1 PLSDSTNDNDEE*AVW**LVL**S*GV***IF****DG*S*Q**K**A**I*********A*P**P***Q******S*I****S**P**H
Bean2 D*SSSDDDENDD*S*L**LV**A***T*A**M*****G***Q*VA**A**********A*S**Q**********L*******P***
Barley2 DE**DD*DDEGHEDWREAYGSHLQL*V***TR*****G***A*******LI*******L*C*P**A*********A*********A**R
Corn ....
Rice --DGSDDEEEEQEDWRELYGSHLQL*V**P*R*A*N*G***A*T*****LI*******L*C*P**AEL********AA****A***A**R
Barley1 -DS*SDDEDSVPPDWRSLYSPR--LDV****K*****A*S*A*VK*H*AL**********P**P***S**********A***
Sbean1 DSSSDDEDQNDDASFL**L*Q*A***T*A*L******G***Q**P**A****F*******A****P*********S**R******P***
Sbean2 DSSSDEEEDTTLN--L*DL***GTT***S*IF****DG*S*H**Q**S*L**********P**P**********P**R

Figure 3.5.

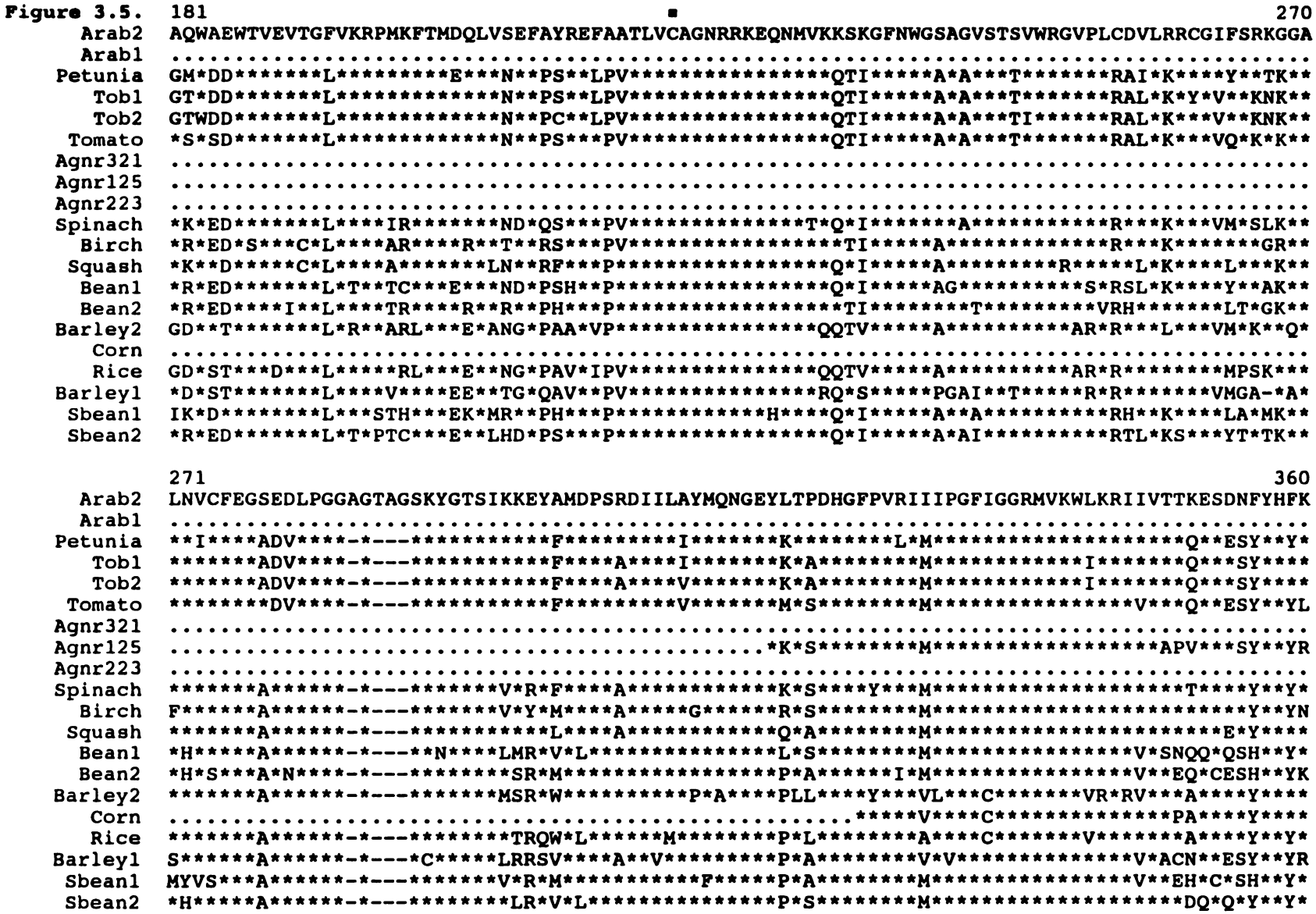

181
270
Arab2 AQWAEWTVEVTGFVKRPMKFTMDQLVSEFAYREFAATLVCAGNRRKEQNMVKKSKGFNWGSAGVSTSVWRGVPLCDVLRRCGIFSRKGGA
Arab1
Petunia
Tob1
Tob2
Tomato
Agnr321
Agnr125
Agnr223
Spinach
Birch
Squash
Bean1
Bean2
Barley2
Corn
Rice
Barley1
Sbean1
Sbean2
271
360
Arab2 LNVCFEGSEDLPGGAGTAGSKYGTSIKKEYAMDPSRDIILAYMQNGEYLTPDHGFPVRIIIPGFIGGRMVKWLKRIIVTTKESDNFYHFK
Arab1
Petunia
Tob1
Tob2
Tomato
Agnr321
Agnr125
Agnr223
Spinach
Birch
Squash
Bean1
Bean2
Barley2
Corn
Rice
Barley1
Sbean1
Sbean2

Figure 3.5.


361
I1
I2
450
Arab2 DNRVLPSLVDAELADEEGWWYKPEYIINELNINSVITTPCHEEILPINAFTTQRPYTLKGYAYSGGGKKVTRVEVTVDGGETWNVCALDH
Arab1
Petunia
Tob1
Tob2
Tomato
Agnr321
Agnr125
Agnr223
Spinach
Birch
Squash
Bean1
Bean2
Barley2
Corn
Rice
Barley1
Sbean1
Sbean2
451
I3
540
Arab2 QEKPNKYGKFWCWCFWSLEVEVLDLLSAKEIAVRAWDETLNTQPEKMIWNLMGMMNNCWFRVKTNVCKPHKGEIGIVFEHPTLPGNESGG
Arab1
Petunia
Tob1
Tob2
Tomato
Agnr321
Agnr125
Agnr223
Spinach
Birch
Squash
Bean1
Bean2
Barley2
Corn
Rice
Barley1
Sbean1
Sbean2

Figure 3.5.

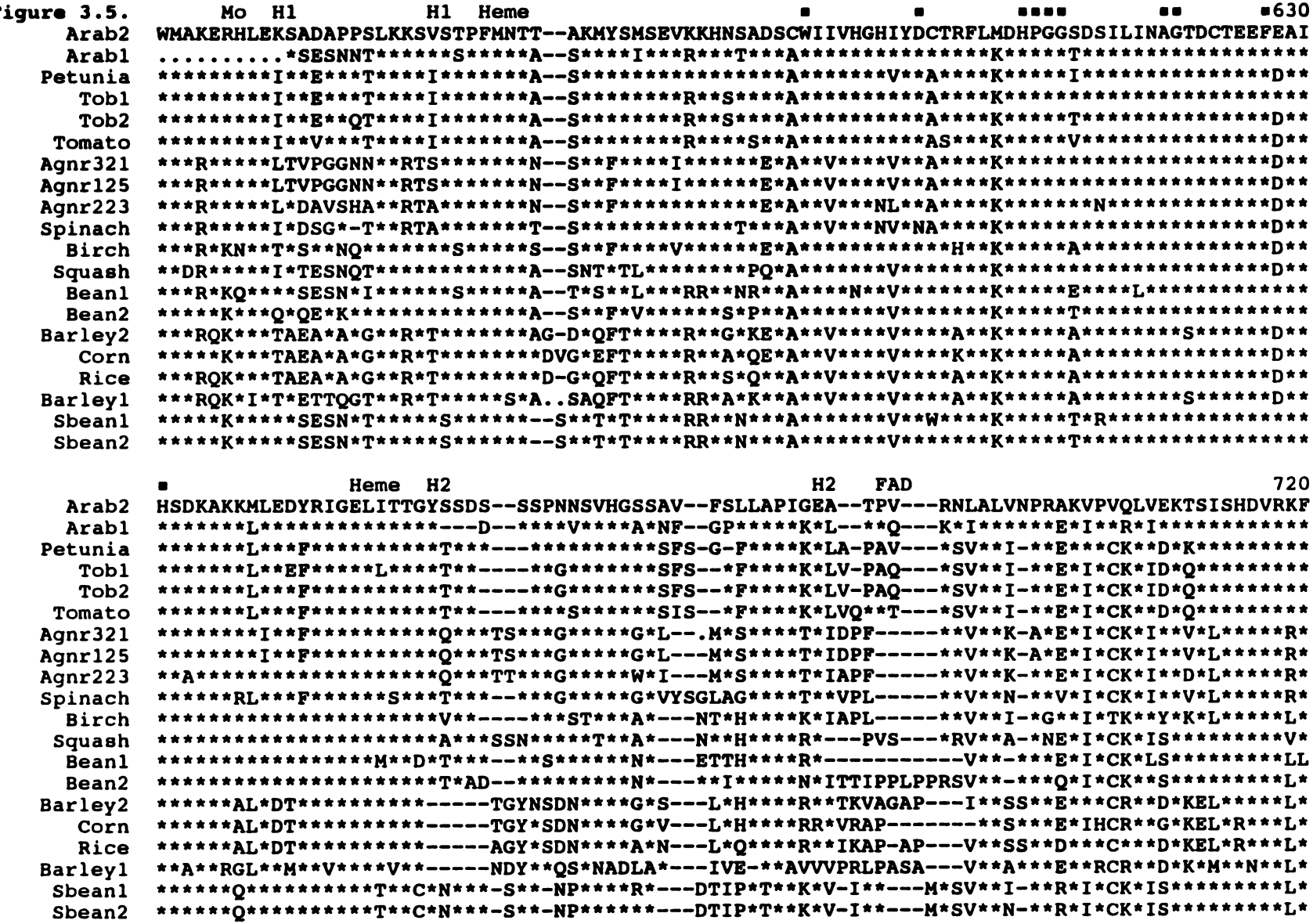

Mo H1 H1 Heme 630
Arab2 WMAKERHLEKSADAPPSLKKSVSTPFMNTT--AKMYSMSEVKKHNSADSCWIIVHGHIYDCTRFLMDHPGGSDSILINAGTDCTEEFEAI
Arab1
Petunia
Tob1
Tob2
Tomato
Agnr321
Agnr125
Agnr223
Spinach
Birch
Squash
Bean1
Bean2
Barley2
Corn
Rice
Barley1
Sbean1
Sbean2
Heme H2 H2 FAD 720
Arab2 HSDKAKKMLEDYRIGELITTGYSSDS--SSPNNSVHGSSAV--FSLLAPIGEA--TPV---RNLALVNPRAKVPVQLVEKTSISHDVRKF
Arab1
Petunia
Tob1
Tob2
Tomato
Agnr321
Agnr125
Agnr223
Spinach
Birch
Squash
Bean1
Bean2
Barley2
Corn
Rice
Barley1
Sbean1
Sbean2

Figure 3.5.

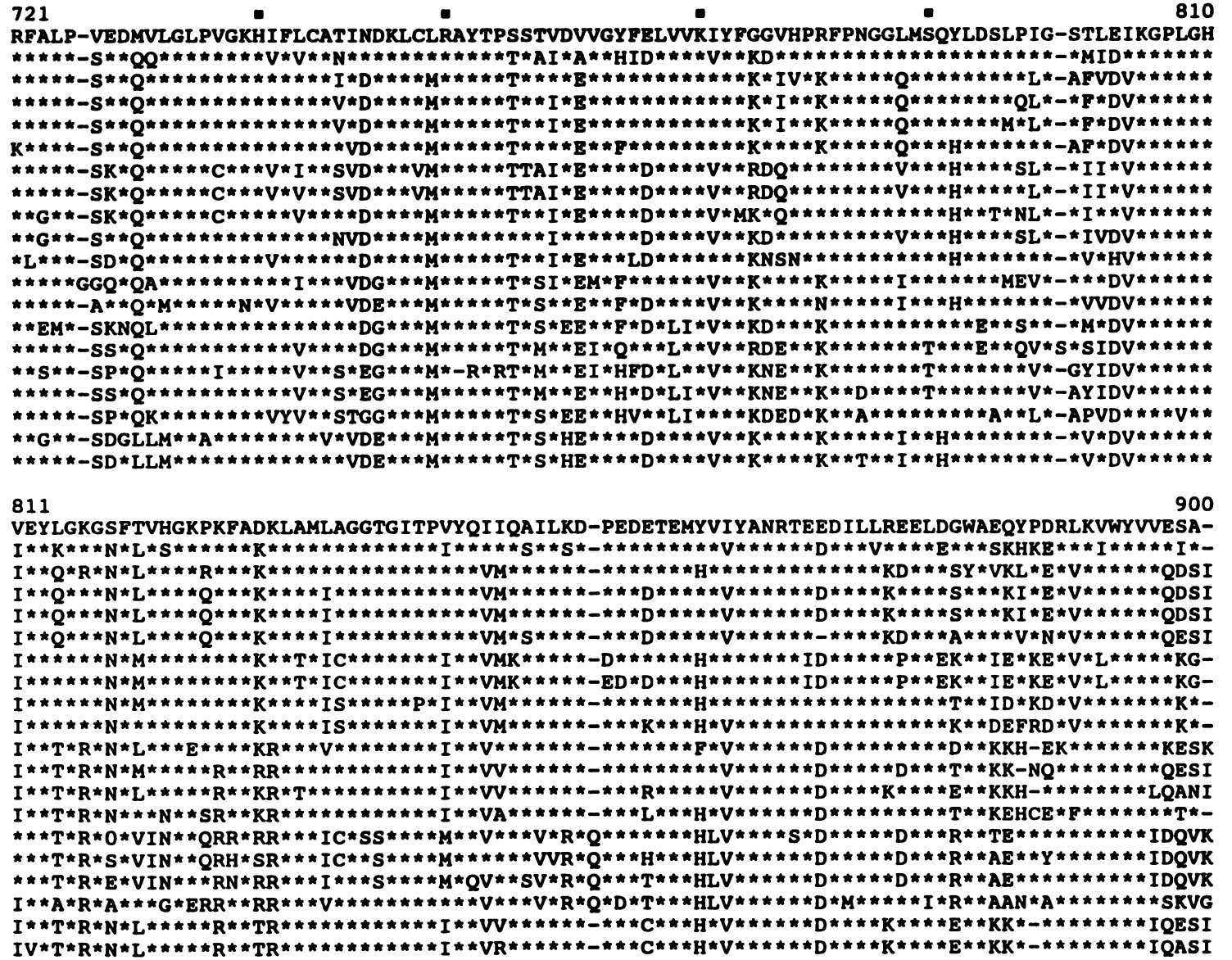
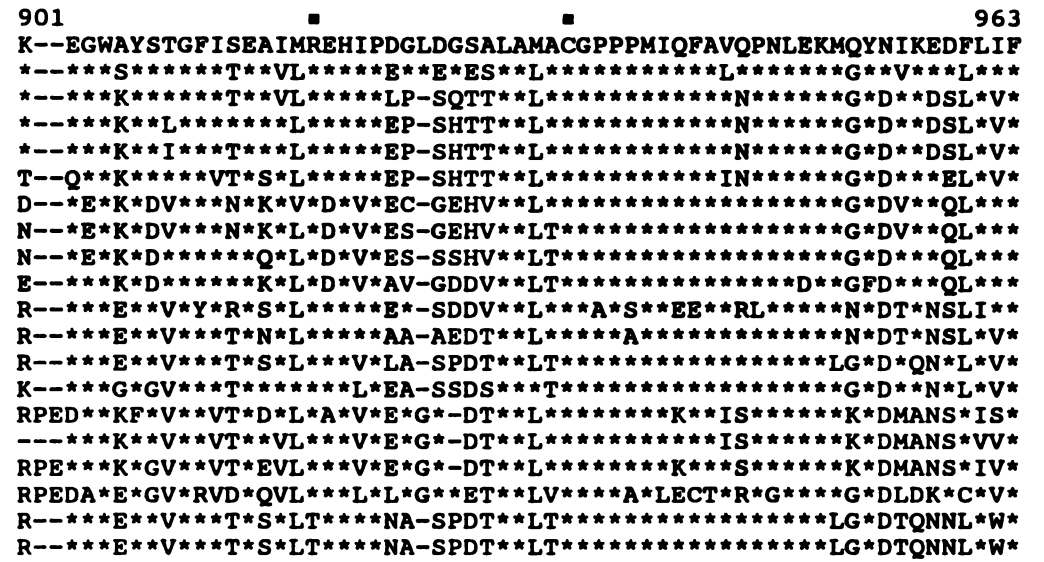

721
810
Arab2 RFALP-VEDMVLGLPVGKHIFLCATINDKLCLRAYTPSSTVDVVGYFELVVKIYFGGVHPRFPNGGLMSQYLDSLPIG-STLEIKGPLGH
Arab1 *****-S**QQ********V*V**N***********T*AI*A**HID****V**KD**********-*MID*******
Petunia *****-S**Q**********I*D****M*****T****E**********K*IV*K*****Q********L*-AFVDV******
Tob1 *****-S**Q**********V*D****M*****T**I*E**********K*I**K*****Q********QL*-*F*DV******
Tob2 *****-S**Q**********V*D****M*****T**I*E**********K*I**K*****Q*******M*L*-*F*DV******
Tomato K****-S**Q**********VD****M*****T****E**F*********K****K*****Q***H*******-AF*DV******
Agnr321 *****-SK*Q*****C***V*I**SVD***VM*****TTAI*E****D****V**RDQ********V***H****SL*-*II*V******
Agnr125 *****-SK*Q*****C***V*V**SVD***VM*****TTAI*E****D****V**RDQ**********V***H*****L*-*II*V******
Agnr223 **G**-SK*Q*****C*****V****D****M*****T**I*E****D****V*MK*Q**********H**T*NL*-*I**V******
Spinach **G**-S**Q**********NVD****M*******I******D****V**KD*********V***H****SL*-*IVDV******
Birch *L***-SD*Q*********V******D****M*****T**I*E***LD*******KNSN**********H*******-*V*HV******
Squash *****GGQ*QA**********I***VDG***M*****T*SI*EM*F******V**K****K*****I*******MEV*-***DV******
Bean1 *****-A**Q*M*****N*V*****VDE***M*****T*S**E**F*D****V**K****N*****I***H*******-*VVDV******
Bean2 **EM*-SKNQL**********DG***M*****T*S*EE**F*D*LI*V**KD***K**********E**S**-*M*DV******
Barley2 *****-SS*Q**********V****DG***M*****T*M**EI*Q***L**V**RDE**K*******T***E**QV*S*SIDV******
Corn **S**-SP*Q*****I*****V**S*EG***M*-R*RT*M**EI*HFD*L**V**KNE**K*******T*******V*-GYIDV******
Rice *****-SS*Q**********V**S*EG***M*****T*M**E**H*D*LI*V**KNE**K**D****T*******V*-AYIDV******
Barley1 *****-SP*QK********VYV**STGG***M*****T*S*EE**HV**LI****KDED*K**A*********A**L*-APVD****V**
Sbean1 **G**-SDGLLM**A********V*VDE***M*****T*S*HE****D****V**K****K*****I**H********-*V*DV******
Sbean2 *****-SD*LLM**********VDE***M*****T*S*HE****D****V**K****K**T**I**H********-*V*DV******
811
900
Arab2 VEYLGKGSFTVHGKPKFADKLAMLAGGTGITPVYQIIQAILKD-PEDETEMYVIYANRTEEDILLREELDGWAEQYPDRLKVWYVVESA-
Arab1 I**K***N*L*S******K**********I*****S**S*-*********V******D***V****E***SKHKE***I*****I*-
Petunia I**Q*R*N*L****R***K***************VM******-*******H**********KD***SY*VKL*E*V******QDSI
Tob1 I**Q***N*L****Q***K****I**********VM******-***D*****V******D****K****S***KI*E*V******QDSI
Tob2 I**Q***N*L****Q***K****I**********VM******-***D*****V******D****K****S***KI*E*V******QDSI
Tomato I**Q***N*L****Q***K****I**********VM*S****-***D*****V******-****KD***A****V*N*V******QESI
Agnr321 I******N*M********K**T*IC*******I**VMK*****-D******H*******ID*****P**EK**IE*KE*V*L*****KG-
Agnr125 I******N*M********K**T*IC*******I**VMK*****-ED*D***H*******ID*****P**EK**IE*KE*V*L*****KG-
Agnr223 I******N*M********K****IS*****P*I**VM******-*******H*****************T**ID*KD*V*******K*-
Spinach I******N**********K****IS*******I**VM******-***K***H*V****************K**DEFRD*V*******K*-
Birch I**T*R*N*L***E****KR***V********I**V*******-*******F*V******D*********D**KKH-EK*******KESK
Squash I**T*R*N*M*****R**RR***********I**VV******-*********V******D*****D***T**KK-NQ********QESI
Bean1 I**T*R*N*L*****R**KR*T**********I**VV******-***R*****V******D****K****E**KKH-********LQANI
Bean2 I**T*R*N***N**SR**KR***********I**VA******-***L***H*V******D*********T**KEHCE*F*******T*-
Barley2 ***T*R*O*VIN**QRR*RR***IC*SS****M**V***V*R*Q*******HLV****S*D*****D***R**TE**********IDQVK
Corn ***T*R*S*VIN**QRH*SR***IC**S****M******VVR*Q***H***HLV******D*****D***R**AE**Y*******IDQVK
Rice ***T*R*E*VIN***RN*RR***I***S****M*QV**SV*R*Q***T***HLV******D*****D***R**AE**********IDQVK
Barley1 I**A*R*A***G*ERR**RR***V**********V***V*R*Q*D*T***HLV******D*M*****I*R**AAN*A********SKVG
Sbean1 I**T*R*N*L*****R**TR***********I**VV******-***C***H*V******D****K****E**KK*-********IQESI
Sbean2 IV*T*R*N*L*****R**TR***********I**VR******-***C***H*V******D****K****E**KK*-********IQASI

Figure 3.5.


901
963
Arab2 K--EGWAYSTGFISEAIMREHIPDGLDGSALAMACGPPPMIQFAVQPNLEKMQYNIKEDFLIF Crawford et al., 1988
Arab1 *--***S******T**VL*****E**E*ES**L**********L*******G**V***L*** Cheng, et al., 1988
Petunia *--***K******T**VL*****LP-SQTT**L**********N******G*D**DSL*V* Salanoubat and Ha, 1993
Tob1 *--***K**L*******L*****EP-SHTT**L**********N******G*D**DSL*V* Vaucheret et al., 1989
Tob2 *--***K**I***T***L*****EP-SHTT**L**********N******G*D**DSL*V* Vaucheret et al., 1989
Tomato T--Q**K*****VT*S*L*****EP-SHTT**L**********IN******G*D***EL*V* Daniel-Vedele et al., 1989
Agnr321 D--*E*K*DV***N*K*V*D*V*EC-GEHV**L*****************G*DV**QL***
Agnr125 N--*E*K*DV***N*K*L*D*V*ES-GEHV**LT****************G*DV**QL***
Agnr223 N--*E*K*D******Q*L*D*V*ES-SSHV**LT****************G*D***QL***
Spinach E--***K*D******K*L*D*V*AV-GDDV**LT**************D**GFD***QL*** Prosser and Lazarus, 1990
Birch R--***E**V*Y*R*S*L*****E*-SDDV**L***A*S**EE**RL*****N*DT*NSLI** Friemann et al., 1992
Squash R--***E**V***T*N*L*****AA-AEDT**L*****A**********N*DT*NSL*V* Hyde et al., 1991
Bean1 R--***E**V***T*S*L***V*LA-SPDT**LT****************LG*D*QN*L*V* Hoff et al., 1991
Bean2 K--***G*GV***T*******L*EA-SSDS***T****************G*D**N*L*V* Jensen et al., 1994
Barley2 RPED**KF*V**VT*D*L*A*V*E*G*-DT**L********K**IS******K*DMANS*IS* Schnorr et al., 1991
Corn ---***K**V**VT**VL***V*E*G*-DT**L**********IS******K*DMANS*VV* Gowri and Campbell, 1989
Rice RPE***K*GV**VT*EVL***V*E*G*-DT**L********K***S******K*DMANS*IV* Choi et al., 1989
Barley1 RPEDA*E*GV*RVD*QVL***L*L*G**ET**LV****A*LECT*R*G****G*DLDK*C*V* Miyazaki et al., 1991
Sbean1 R--***E**V***T*S*LT****NA-SPDT**LT****************LG*DTQNNL*W* Wu et al., 1995
Sbean2 R--***E**V***T*S*LT****NA-SPDT**LT****************LG*DTQNNL*W* Wu et al., 1995

isolated for comparison. Agnr125 and Agnr321 have highly homologous sequences in the 3'-untranslated region. Of the nine Agnr1 clones sequenced, Agnr125 had the longest 3' end and the only putative polyA tail. The 3'-untranslated sequences of the three Agnr3 clones are shown in Figure 3.6. Agnr321 had the longest sequence, but two shorter clones also carried putative polyA tails. However, the putative polyadenylation signals were not always obvious.

Subcloning DNA fragments from A. githago NR clones. Because of the high sequence homology between the three *A. githago* NR clones, smaller DNA fragments that might be gene specific for differentiating the NR mRNAs were subcloned. Figure 3.7 shows the locations chosen for obtaining subclones surrounding the hinge 1 region and Table 3.2 shows the nucleotide sequence homology between the subclones. The sequences of the subclones are identified in Figure 3.5. Agnr1-1 and Agnr3-1 shared very high homology and therefore could not be used as gene-specific probes. Agnr2-1 had less sequence homology and under stringent conditions did not cross-hybridize appreciably with the other two NRs (discussed in more detail in Chapter 4).

```
Agnr321  TGAGAGATTAGCCCACTTAATTTATTCTTAAATTTGGTTAATTAATTAATAA
Agnr324  TGA**************************************************
Agnr319  TGA**************************************************


Agnr321  TAATTTAAAAAATCTATTACTTATAATATTTATGTTATTGAAATTGTATATG
Agnr324  **************************************************
Agnr319  ****************************************AAAAAAAA


Agnr321  TTATTTTAATTAATTTGGATTTTTAAGTTGGTTTATTTATGTACATATATAA
Agnr324  **************************************************

Agnr321  AAAGAGTATATTTTGTAGTTTTCGTGTTTTAAATGAAAGTACAAGGAAGAAA
Agnr324  ******************************AAAAAAAAAAA


Agnr321  TTCCTATGTGTGAAGGTTATAGCTAAAAAAAA
```

Figure 3.6. The 3'-untranslated regions of three *A. githago* Agnr3 cDNA clones.

The 3'-untranslated regions of three *A. githago* cDNA clones corresponding to Agnr3 are compared. A fourth clone had the same 3'-end as Agnr321. Asterisks represent identical nucleotides to Agnr321. The stop codons are underlined.

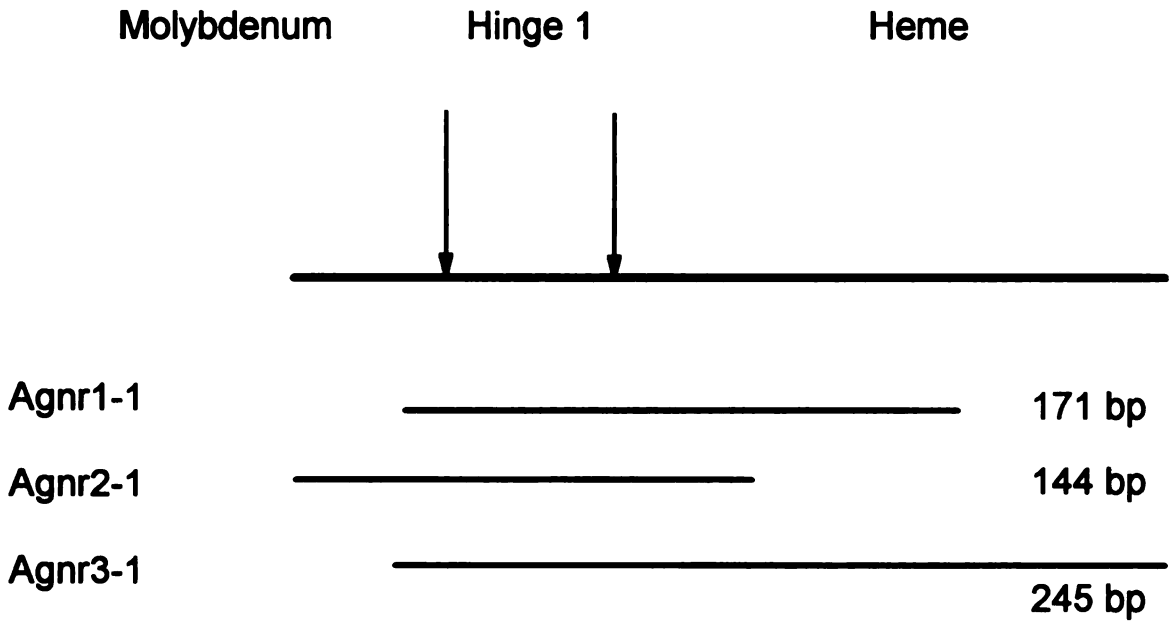


Figure 3.7. The location and lengths of three *A. githago* NR subclones.

DNA fragments surrounding the hinge 1 region were subcloned from the three *A. githago* nitrate reductase clones in an attempt to generate gene-specific probes.

Table 3.2. Nucleotide sequence homology of three *A. githago* NR subclones.

The sequence homology is calculated as a percentage of perfect matches between the subclone (Agnr1-1, Agnr2-1, Agnr3-1) and the cDNA clones over the length of the subclone.

	% Homology		
	Agnr1	Agnr2	Agnr3
Agnr1-1	——	77	95
Agnr2-1	78	——	78
Agnr3-1	95	80	——

DISCUSSION

Three *A. githago* NR partial cDNA clones that show high sequence homology to other plant NRs have been isolated. The greatest homology between *A. githago* and the seventeen NR sequences available was to spinach, which is in agreement with spinach being the most closely related of these plants to *A. githago,* both in the order Caryophyllales. Agnr1 and Agnr3 show very high sequence homology to each other in the coding and 3'-untranslated regions. *A. githago* is a tetraploid plant (2n = 48; Firbank, 1988). While the origin of this species is unknown, it has been suggested that *A. githago* may have arisen as a polyploid of *Agrostemma gracilis* (2n = 24; Firbank, 1988). Agnr1 and Agnr3 may have evolved from the same *A. gracilis* ancestral gene, while Agnr2 is a second NR gene. However, since there is greater sequence homology in the 3' half of NR genes, full length clones are needed to determine if the high sequence homology extends into the 5'-half of the genes.

There are many conserved amino acids in NR believed to be important in enzyme activity. Figure 3.5 identifies the positions of these amino acids. In the molybdenum domain, the cysteine is believed to be associated with the binding of the molybdenum cofactor (Neame and Barber, 1989). One of several other conserved cysteines in this domain may be involved in forming an interchain disulfide bridge (Hyde *et al.*, 1989). In the heme binding domain, ten residues are conserved in all plant NRs and in all other members of the cytochrome b5 superfamily. The two conserved histidines are involved in heme binding (Meyer *et al.*, 1991). In the flavin binding domain, the histidine, cysteine, lysine, two

arginines, and serine/threonine are all believed to be involved at the NADH-binding site (for a review, see Rouzé and Caboche, 1992). All of these amino acids involved with the NR activity are also conserved in the three *A. githago* clones.

Nitrate reductase is a key enzyme in nitrogen metabolism in higher plants. The importance of NR may be reflected by the presence of more than one NR gene in many higher plants, including the relatively small genome of *A. thaliana*. In some plants, the genes are differentially expressed in different organs. For example, in *Phaseolus vulgaris* one gene is expressed exclusively in the roots, while a second gene is expressed in the roots and leaves (Jensen *et al.*, 1994). In other plants such as barley, the requirement for NADH or NAD(P)H as the electron donor biochemically differentiates between NRs (Dailey *et al.*, 1982). NR proteins may also be active under different pH optima (Streit *et al.*, 1985) or have different kinetics (Kleinhofs *et al.*, 1988). NR activity is regulated by both nitrate and cytokinins in *A. githago* embryos. The three NR cDNA clones will be used to examine the regulation of NR by nitrate and cytokinins.

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CHAPTER 4

Cytokinin and Nitrate Regulation of Nitrate Reductases in *Agrostemma githago* Embryos

ABSTRACT

Northern blot analysis and reverse transcription-polymerase chain reaction (RT-PCR) analysis were used to study the expression of the three nitrate reductase transcripts in *A. githago*. All three NR clones hybridized to a 3.5-kb mRNA. Agnr1 and Agnr3 were both regulated in a similar manner. The mRNA levels were transiently increased up to ten fold by both cytokinin and nitrate in a time-dependent manner similar to but preceding the induction of NR activity. Cytokinin plus nitrate did not show an additive effect on NR Agnr1 mRNA even when NR activities were additive. Both cytokinin and nitrate increased these mRNA levels in both roots and cotyledons, but the increase was observed in cotyledons earlier than in roots. The decrease in nitrate-regulated mRNA levels over time was initially due to a decrease in levels in the cotyledons.

Agnr2 was much less abundant than were the other two NR mRNAs. Agnr2 mRNA level was not increased by cytokinin but was increased by nitrate predominantly in the roots. In control embryos, there were low levels of all three mRNAs that decreased with time of incubation.

The length of seed imbibition affected the levels of the three NR mRNAs in isolated embryos. The levels of the three NR messages in embryos isolated

from seeds imbibed for 12 h but not incubated on media were at least as great as the maximum message levels obtained by cytokinin or nitrate incubation.

Nuclear runoff transcription studies showed that the increase of Agnr1 and Agnr3 mRNA levels by cytokinin were not due to increased rates of transcription. The increase in these mRNA levels by nitrate was due, in part, to increased rates of transcription. Due to the low levels of Agnr2 mRNA, it could not be determined how expression of this mRNA was regulated.

INTRODUCTION

Most plants assimilate nitrogen in the form of nitrate. Because the rate-limiting and regulated step of nitrate assimilation is catalyzed by nitrate reductase (NR), much research has focussed on NR and its regulation. NR is regulated by nitrate in all plants. NR may also be regulated by light, circadian rhythms, CO_2, reduced nitrogen compounds, sucrose, plant hormones, and a plastidic factor (for reviews, see Rouzé and Caboche, 1992; Hoff *et al.,* 1994; Crawford, 1995).

Nitrate reductase cDNA or genomic clones have been identified in at least twelve plant species (Figure 3.5). More than one gene is present in many of these plants, including in the relatively small genome of *Arabidopsis thaliana* (Cheng *et al.*, 1988). These clones have allowed the study of NR gene expression in these and trangenic plants.

In most plants, NR cDNA clones hybridize to 3 to 3.7-kb mRNAs. These mRNAs are typically present at low levels in the absence of nitrate and increase in response to nitrate and other signals. In time course experiments of nitrate treatment, both NR activity and NR mRNA expression are transiently increased (*e.g.*, Kleinhofs *et al.*, 1988; Cheng *et al.*, 1991; Jensen *et al.*, 1994).

It is of particular interest to determine the regulation of multiple NR genes from the same plant. In soybean, three NR proteins are active under different pH optima (Streit *et al.*, 1985). In barley, *e.g.*, the requirement for NADH or NADPH as the electron donor biochemically differentiates between NRs (Dailey *et al.*, 1982). However, many higher plants have only one identified form of NR

protein but more than one NR gene. In several plants, the genes are differentially expressed in different organs. In *Phaseolus vulgaris*, *e.g.*, one gene is expressed exclusively in the roots (Hoff *et al.*, 1991), whereas a second gene is expressed in the roots and leaves (Jensen *et al.*, 1994). In rice and *A. thaliana*, two NR genes are expressed with very different kinetics (Kleinhofs *et al.*, 1988; Cheng *et al.*, 1991).

The mechanisms of regulation of NR are being examined. Nuclear runoff transcription studies have shown at least partial transcriptional regulation of NR by nitrate in soybean (Callaci and Smarrelli, 1991) and corn (Lillo, 1991), and transcriptional regulation by cytokinin in barley (Lu *et al.*, 1990). The circadian rhythms in *A. thaliana* were shown to be post-transcriptionally regulated (Pilgrim *et al.*, 1993). Studies using transgenic plants or mutants have shown multiple levels of regulation. Nitrate may regulate gene expression at the transcriptional or post-transcriptional levels. Light may regulate gene expression at the transcriptional, post-transcriptional, or post-translational levels. Circadian rhythms may regulate expression both transcriptionally and post-transcriptionally, whereas sucrose regulates at the transcriptional level (for reviews, see Rouzé and Caboche, 1992; Hoff *et al.*, 1994; Crawford, 1995).

Cytokinins have been shown to regulate gene expression both at the transcriptional and post-transcriptional levels (see Chapter 1). In barley, cytokinins have been shown to regulate NR expression, at least partly, at the transcriptional level (Lu *et al.*, 1990). The goal of this work was to examine the expression of the three *A. githago* NR genes in time-course experiments and in

specific tissues of the embryos as influenced by nitrate and cytokinin. Regulation by nitrate and cytokinin was examined with nuclear runoff transcription experiments to determine whether regulation was at the transcriptional level.

MATERIALS AND METHODS

Plant material, Incubation procedures, Tissue extracton, NADH-NR assay, Radiolabelling probes, and other Standard molecular biological methods. See Materials and Methods, Chapter 3.

RNA extraction. Total RNA was extracted as described in Chapter 3. The RNA was DNAse I treated to remove any DNA that could interfere with the reverse transcription-polymerase chain reaction (RT-PCR) analysis.

Reverse transcription-polymerase chain reaction. A non-degenerate oligonucleotide common to all three *A. githago* NR clones was synthesized and used as the 5' primer in RT-PCR amplification of all three NR mRNAs. HK52 (5'-TGGTGTTTTTGGTCATTAGAAGT-3') overlaps the degenerate oligonucleotide HK35 (see Chapter 3 for location) and has a similar melting temperature as the gene-specific oligonucleotides used as 3' primers. PCR amplification with HK52 and the gene-specific oligonucleotides HK38, HK39, or HK76 (sequences in Materials and Methods, Chapter 3) generates a 286-bp DNA fragment. RT-PCR amplification from the sense RNAs transcribed from the three NR cDNA clones (Agnr1, Agnr2, Agnr3; Chapter3) was used to determine optimum conditions and show specificity of the gene-specific oligonucleotides in RT-PCR amplification. RNA was reverse transcribed into cDNA with the NR gene-specific oligonucleotides. A 33.5 μl mixture containing varying amounts of total RNA in 10 μl, 1-20 μl internal standard (see below), and 0.82 μg gene-specific oligonucleotide (HK39, HK38, or HK76) was incubated at 65°C for 5 min and then cooled on ice. Following this, 14 U RNAsin RNAse inhibitor

(Promega, Madison, WI) and 100 U M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD) were added to the cDNA in a total volume of 50 μl containing 0.5 mM of each dNTP, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 10 mM DTT, and 3 mM $MgCl_2$. This mixture was incubated at 37°C for 1 h and the reaction stopped by incubation at 95°C for 10 min. Ten μl of the RT reaction was used in a 50 μl PCR reaction. A 50 μl PCR reaction was run in standard PCR buffer containing 1 μM HK52 and 1 μM of the gene-specific oligonucleotide under the following conditions: 3 min at 94°C and then 22 cycles of 1 min at 94°C, 2 min at 63°C, and 2 min at 72°C. In the last cycle the extension time was prolonged to 9 min. Ten to 20 μl of the reaction mixtures were separated in 1.2% agarose gels, blotted onto Hybond N^+ nylon membranes (Amersham, Arlingon Heights, IL), and the membranes were baked at 80°C for 1 to 2 h. Controls without reverse transcriptase added were always performed in parallel to check for contamination from the RNAs or stock solutions.

Southern blots were hybridized with the 495-bp random-primed labelled insert of Agpcr1 (Chapter 3) or end-labelled HK40 (a non-degenerate internal oligonucleotide common to all three *A. githago* NR clones: 5'-TGTGTTCGAGCATCCGAC-3'). Autoradiography was performed using Hyperfilm-MP (Amersham). The signals were quantitated with a phosphorimager (Molecular Dynamics, Sunnyvale, CA). Experiments were repeated two to three times with each set of RNAs.

Cross-hybridization of A. githago NR clones. Because of the high degree

of sequence identity between the NR clones, smaller DNA fragments surrounding the hinge 1 region were subcloned for all three NR clones, corresponding to Agnr1-1, Agnr2-1, and Agnr3-1 (see Figure 3.7 and Table 3.2). Sense strand RNAs were produced by *in vitro* transcription from the Agnr1 and Agnr2 (Chapter 3) linearized plasmids. The templates were removed by DNAse I digestion. The RNAs were extracted, precipitated, suspended in water, and their concentrations were measured. Equivalent amounts of sense RNAs were serially diluted, blotted, and hybridized with antisense RNA probes.

RNA probes. Agnr1-1, Agnr2-1, and Agnr3-1 were linearized by digestion with *Eco*RI, *Xba*I, or *Xba*I, respectively. T7 or T3 polymerase was used to synthesize antisense radiolabelled RNA probes from the linearized templates using [^{32}]PUTP, 800 Ci/mmol (New England Nuclear). Probes were DNAse I treated to remove the DNA template.

RNA blot analysis. Total (Figure 4.8) or poly(A)$^+$ (Figure 4.6) RNA was separated on 1% agarose-formaldehyde gels. Ethidium bromide staining of the ribosomal bands was used to confirm equal loading of total RNAs. RNA was transferred to Hybond N nylon membranes (Amersham). The membranes were baked at 80°C for 1 to 2 h and prehybridized at 65°C in 5X SSPE, 10X Denhardt's solution, 0.5% SDS, 0.25 mg/ml salmon sperm DNA, and 50% formamide (20X SSPE: 3.6 M NaCl, 0.2 M sodium phosphate, 0.02 M EDTA, pH 7.7). ^{32}P-labelled antisense strand RNA probes were hybridized at 65°C in the above prehybridization buffer. The final wash of the filters was in 0.2X SSC, 0.1% SDS at 65°C.

Internal Standards (IS). Controls containing the same primer sites as target mRNAs but generating different size DNA fragments from RT-PCR were constructed. For IS1 and IS3, DNA fragments that spanned an intron were PCR amplified from genomic clones (Chapter 5) with HK52 and the gene-specific oligonucleotide (HK39 or HK76) and ligated into the *Sma*I site of pBluescript SK$^-$. IS2 was generated by using an Agpcr2 (Chapter 3) clone that was missing part of the 3′ sequence. This clone was linearized by digestion with *Sma*I. The *Sma*I restriction site lies between HK52 and the gene-specific oligonucleotide HK38. A 300-bp unrelated DNA sequence was ligated into this *Sma*I site to change the distance between HK52 and HK38. This larger insert was released from pCRII by digestion with *Eco*RI and was ligated into the *Eco*RI site of pBluescript SK$^-$. Each of the IS clones was linearized and sense strand RNAs were synthesized and added to the RT-PCR reactions as internal standards. Test experiments were done to determine the amounts of internal standard RNAs needed to produce PCR products in similar amounts as the target mRNAs. IS1, IS2, and IS3 generate DNA fragments from RT-PCR of approximately 1100 bp, 586 bp, and 1200 bp, respectively.

Preparation of nuclei. Nuclei prepared from 1.5 to 2.0 g embryos were suspended in 20 ml of nuclear suspension buffer A and 0.5% Triton X-100 (buffer A: 20 mM MES buffer, pH 5.7, 2.5% Ficoll 400, 2.5% dextran 40,000, 50 mM KCl, 0.44 M sucrose, 0.5 mM EDTA, 1% thiodiglycol, 0.5 mM spermidine, 0.1 mM spermine, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin). The nuclei were consecutively filtered through 4

layers of cheesecloth, 2 layers of miracloth, and a 20 μm filter. The nuclei were collected by centrifugation for 15 min at 2500 rpm in an HB-4 rotor (Du Pont Sorvall, Wilmington, DE), resuspended in 5 ml buffer A, and applied to a Percoll gradient. The gradients consisted of 5 ml each of 1.8 M sucrose, 80% Percoll, and 60% Percoll (pH ~6.0 in buffer A). The gradients were centrifuged for 20 min at 5000 rpm in an HB-4 rotor, and the nuclei were collected from the 80% Percoll/1.8 M sucrose interface. (The nuclei from the 60%/80% Percoll interface were much fewer in number and therefore discarded.) The nuclei were washed with buffer A plus 50% glycerol to remove the Percoll and centrifuged for 10 min at 3000 rpm in an HB-4 rotor. The nuclei were counted with a hemocytometer and the volume adjusted such that 50-μl aliquots, containing 1 to 3 X 10^6 nuclei, could be stored at -80°C. All treatments were performed at 0 to 4°C. The nuclei yields were 2 to 4 X 10^6 nuclei/g tissue and >95% of the nuclei appeared intact.

Transcription in isolated nuclei. The reaction mixture for *in vitro* nuclear runoff transcription contained: 100 mM Hepes buffer, pH 7.9, 50 mM $MgCl_2$, 500 mM $(NH_4)_2SO_4$, 20% glycerol, 2 mM DTT, 100 μM ATP, GTP, CTP, 150 μC [^{32}]PUTP (3000 Ci/mmol, New England Nuclear), 72 units of RNAse inhibitor (Boehringer Mannheim Biochemicals, Indianapolis, IN), and 50 μl nuclei in a total volume of 100 μl. The reaction was incubated at 30°C for 20 min and then treated with DNAse I for 5 min. The transcribed RNA was phenol extracted, precipitated, suspended in water, and counted in a scintillation counter. The reaction mixture typically contained 1 to 3 X 10^6 nuclei and yielded 1 to 2 X

10^8 cpm incorporated into RNA. All treatments were performed under RNAse-free conditions. The transcriptional competence of each of the nuclear preparations was tested by measuring the incorporation of [^{32}]PUTP at 5-min intervals during the transcription reaction. One-μl aliquots of the transcribed RNA were spotted in duplicate onto DE81 filters and incorporated counts determined according to Maniatis *et al.* (1982).

DNA slot blot hybridization. To quantitate levels of NR transcript produced in the nuclear runoff assays, hybridizations were carried out in DNA excess by applying 5 μg of linearized Agnr1, Agnr2, and Agnr3 plasmid DNA (Chapter 3) to nitrocellulose using a slot blot apparatus (Bio-Rad, Hercules, CA). Linearized pBluescipt SK$^-$ and pRL5 were used as negative and positive controls, respectively. pRL5 is a pBluescript plasmid containing a 1.2 kb insert coding for a rice ribosomal 5S RNA-binding protein (Kim and Wu, 1993). Blots were prehybridized in 5X SSC, 10X Denhardt's solution, 0.1 M potassium phosphate, pH 6.8, 100 μg/ml salmon sperm DNA, 10% dextran sulfate, and 30% formamide for at least 24 h at 51 °C. Blots were hybridized in the same buffer with equivalent counts from each transcription assay, typically 40 to 100 X 10^6 cpm, for at least 48 h at 51 °C. The negative controls typically gave no signal, and relative amounts of NR mRNAs were related to the signals from the positive control. Two to three experiments were done with each set of nuclear preparations.

RESULTS

Cross-hybridization of A. githago nitrate reductase clones. Because of the high sequence homology between the three nitrate reductase cDNA clones, smaller DNA fragments surrounding the hinge 1 region were subcloned in an attempt to generate gene-specific probes (Chapter 3). Using *in vitro* transcribed sense RNAs from Agnr1 and Agnr2, it was determined that Agnr1-1 and Agnr2-1 antisense RNA probes showed less than 1% cross-hybridization (Figure 4.1) and Agnr2-1 could be used as a gene-specific probe. Agnr1-1 and Agnr3-1 showed 10% cross-hybridization (data not shown) and could not be used as gene-specific probes.

Regulation of NR mRNAs.

RT-PCR. Because it was not possible to differentiate between all three NR clones, and because the NR mRNA levels were of low abundance, RT-PCR analysis was used to study the regulation of NR mRNAs in *A. githago*. The gene-specific oligonucleotides (HK39, HK38, HK76, Chapter 3) were used for priming the reverse transcription reactions, as well as for PCR amplification. Synthetic RNAs served as internal standards for both reverse transcription and PCR amplification. The internal standards contained the same primer sequences as the target mRNAs but yielded PCR products of different sizes. The addition of the internal standards to each RNA sample also served as controls for sample-to-sample and tube-to-tube variations. RT-PCR analysis, illustrated in Figure 4.2, allowed for the relative quantitation of target mRNAs.

An example of how mRNAs were quantitated is shown in Figure 4.3.

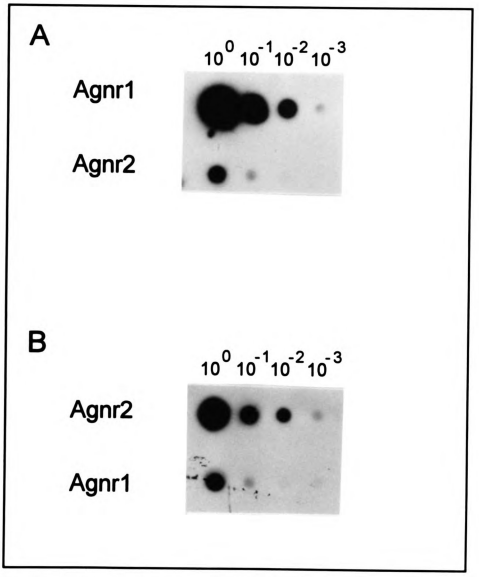


Figure 4.1. Cross-hybridization of Agnr1-1 and Agnr2-1.

The cDNA clones Agnr1 and Agnr2 (Chapter 3) were used to produce sense strand RNAs by *in vitro* transcription. Equivalent amounts of sense RNAs were serially diluted, blotted, and probed with [^{32}P]-labelled antisense RNA prepared from either (A) Agnr1-1 or (B) Agnr2-1.

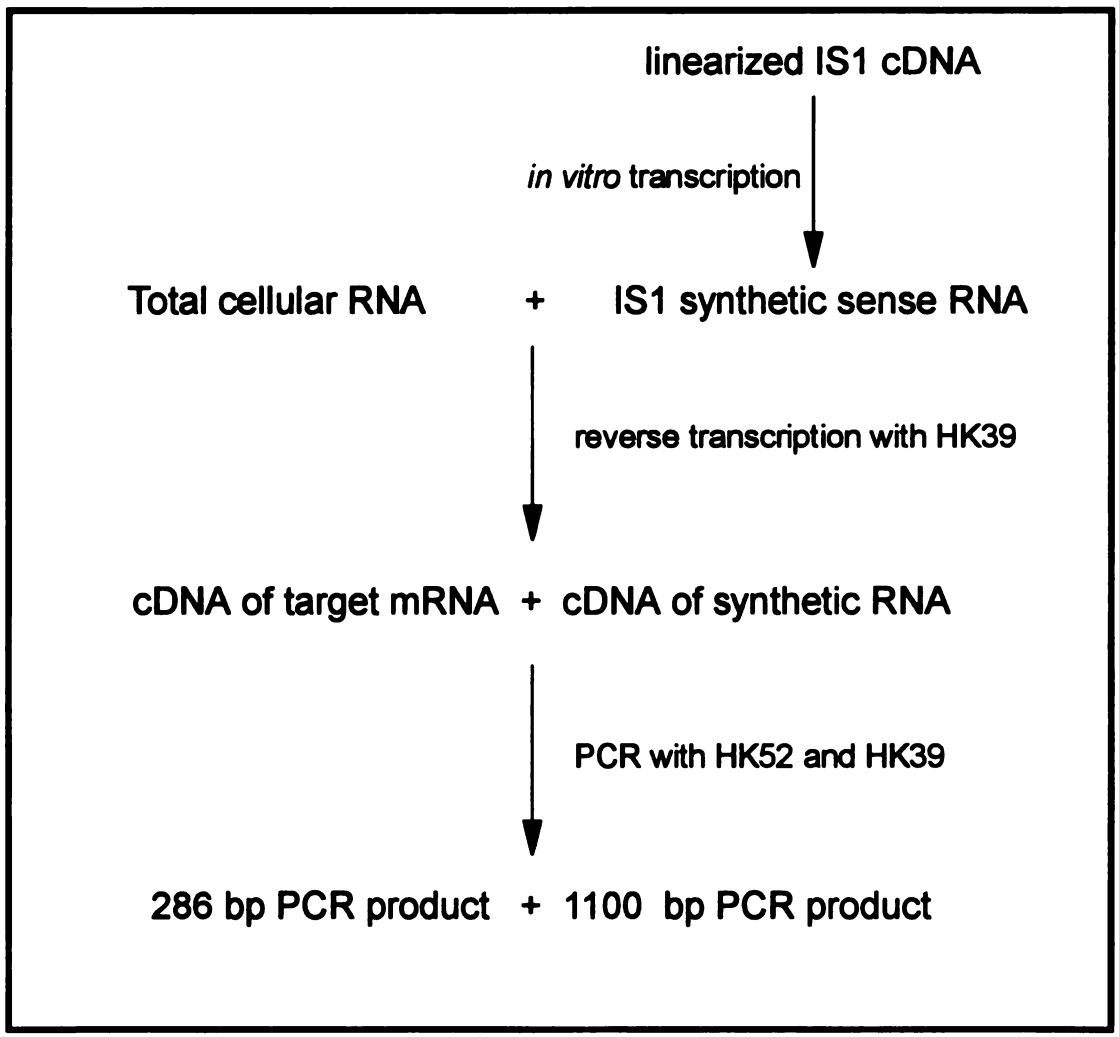


Figure 4.2. Schematic representation of RT-PCR.

An example of RT-PCR analysis is shown. An internal standard (IS1) containing the same primer sites as the targeted mRNA is generated by *in vitro* transcription from a linearized plasmid DNA. The IS1 RNA is added to the total RNA in various amounts, and both are reverse transcribed using the gene-specific oligonucleotide HK39 as primer. A fraction of the synthesized cDNA is amplified by PCR, resulting in amplified products of 286 bp from the total RNA and 1100 bp from the IS1 RNA.

Figure 4.3. Quantitative analysis of NR mRNA by RT-PCR analysis.

(A) Ethidium bromide staining of PCR products separated in 1.2% agarose. Total RNAs isolated from embryos incubated for 4 h in water (C), 5 µM BA (B), or 50 mM nitrate (N) were analyzed. 1, 2, 10, or 20 µl of IS1 sense RNA was added to 0.5 µg of total RNA for RT-PCR analysis. The sizes of the NR products and the IS1 products are 286 bp and 1100 bp, respectively. M=molecular weight markers, bp. (B) The gel in (A) was blotted and hybridized with the random-primed labelled insert of Agpcr1 (Chapter 3). The autoradiogram of this filter is shown. (C) Relative signal intensities were quantitated using a phosphorimager, and the relative abundances of NR and IS1 signals were plotted against amount of IS1 RNA added to the reaction. Lines and intercepts were determined by linear regression.

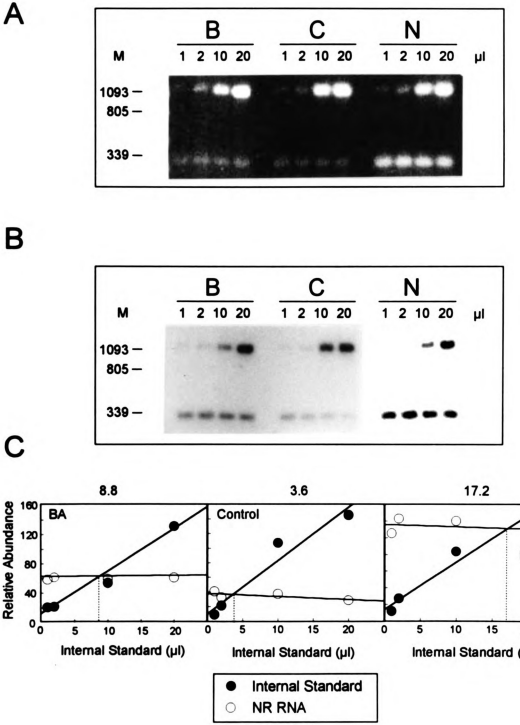


Figure 4.3. Quantitative analysis of NR mRNA by RT-PCR analysis.

Varying amounts of internal standard RNA were added to a defined amount of total RNA for each sample analyzed. RT-PCR was performed and an aliquot was separated on an agarose gel (Figure 4.3A). The gel was blotted, hybridized with the necessary probe (Figure 4.3B), and signals quantitated with a phosphorimager. The relative signal intensities from both the target and the internal standard DNA products were plotted against the amount of internal standard RNA added (Figure 4.3C). The intercepts of the linear regression lines were used to determine the amounts of target mRNA in the samples relative to the amount of internal standard.

Since PCR amplification is an exponential process, the first step was to determine the optimum number of cycles for PCR amplification. Based on published results (Chelly *et al.*, 1988; Wang *et al.*, 1989; Simson *et al.*, 1992; Chang *et al.*, 1993), 16-30 cycles, in increments of two cycles, were tested for the amplification of target mRNAs. The amounts of products were exponentially related up to 26 cycles before reaching a plateau (data not shown), and 22 cycles were chosen for all other experiments.

Another requirement for RT-PCR analysis is that the amounts of RNA used are linearly related to the amount of amplified DNA obtained. Initial experiments using serial dilutions of RNA helped to determine the amounts of total RNA needed to remain within a linear range. Within this range, 2 to 3 concentrations of RNA were tested in RT-PCR. One example of this is shown in Figure 4.4. The addition of 5 times the total RNA resulted in 5 times the relative signal. Each time new RNA was isolated, random samples were tested

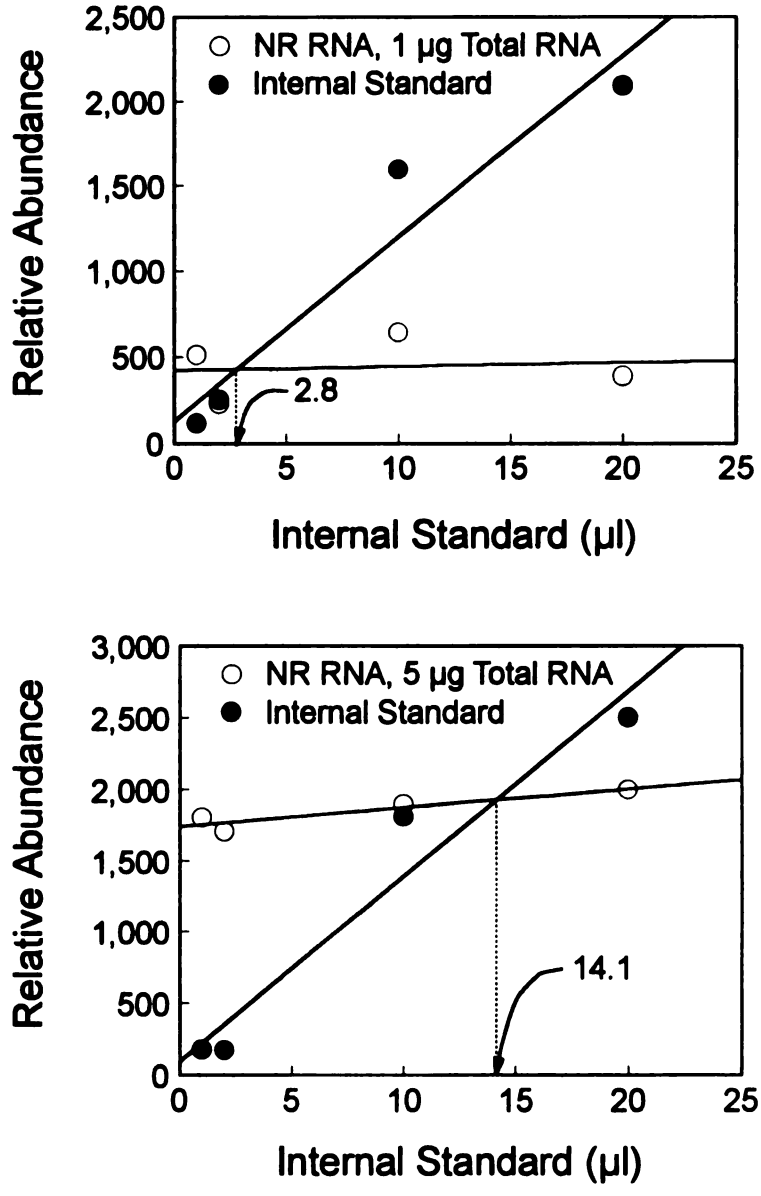


Figure 4.4. RT-PCR with varying amounts of total RNA.

Agnr1 and IS1 RNAs were amplified by RT-PCR. Either 1 µg or 5 µg of total RNA was analyzed. Ten µl of the PCR reaction was separated by agarose gel electrophoresis, blotted onto Hybond N$^+$ nylon membrane, and probed with the random-primed labelled insert of Agpcr1 (Chapter 3). Relative signal intensities were quantified on the phosphorimager, and the relative abundances of NR and IS1 signals were plotted against the amount of IS1 RNA added to the reaction. Lines and intercepts were determined by linear regression.

in this manner over a five to tenfold range. The results not only showed that the amounts of RNA used were linearly related to the amounts of amplified DNA obtained, but they also indicated there were no contaminants in the RNA samples that might have interfered with the RT-PCR analysis.

Time-course experiments. NR activity was regulated by cytokinin and nitrate in a time-dependent manner, and a combination of cytokinin plus nitrate showed an additive effect on activity (Figure 4.5). This additive effect was not always seen for time points beyond 12 h, however. Kuznetsov (1979) also observed an additive effect on NR activity in *A. githago* embryos by cytokinin and nitrate for early time points but not for later time points and suggested that carbohydrate starvation was responsible for experimental data that were below the expected values. Variations between seed lots and experimental variations showed differences in the increase in NR activity, but overall the trends were always the same. Nitrate-enhanced NR activity increased within 2 h, peaked at 6 to 12 h, and declined quite rapidly thereafter. Cytokinin-enhanced NR activity typically did not increase until 6 h, peaked at 12 to 18 h, and stayed more elevated than did nitrate-enhanced NR activity after 24 h. The increase in NR mRNAs was expected to precede the increase in NR activity and to be transient. Therefore, 6, 12, and 18 h of treatment were examined in initial experiments.

Because of cross-hybridization, northern blot analysis with the Agnr1-1 RNA probe detects both Agnr1 and Agnr3 mRNAs (see p. 106). Northern blot analysis showed a transient increase in Agnr1 + Agnr3 mRNA levels by both

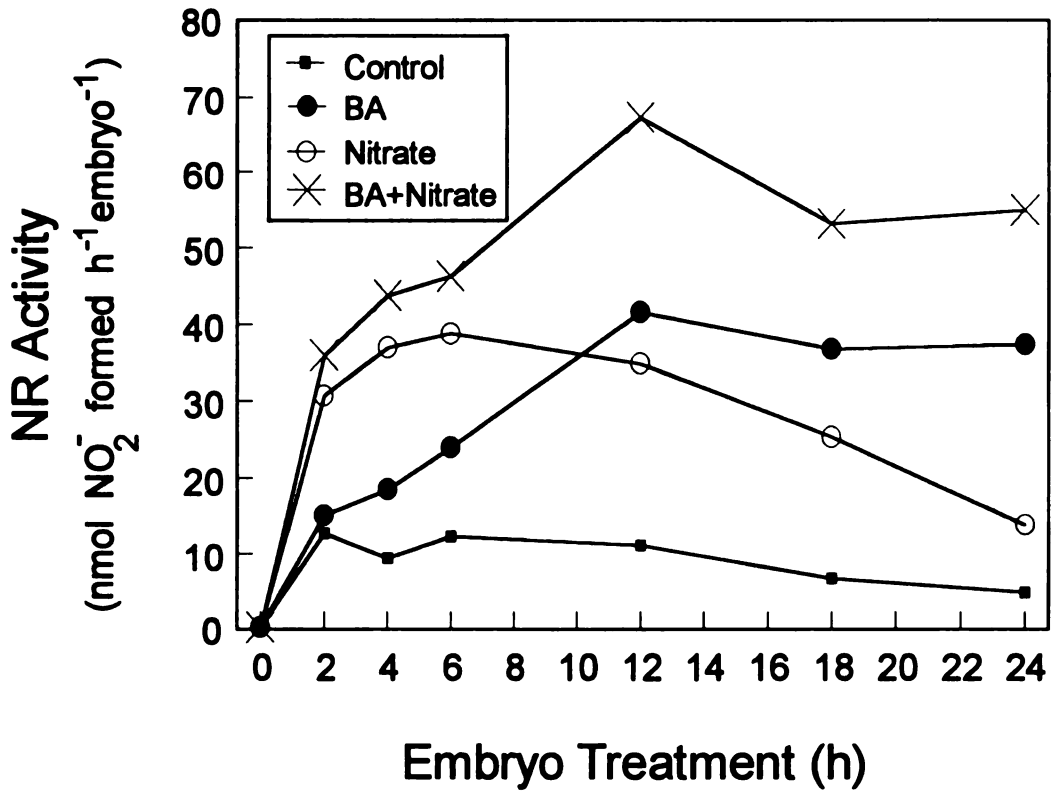


Figure 4.5. Time course of nitrate reductase activity.

Seeds were imbibed for 12 h at RT. The embryos were then isolated and incubated at 30°C in water (control), 5 µM BA, 50 mM nitrate, or 5 µM BA plus 50 mM nitrate for the indicated times. Each point represents the average of triplicate samples, 4 embryos per sample.

cytokinin and nitrate treatments (Figure 4.6). The level of Agnr1 + Agnr3 mRNAs decreased faster with the nitrate treatment than it did with the cytokinin treatment. A low level of Agnr1 + Agnr3 mRNAs was present in the controls and decreased with time. A cytokinin plus nitrate treatment did not show an additive effect on the Agnr1 + Agnr3 mRNA levels, which was observed in two similar experiments. The zero time point showed a relatively high level of the mRNAs (139%) even though NR activity was low. A similar experiment with Agnr2 showed only very weak signals in the control, 6 h nitrate, and 6 h nitrate plus cytokinin (data not shown).

Based on these results, RT-PCR analysis was performed on embryos treated for 2 to 12 h (Figure 4.7). Agnr1 and Agnr3 showed similar results in two experiments. Nitrate-regulated mRNA levels increased by 2 h, peaked at about 4 h, and decreased to near control levels by 12 h. Cytokinin-regulated mRNA levels increased by 2 h, peaked at 4 to 6 h, and decreased by 12 h but still remained at 3 to 5 times the control level. Cytokinin and nitrate enhanced Agnr1 and Agnr3 mRNA levels a maximum of 8 to 10 times. The mRNA levels at the zero time point were relatively high, while the mRNA levels in the isolated embryos incubated in water were low and decreased with time. Northern blot analysis with total RNAs from one experiment using the Agnr3-1 RNA probe showed similar results as the RT-PCR analysis for Agnr1 and Agnr3 mRNAs (data not shown).

Localization of NR mRNAs. Enhancement of NR activity by cytokinins is localized primarily in the cotyledons, whereas enhancement by nitrate is

Figure 4.6. Northern blot analysis of Agnr1 and Agnr3 mRNAs in *A. githago* embryos.

(A) Time course of NR activity (see Figure 4.5 legend). (B) poly(A)$^+$ RNA was isolated from embryos analyzed in (A). Five µg RNA was separated by electrophoresis on a 1% agarose-formaldehyde gel, blotted onto Hybond N nylon membrane, and hybridized with Agnr1-1 RNA probe (Chapter 3). The autoradiogram was exposed for 4 d with two intensifying screens at -80°C. (C) Relative signal intensities were quantitated using a phosphorimager and plotted as percent relative abundances. The signal from the 6 h of BA plus nitrate treatment was set at 100%. The signal from the zero time point had a relative abundance of 139% (data not plotted). Because of cross-hybridization, the Agnr1-1 probe detected Agnr3 mRNA as well as Agnr1 mRNA.

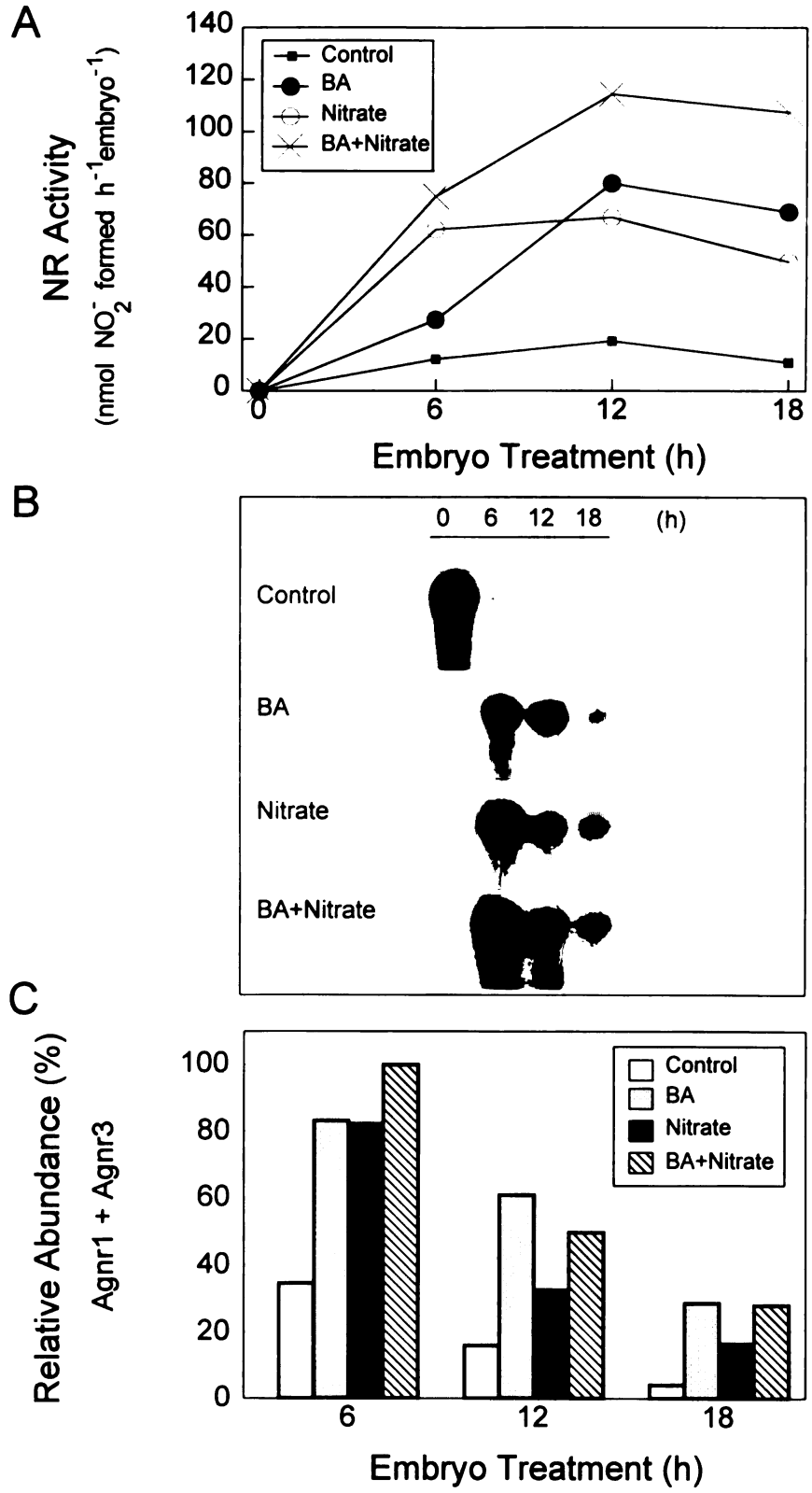


Figure 4.6. Northern blot analysis of Agnr1 and Agnr3 mRNAs in *A. githago* embryos.

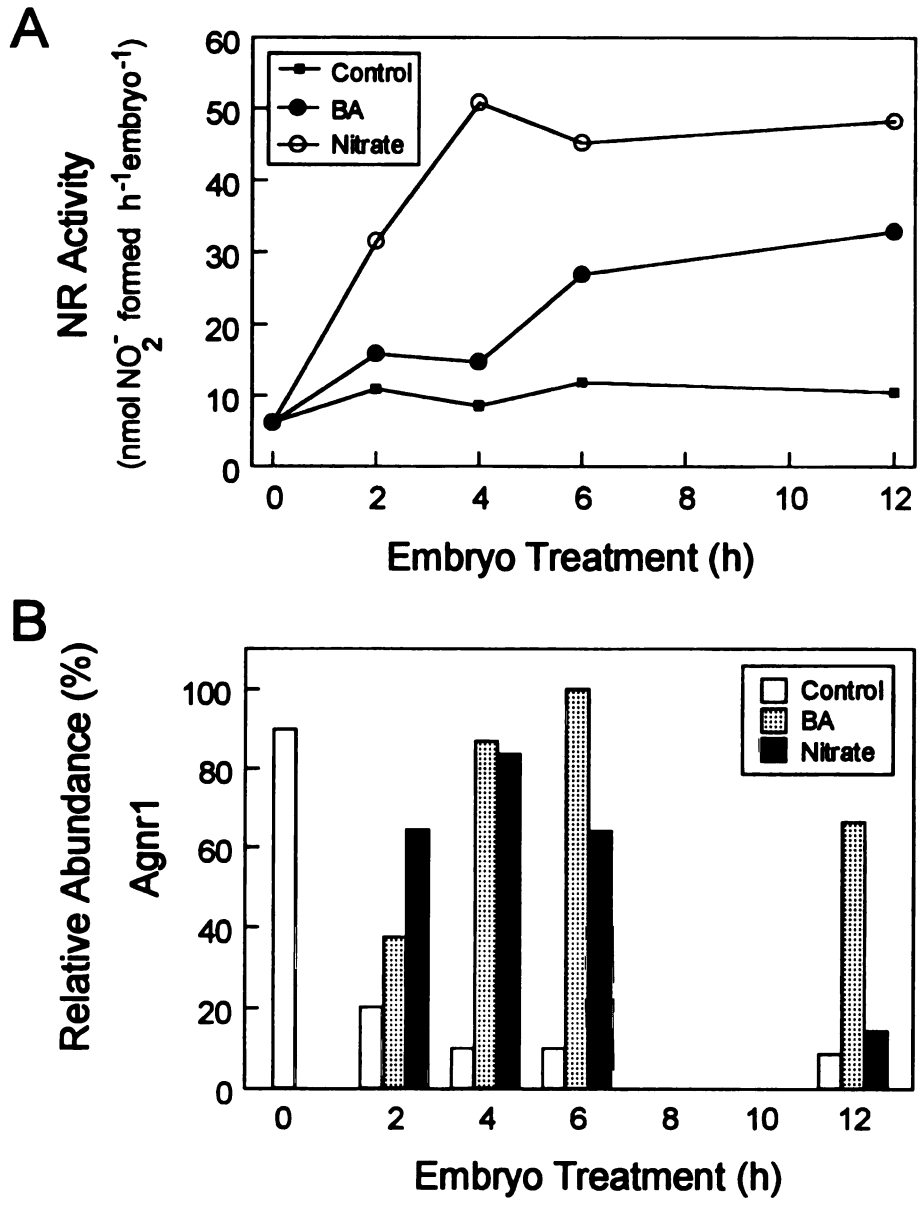


Figure 4.7. Quantitative analysis of Agnr1 mRNA in *A. githago* embryos.

(A) Time course of NR activity (see Figure 4.5 legend). (B) Total RNAs were isolated from embryos analyzed in (A) and 0.5 µg was used for RT-PCR analysis of Agnr1 mRNA. Percent relative abundance is plotted. Agnr1 and Agnr3 showed similar results in two experiments. Similar results were seen by Northern blot analysis with an Agnr3-1 RNA probe and 20 µg total RNA.

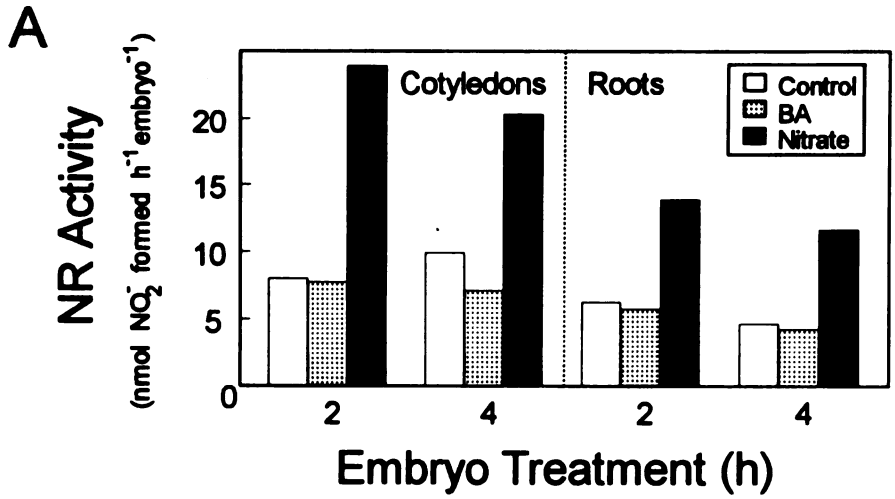
localized in both roots and cotyledons (Table 2.1). The localization of the NR mRNAs was also examined. Cytokinins ususally increased NR activity by 6 h (Figures 4.6, 4.7) or later, but increased NR mRNA levels by 2 h (Figure 4.7B). Therefore, when NR mRNA levels were examined in cotyledons and roots at 2 and 4 h of treatment, nitrate had increased NR activity in cotyledons and roots, but cytokinin had not increased NR activity yet (Figure 4.8A). Unlike NR activity, cytokinins increased Agnr1 and Agnr3 mRNA levels in both roots and cotyledons, with the increase in cotyledons observed sooner than that in roots (Figure 4.8C, D). Nitrate also increased Agnr1 and Agnr3 mRNA levels in cotyledons earlier than in roots. The nitrate-regulated mRNA levels in the cotyledons started to decrease by 4 h, perhaps responsible for the decrease in nitrate-regulated mRNA levels in the embryos. This would agree with earlier experiments showing that the decrease in NR activity was due to a loss of activity in the cotyledons (Table 2.1). Northern blot analysis (Figure 4.8C) and RT-PCR analysis (Figure 4.8D) showed similar results.

Agnr2 mRNA levels were regulated by nitrate but not cytokinins. Northern blot analysis with total RNAs from 6, 12, and 18 h of treatment showed only weak signals in the control, 6 h nitrate, and 6 h nitrate plus cytokinin treatments using the Agnr2-1 RNA probe (data not shown). Nitrate regulation was localized predominantly in the roots (Figure 4.9). Although the overall level of Agnr2 mRNA was much lower in the cotyledons, some regulation by nitrate may also be present in the cotyledons.

The effect of imbibition time on NR mRNAs. Seeds are typically imbibed

Figure 4.8. Quantitative analysis of Agnr1 and Agnr3 mRNAs in roots and cotyledons of *A. githago.*

(A) Seeds were imbibed for 12 h at RT. The embryos were then isolated and incubated at 30°C for 2 or 4 h in water (control), 5 µM BA, or 50 mM nitrate. Embryos were then separated into cotyledons and roots. Results represents the average of triplicate samples, 8 cotyledon pairs or 8 roots per sample. (B) Total RNA was isolated from cotyledons and roots from (A). Twenty µg of total RNAs were separated on a 1% agarose-formaldehyde gel. The gel was blotted onto Hybond N nylon membrane and hybridized with the Agnr3-1 RNA probe (Chapter 3). The autoradiogram was exposed for 14 d with two intensifying screens at -80°C. The size of the hybridizing band was 3.5 kb. C=water control; B=5 µM benzyladenine; N=50 mM nitrate. 2 and 4 represent h of treatment. c=cotyledons; r=roots. (C) The autoradiogram from (B) was quantitated using a phosphorimager and signals plotted as percent relative abundance. Because of cross-hybridization, the Agnr3-1 RNA probe detected Agnr1 mRNA as well as Agnr3 mRNA. (D) RT-PCR analysis of Agnr1 mRNA using 0.5 µg of each total RNA from (A). Percent relative abundance is plotted. Similar results were seen with RT-PCR analysis of Agnr3 mRNA.



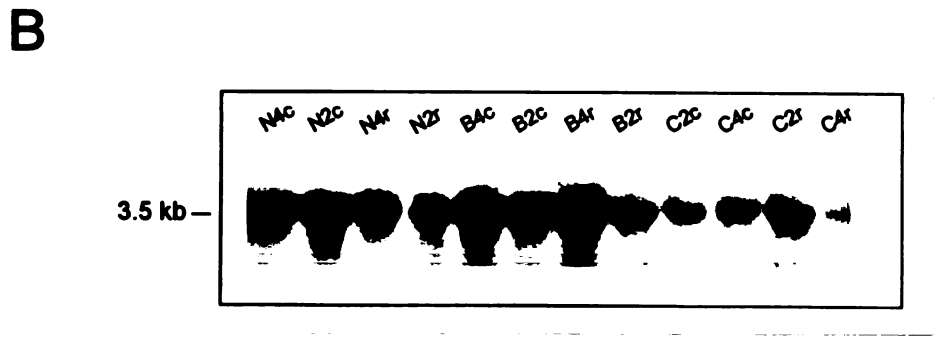
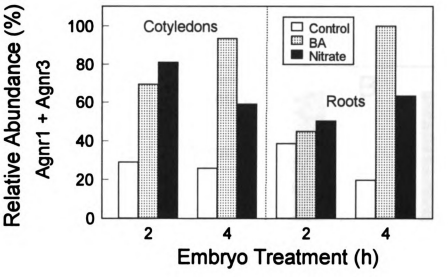


Figure 4.8. Quantitative analysis of Agnr1 and Agnr3 mRNAs in roots and cotyledons of *A. githago*.



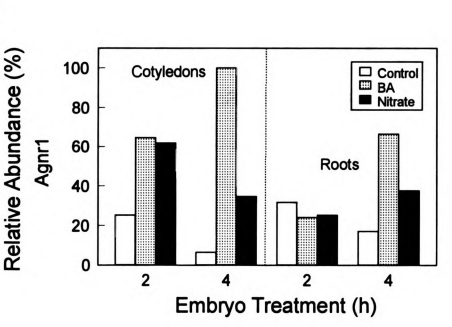


Figure 4.8 cont... Quantitative analysis of Agnr1 and Agnr3 mRNAs in roots and cotyledons of *A. githago*.

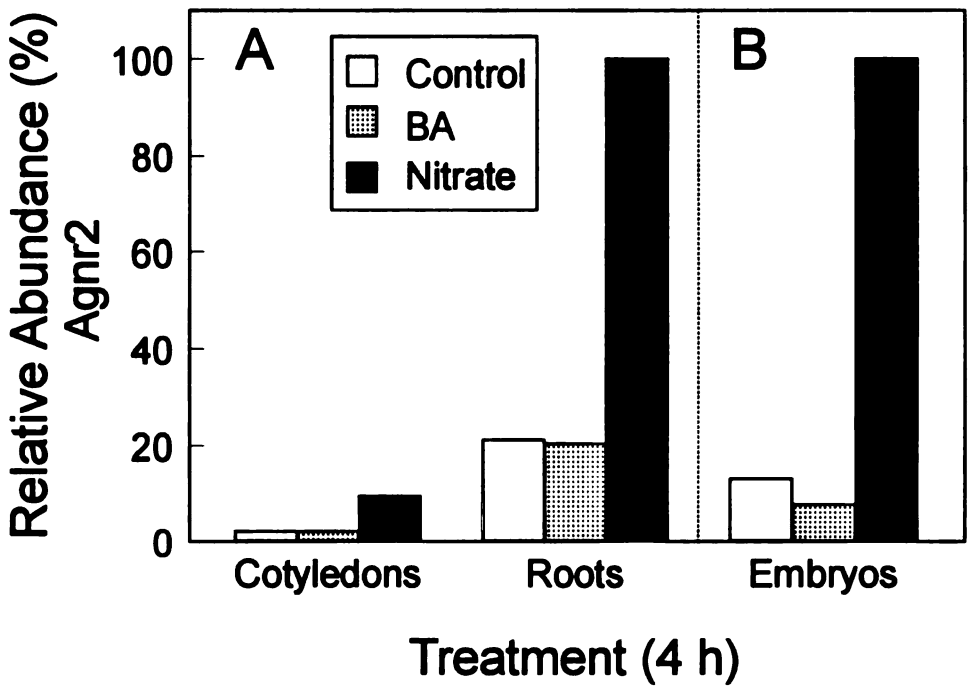


Figure 4.9. Quantitative analysis of Agnr2 mRNA in *A. githago.*

Ten µg of total RNA was used for RT-PCR analysis of Agnr2 in (A) roots and cotyledons, or (B) embryos. The same RNAs used for 4 h treatments of roots and cotyledons in Figure 4.8 were used for this analysis. Percent relative abundance was based on the maximum signal in each of the experiments.

for 12 h before embryo isolation and treatment. The zero time point has very low NR activity levels, but was found to have mRNA levels similar to maximum levels in cytokinin- or nitrate-treated embryos (Figures 4.6, 4.7). The length of imbibition may effect NR mRNAs. Seeds were imbibed for 8, 12, or 16 h, and the NR mRNA levels measured in the isolated embryos. For all three NRs, maximum mRNA levels were found at 12 h imbibition followed by a decrease in mRNA levels at 16 h imbibition (Figure 4.10). NR activity was changed little during this length of imbibition (Figure 4.10).

Transcriptional regulation of NR mRNAs. Nuclear runoff transcription assays were performed to assess whether the increases in NR mRNA levels by cytokinin and nitrate were due, at least in part, to the enhancement of rates of transcription. Based on results from the time course experiments, nuclei were isolated from embryos treated for 4 h in water, 5 μM BA or 50 mM nitrate. Nuclei were also isolated from embryos isolated from seeds imbibed for 8, 12, or 16 h. The transcriptional competence of isolated nuclei was tested and found to be similar for all treatments. An example is shown in Figure 4.11.

Two nuclear runoff transcription experiments showed similar results (Figure 4.12A, B). Although there was an increase in the level of Agnr1 mRNA (Figure 4.12A) or Agnr1 + Agnr3 mRNAs (Figure 4.12B) in cytokinin-treated embryos, there were no increases in the transcription rates of Agnr1 + Agnr3 (Figure 4.12A, B). Nitrate increased the transcription rates of Agnr1 + Agnr3, but not to the same extent as the increase in Agnr1 (Figure 4.12A) or Agnr1 + Agnr3 (Figure 4.12B) mRNA levels. This suggests that the increases

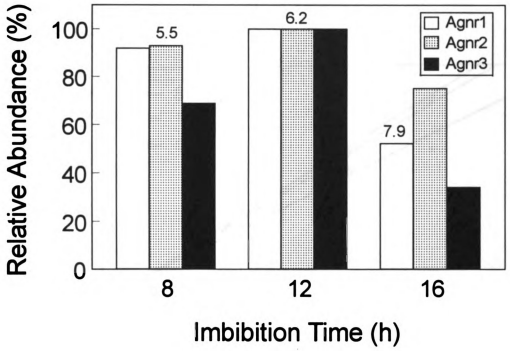


Figure 4.10. Quantitative analysis of NR mRNAs in *A. githago* embryos isolated from seeds imbibed for various times.

Seeds were imbibed for 8, 12, or 16 h at RT. The embryos were then isolated and immediately frozen. NR activities, representing the average of triplicate samples, 4 embryos per sample, are indicated above the bars (nmol NO_2^- formed h^{-1} $embryo^{-1}$) . Percent relative abundance was set at 100% for each mRNA at 12 h and cannot be compared between the three mRNAs. Ten µg total RNA was used for RT-PCR analysis of Agnr2, and 0.5 µg total RNA was used for RT-PCR analysis of Agnr1 and Agnr3. Similar results were obtained in two experiments.

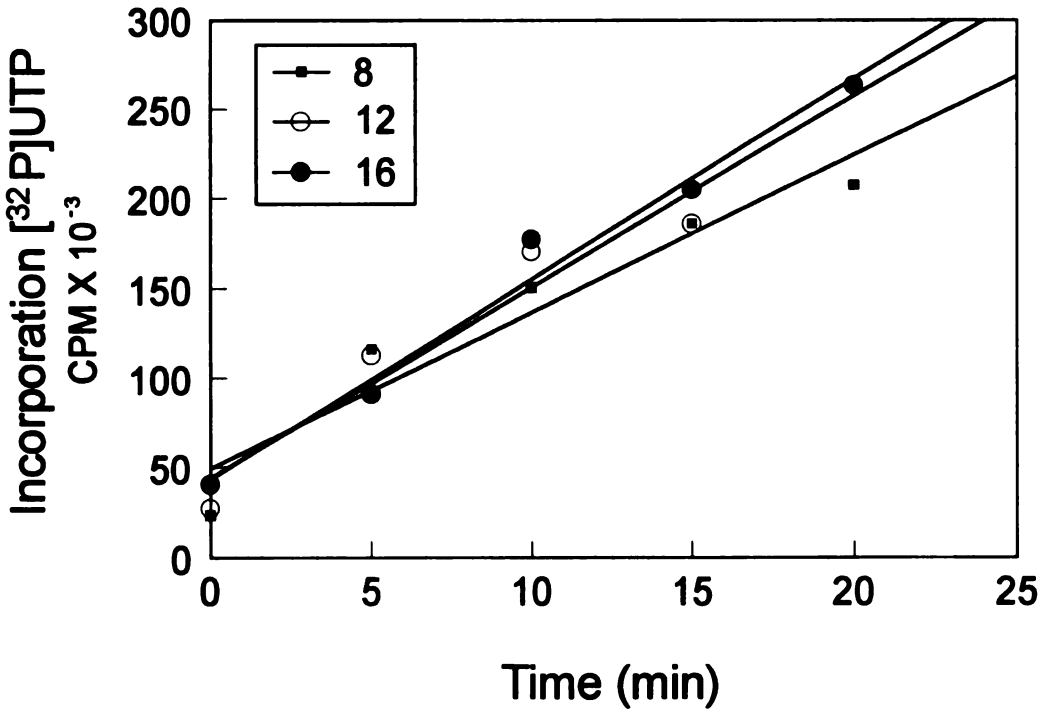


Figure 4.11. Transcriptional competence of isolated nuclei.

Incorporation of [^{32}P]UTP into RNA was measured for nuclei isolated from embryos isolated from seeds imbibed for 8, 12, or 16 h. Each point is the average of duplicate samples using 2 X 10^6 nuclei and 150 µC [^{32}P]UTP in a standard *in vitro* transcription reaction mixture. Lines are drawn by linear regression. Similar results were observed in all experiments with all isolated nuclei.

Figure 4.12. Nuclear runoff transcription analysis.

Embryos were treated for 4 h in water (control), 5 μM BA, or 50 mM nitrate (A, B) or embryos were isolated from seeds imbibed for 8, 12, or 16 h (C). NR activities (nmoles nitrite/h embryo) were measured and relative levels can be compared between (B) and (C). In (B) and (C), 20 μg total RNA was analyzed by Northern blot analysis with the Agnr3-1 RNA probe, and relative levels can be compared between (B) and (C). Because of cross-hybridization, the Agnr3-1 probe detected Agnr1 as well as Agnr3 mRNA. In (A), 0.5 μg total RNA was analyzed by RT-PCR analysis for Agnr1 mRNA levels (similar results were seen by RT-PCR analysis for Agnr3). [^{32}P]-labelled RNAs were synthesized using nuclei isolated from the embryos. The reaction mixtures contained 2 X 10^6 nuclei. One hundred, 50, or 33 million cpm (A, B, and C, respectively) were hybridized to the linearized Agnr1 plasmid DNA (Chapter 3) immobilized to nitrocellulose. Because of differing amounts of radioactive RNA used in the hybridizations, relative transcription levels cannot be compared between experiments. Similar results were observed when linearized Agnr3 plasmid was immobilized to nitrocellulose.

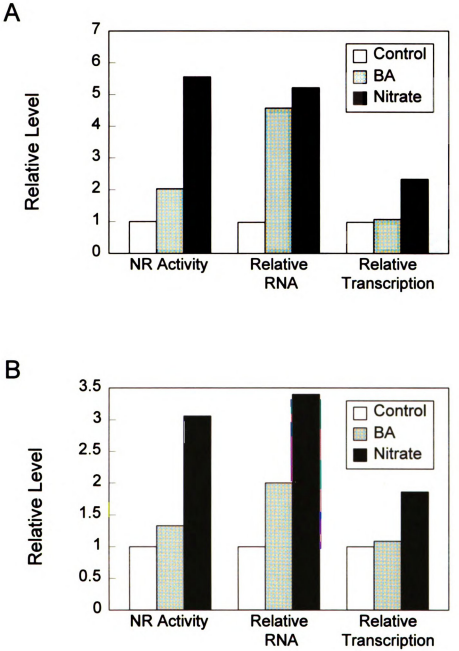


Figure 4.12. Nuclear runoff transcription analysis.

C

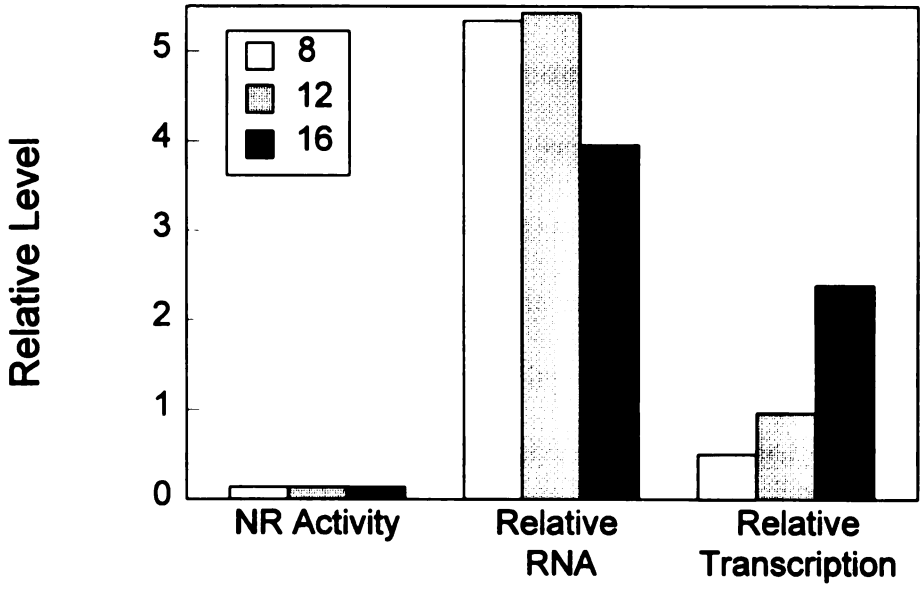


Figure 4.12 cont... Nuclear runoff transcription analysis.

in mRNA levels by nitrate are partly due to increases in the rates of transcription. Insufficient signal was obtained with Agnr2 for quantitation.

Although there were very low levels of enzyme activity in embryos that had not been treated (zero time point), there were high levels of all three NR mRNAs. The mRNA levels varied dependent on the time of seed imbibition (Figure 4.10). Figure 4.12C shows an increase in the transcription rate of Agnr1 + Agnr3 with longer imbibition times.

Nuclear runoff transcription analysis is most reliable when there is a large increase in mRNA levels. The increases in NR mRNA levels observed in these experiments were only a few fold. Therefore, although nuclear runoff analysis may give a general idea of the mechanisms of regulation, further analysis such as promoter analysis is required.

DISCUSSION

The NR genes in *A. githago* were differentially regulated. Agnr2 mRNA levels were much less abundant than Agnr1 or Agnr3 mRNA levels. Agnr2 mRNA levels were not regulated by cytokinin, but were increased by nitrate, predominantly in the roots. Agnr1 and Agnr3 mRNAs were regulated in a similar manner. Their mRNA levels were increased by cytokinin and nitrate in both roots and cotyledons, but the increases were earlier in the cotyledons. Cytokinin and nitrate did not show an additive effect on the mRNA levels even when NR activities were additive. All the mRNA levels were transiently enhanced a maximum of 8 to 10 times, preceding changes in NR activity.

Regulation of NR in *A. githago* appears to be complex and may vary during imbibition, embryo isolation, and embryo treatment, and vary between tissues. All three mRNAs were present at relatively high levels in embryos at time zero, *i.e.* at the end of the imbibition period when embryos were isolated from seeds, but NR activity was very low (Figures 4.6, 4.7). The mRNA levels varied dependent on the imbibition time, while there were little changes in NR activity over the length of imbibition (Figure 4.10). This indicated that in imbibed seeds there were high levels of mRNAs that were either not translated, or if translated, the proteins were inactive. Nuclear runoff experiments indicated that there were changes in the rates of transcription of Agnr1 and Agnr3 during the imbibition period (Figure 4.12C). Within 2 h of embryo isolation and incubation in water, the mRNA levels were markedly decreased while NR activity had increased slightly (Figure 4.7). This indicated that the

isolation of the embryos lead to a significant decrease in the NR mRNA levels. This decrease in mRNA levels might be due to a decrease in the rate of transcription of the mRNAs and/or a decrease in the mRNA stabilities.

Incubation of isolated embryos with cytokinin or nitrate lead to an increase in NR mRNA levels, preceding the increase in NR activity. The yield of RNA was typically 800-1400 μg/g fresh weight for all samples and did not differ with various treatments. Therefore, cytokinin and nitrate could increase the rate of transcription of the mRNAs and/or increase the mRNA stabilities. Nitrate regulated Agnr1 and Agnr3 mRNA levels, at least partly, at the transcriptional level (Figure 4.12A, B). It would appear that nitrate also regulates mRNA levels post-transcriptionally. Transcriptional regulation of Agnr1 and Agnr3 mRNAs by cytokinins was not observed. Because the changes in mRNA levels were relatively small, promoter analysis is necessary to determine with certainty the involvement of transcription in regulation of NR. It is not known whether there is translation of mRNAs carried over from the seeds, or whether there is only translation of newly transcribed mRNAs.

Cytokinin was shown to increase NR activity primarily in the cotyledons (Table 2.1). However, cytokinin increased Agnr1 and Agnr3 mRNA levels in both roots and cotyledons (Figure 4.8). Since Agnr1 and Agnr3 mRNAs were much more abundant than Agnr2 mRNA, they are likely responsible for a major part of the NR activity. It is possible the general response to cytokinin is to increase NR mRNA levels in all tissues, but only in the cotyledons does cytokinin or other factors allow expression of the protein. Agnr2 mRNA levels

were increased by nitrate primarily in the roots, but at a much lower level than Agnr1 or Agnr3 mRNAs.

Because it was not possible to differentiate between all three NR clones, and because the NR mRNA levels were of low abundance, RT-PCR analysis was used to study the regulation of NR mRNAs in *A. githago*. PCR amplification is an exponential process; the extent of amplification (N) is given by the equation $N = N_o(1 + eff)^n$, where N_o is the initial amount of material, eff is the efficiency, and *n* is the number of cycles (Wang *et al.*, 1989). Small differences in efficiency can lead to large differences in the yield of product. Construction of internal standards that contain the same primers as target mRNAs for RT and PCR allow co-amplification of RNAs with the same efficiencies and provide controls for sample-to-sample and tube-to-tube effects. Regardless of the amount of RNA used for RT-PCR analysis, the RNA was always added in a 10 μl volume. Addition of smaller volumes were found to have greater variation in results, probably due to inaccuracies in pipetting.

The RT-PCR analysis used here should allow for quantification of target mRNAs. However, I found that over time the relative signals from the internal standard RNAs increased significantly even though the amounts of the RNAs were the same. Therefore, although relative RNA amounts could be compared between RNA samples within an experiment, absolute RNA amounts could not be used. Similar results have been observed with RT-PCR analysis of other mRNAs (Sergei Mekhedov, personal communication), but the reasons behind these results are unknown. Therefore, quantitation of the NR mRNA levels

allowed only relative comparisons and target mRNAs could not be quantified. However, RNA blot analysis showed similar results to RT-PCR analysis, confirming the results of this analysis.

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CHAPTER 5

The Isolation of Nitrate Reductase Genomic Clones from *Agrostemma githago*

ABSTRACT

Genomic clones corresponding to two of the three nitrate reductase cDNA clones previously identified have been isolated. λAgnr1 contains a 13.5-kb insert that spans the entire coding region of Agnr1 and 5 kb of sequence 5' to the coding region. λAgnr3 contains an 11-kb insert that is lacking 1 kb of the 3' end of the coding region, but contains 7 kb of sequence 5' to the coding region.

λAgnr3 contains three introns at conserved positions of introns in other NR genes. Intron 1, intron 2, and intron 3 are 88 bp, 1000 bp, and 900 bp, respectively. λAgnr1 contains intron 3, which is 800 bp, and must also contain one or more as yet unidentified introns. Southern blot analysis suggests that no other unidentified NR genes exist in *A. githago.*

INTRODUCTION

Plants obtain most of their nitrogen in the form of nitrate. Because the rate-limiting and regulated step of nitrate assimilation is catalyzed by nitrate reductase (NR), much research has focussed on NR and its regulation (for reviews, see Rouzé and Caboche, 1992; Hoff *et al.*, 1994; Crawford, 1995).

NR is a multifunctional enzyme containing one each of a molybdenum cofactor, heme, and FAD prosthetic groups for each subunit of the homodimer. Ten higher-plant NR genes have been cloned, including two from bean (Hoff *et al.*, 1991; Jensen *et al.*, 1994) and two from barley (Miyazaki *et al.*, 1991; Schnorr *et al.*, 1991). Although the sizes and numbers of introns vary, the positions of the introns are conserved. Three introns are present in the molybdenum binding domain. Only in bean has a fourth intron been found, located between the heme and FAD binding domains (Jensen *et al.*, 1994). Except for the bean NR2 gene that contains four introns, higher plant NR genes contain either one, two or three introns, all located in the three conserved positions in the molybdenum binding domain. The goal of this work was to isolate and characterize genomic clones corresponding to the three NR cDNA clones from *A. githago*.

MATERIALS AND METHODS

Plant Material. A. githago seeds were sterilized and imbibed in water for 2 to 3 days in the dark. The seedlings (3 to 4 cm in length) were used for DNA isolation. For some samples, embryos isolated from seeds imbibed for 12 h at room temperature in the dark were used for DNA isolation.

Isolation of genomic DNA. Tissue was frozen and ground in liquid nitrogen. Five ml of lysis buffer per gram of tissue was added and stirred gently at 4°C for 15 min (lysis buffer: 30 mM EDTA, 50 mM Tris-HCl, pH 8.0, 2% sarkosyl, 0.2% ß-mercaptoethanol, 200 µg/ml ethidium bromide). Cesium chloride was added to the samples (0.9 g/ml). The samples were centrifuged at 17,000 X g in an SS-34 rotor (Du Pont Sorvall) for 20 min at 4°C. The DNA supernatent was filtered through cheesecloth, and the density of the DNA solution was adjusted to 1.55 g/ml. Samples were centrifuged according to Maniatis *et al.* (1982), and genomic DNA was isolated from the gradients.

Construction of an A. githago genomic DNA library. Genomic DNA was partially digested with *Sau*3A by varying the enzyme concentration in a 1 h digestion according to Maniatis *et al.* (1982). The DNA was size fractionated on 5 to 20% potassium acetate gradients. Fractions of 0.5 ml were collected from the gradients, and the DNA from each sample was separated by electrophoresis on 0.5% agarose gels. Fractions containing 9 kb to 23 kb DNA fragments were pooled and precipitated with ethanol. This DNA was ligated into the *Bam*HI site of lambda EMBL3 (Stratagene) according to the manufacturer's instruction. *In vitro* encapsidation of the ligated molecules was

achieved at 22°C during 2 h using commercial extracts (GigapackII Plus, Stratagene). Recombinant phage were grown on *E. coli* strain LE392. One million recombinant phages were amplified according to Stratagene's instructions. An amplified library of $\sim 10^{10}$ recombinant clones was obtained.

Screening of A. githago genomic libraries. Both unamplified and amplified libraries were used for screening. Initially, the radiolabelled insert of Agpcr1 (Materials and Methods, Chapter 3) was used for screening the libraries. Hybridizations were at 58°C to 60°C in 6X SSC, 5X Denhardt's solution, 0.5 M sodium phosphate, 0.5% SDS. Final washes were in 0.2X SSC, 0.5% SDS at 58°C to 60°C. Positive phages were purified through further screening to obtain isolated phages. Phage DNA was isolated according to Maniatis *et al.* (1982) and digested with restriction enzymes. ^{32}P-labelled gene-specific oligonucleotides (HK38, HK39, and HK76) were used to identify the genomic clones. Genomic clones corresponding to Agnr1 and Agnr3 were obtained. Both libraries were further screened with radiolabelled insert of Agpcr2 or *in vitro* transcribed antisense strand RNA from Agnr2-1 in an attempt to isolate a genomic clone corresponding to Agnr2. No clone was obtained.

Analysis of genomic clones. Phage DNAs were digested with restriction enzymes to create partial restriction enzyme maps. Some DNA fragments were subcloned into pBluescript SK$^-$ for further analysis and sequencing.

Polymerase chain reaction. HK52 (Materials and Methods, Chapter 4) and either the gene-specific oligonucleotide HK39 or HK76 (Materials and Methods, Chapter 3) were used for PCR amplification from λAgnr1 or λAgnr3

phage DNA. The resulting DNA fragments were ligated into the *Sma*I site of pBluescript SK$^-$. PCR conditions were as previously described (Materials and Methods, Chapter 3). These clones were also used as templates for *in vitro* transcription of sense RNAs used as internal standards for RT-PCR analysis (Chapter 4).

Southern blot analysis. Genomic DNA samples were digested with restriction enzymes and separated by electrophoresis on 0.6% agarose gels. The gels were treated according to Maniatis *et al.* (1982), and the DNA was transferred to Hybond N$^+$ nylon membranes in 10X SSC. Blots were hybridized with the random-primed labelled insert of Agpcr1 (Chapter 3), and hybridization conditions were the same as used for library screening.

DNA Sequencing. The DNA sequences were determined as in Materials and Methods, Chapter 3, except that the DNAs were not completely sequenced on both strands. Most DNA subclones were sequenced only from the terminal ends.

Standard molecular biological methods. Most other methods were performed according to standard molecular biological techniques (Maniatis *et al.*, 1982; Sambrook *et al.*, 1989).

RESULTS

Isolation of NR genomic clones from A. githago. Approximately 1 X 10^6 recombinant clones from both the amplified and unamplified libraries were screened with the radiolabelled insert of Agpcr1 (Materials and Methods, Chapter 3) at intermediate-stringency conditions that would recognize all three NR clones. Five clones corresponding to Agnr1 and two clones corresponding to Agnr3 were isolated, as identified by hybridization with the gene-specific oligonucleotides (HK39 and HK76). λAgnr1 (13.5-kb insert) and λAgnr3 (11-kb insert) were chosen for further analysis. More than 2 X 10^6 recombinant clones from the unamplified library and 3 X 10^6 recombinants from the amplified library were screened with the insert of Agpcr2 (Materials and Methods, Chapter 3) or the *in vitro* transcribed antisense RNA of Agnr2-1 (Materials and Methods, Chapter 3). No clones corresponding to Agnr2 were found.

Analysis of A. githago NR genomic clones. Restriction enzyme maps were constructed for λAgnr1 and λAgnr3 (Figure 5.1). The two restriction maps were aligned at the common *Sma*I site to show relative positioning between the two maps. Several restriction enzyme fragments were subcloned into pBluescript SK^- and sequenced. λAgnr1 contains the entire coding sequence of Agnr1. λAgnr3 is missing 1 kb of the 3'-terminal end of the coding region of Agnr3. Based on sequence homology to tobacco NR (Vaucheret *et al.*, 1989), λAgnr1 and λAgnr3 contain ~5 kb and ~7 kb, respectively, of sequence upstream from the start of the coding region.

By comparison of the Agnr3 cDNA sequence to the λAgnr3 sequence,

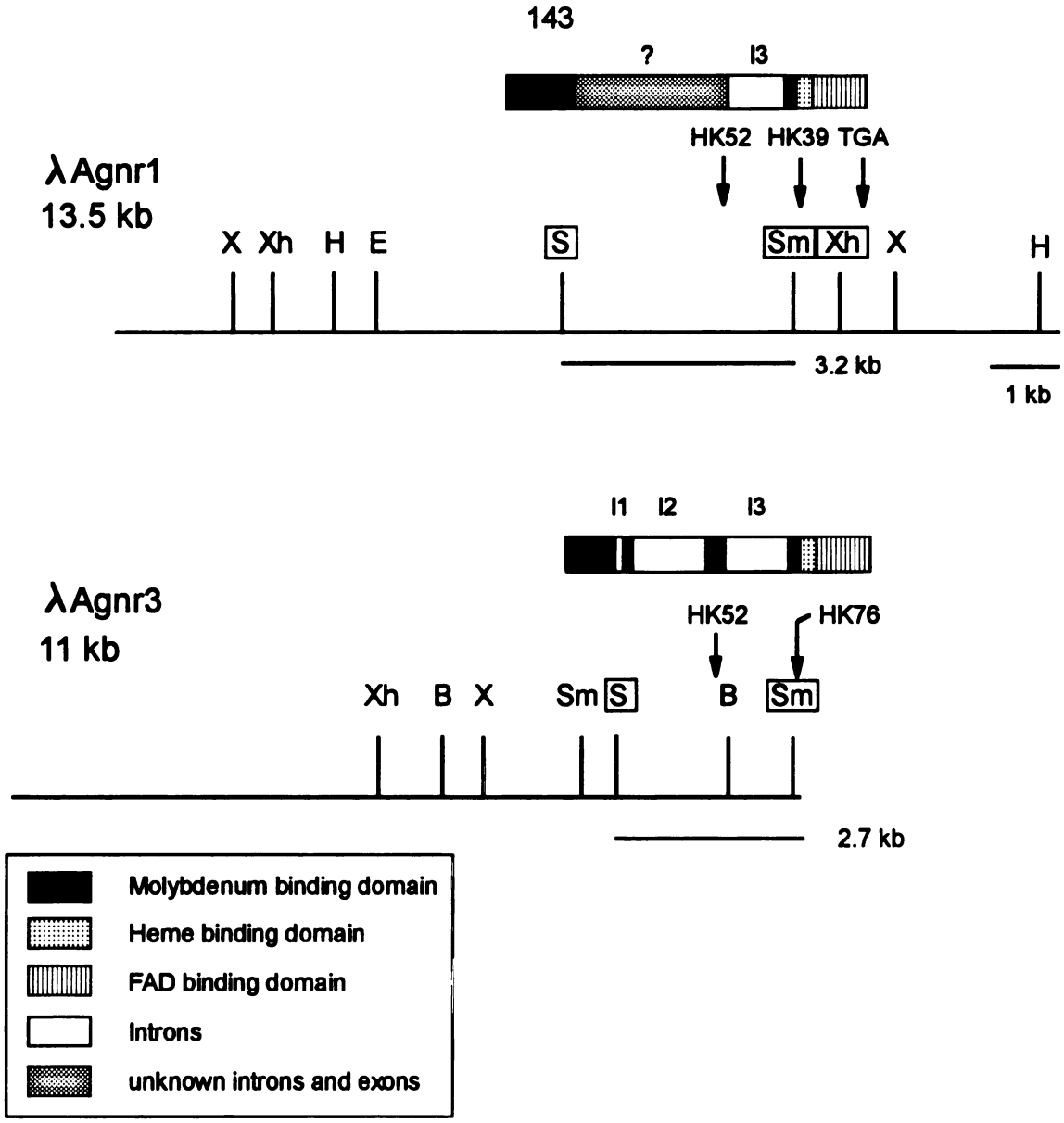

Figure 5.1. Restriction enzyme maps of the 13.5-kb insert of λAgnr1 and the 11-kb insert of λAgnr3.

Restriction enzyme maps of the two genomic clones are shown. Enzymes that are boxed correspond to the same enzymes indicated in Figure 5.2. The positions of the 5' oligonucleotide (HK52) and the gene-specific oligonucleotides (HK39 and HK76) used for PCR amplification are indicated. The organizations of the genes are indicated, with the position of the start of the 5' coding region estimated. The positions of the identified introns are indicated as I1, I2, and I3. Restriction enzyme sites are as follows: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; S, *Sal*I, Sm, *Sma*I; X, *Xba*I, Xh, *Xho*I. Not all restriction enzyme sites are shown. The position of the stop codon in λ Agnr1 is indicated, but is not present in λAgnr3. The 3.2-kb *Sal*I/*Sma*I and the 2.7-kb *Sal*I fragments are referred to in the results.

three introns were identified in λAgnr3 (Figures 5.1, 5.2). All three introns were located in conserved positions for introns in NR genes (Rouzé and Caboche, 1992). Intron 1 was 88 bp. Based on PCR analysis, intron 3 was estimated to be 900 bp (see *PCR analysis* below). The *Sal*I DNA fragment in λAgnr3 (Figure 5.1) is 2.7 kb. The distance between the *Sal*I and *Sma*I restriction enzyme sites in the cDNA clone is 525 bp (Figure 5.2). Therefore, if intron 1 is 88 bp, intron 3 is 900 bp, and the distance from the *Sma*I site to the end of λAgnr3 is 190 bp (Figure 5.1), intron 2 must be 1 kb [*i.e.* (2.7 kb) - (0.525 + 0.088 + 0.9 + 0.19 kb) = 1 kb].

For λAgnr1, intron 3 was estimated by PCR analysis to be 800 bp (see *PCR analysis* below). The distance between the *Sal*I and *Sma*I restriction enzyme sites in λAgnr1 is 3.2 kb (Figure 5.1). The distance between these sites in the cDNA clone is 525 bp (Figure 5.2). Therefore, if intron 3 is 800 bp, there must be another ~1.9 kb of intron sequence between the *Sal*I and *Sma*I sites [*i.e.* (3.2 kb) - (0.525 + 0.8) = 1.9 kb].

Analysis of the nucleotide sequences of the A. githago genomic clones. Partial sequence information was obtained from subclones of the genomic clones. The conserved sequences at the 5′ (G:GT) and 3′ (AG:) splice junctions of higher plants (Brown, 1986) were present at the intron boundaries in *A. githago* genes. Figure 5.3 shows the conservation between the *A. githago* intron splice junctions and the plant consensus splice junction sequences. The locations of the introns within the cDNA sequences (Figure 3.4) and the protein sequences (Figure 3.5) are at conserved locations for NR introns. The average

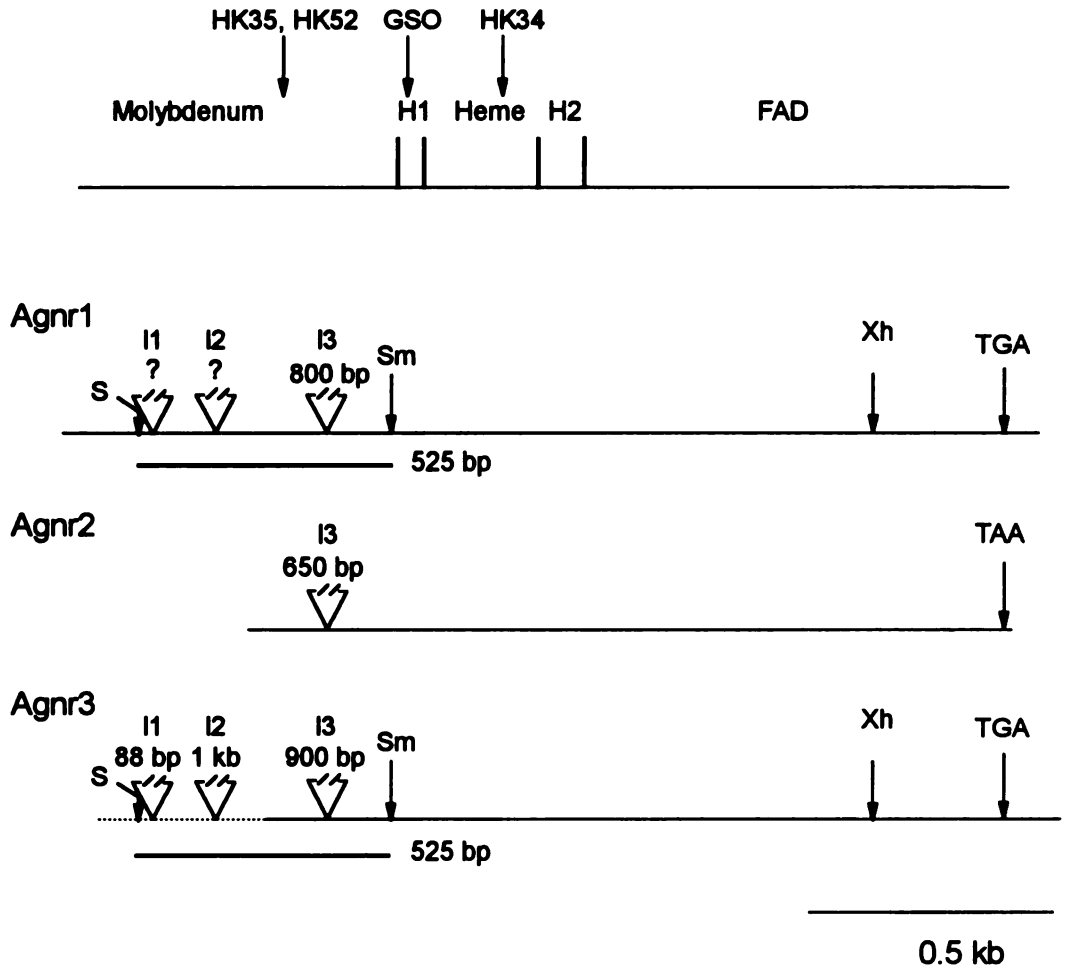

Figure 5.2. Schematic representation of the *A. githago* NR cDNA clones, Agnr1, Agnr2, and Agnr3.

The three *A. githago* NR cDNA clones are aligned. Agnr3 is extended with a dashed line to represent information obtained from λ Agnr3. The positions of the binding domains and the hinge regions (H1, H2) are indicated above. The positions of HK52 and the gene-specific oligonucleotides (GSO) used for PCR amplification from lambda DNA are indicated. The positions of the degenerate oligonucleotides HK35 and HK34 used for initial PCR amplification of NR clones are indicated. Restriction enzyme sites correspond to sites in the genomic clones in Figure 5.1. I1, I2, and I3 are the three introns. I3 is present in all three NR genes. I1 and/or I2 may be present in λ Agnr1, and both I1 and I2 are present in λ Agnr3. The 525-bp *SalI*/*SmaI* fragment is referred to in the results. The sequences of the oligonucleotides are shown in Chapter 3 (HK35, HK34, GSO) and Chapter 4 (HK52).

	5' SPLICE JUNCTION	3' SPLICE JUNCTION
Agnr3		
Intron 1	AAG: GTATAA	TATTTTAATTTGCAG: C
Intron 2	CTG: GTTTGT	?
Intron 3	ATG: GTATGT	TCTAAATGTATGCAG: G
Agnr1		
Intron 3	ATG: GTACGT	TAATCTGTTATGTAG: G
Consensus	$^{C}_{A}$AG: GTAAGT	TTT$_{R}^{T}$TT $_{RRRR}^{TTTT}$TGCAG: G

Figure 5.3. The 5' and 3' splice junction sequences from introns in *A. githago* NR genes.

The 5' and 3' splice junction sequences of the *A. githago* NR genes are aligned on the basis of the conserved GT and AG dinucleotides, respectively. The plant splice junction consensus sequences are also indicated (Brown, 1986). R=G or A.

A+T content of the coding regions of the three *A. githago* NR clones was 53%. In λAgnr3, the A+T content of intron 1, intron 2, and intron 3 was 89%, 72% (over 127 bp), and 65% (over 560 bp), respectively. In λAgnr1, the A+T content of the 409 bp sequenced from I3 was 67%. High A+T content in introns is a general feature of plant genes and may play an important role in splicing of plant introns (Montero *et al.*, 1990; Wiebauer *et al.*, 1988).

PCR analysis. Based on conserved intron positions in other NR genes, it was possible that PCR amplification with HK52 and the gene-specific olionucleotides would span an intron. The distance between HK52 and the gene-specific oligonucleotides in cDNA clones is 286 bp (Figure 5.2). PCR amplification of λAgnr1 and λAgnr3 lambda phage DNA with HK52 and the gene-specific oligonucleotides HK39 and HK76 (Figure 5.1) indicated that λAgnr1 and λAgnr3 contained an intron of 800 bp and 900 bp, respectively. Sequencing of DNA subclones showed that this intron corresponded to the conserved position of the third intron in other NR genes. PCR amplification of lambda phage DNA isolated from the amplified genomic library also showed the presence of this intron. The PCR fragments were ligated into the *Sma*I site of pBluescript SK$^-$ and further analyzed by restriction enzyme digestions and sequencing. HK52 and the gene-specific oligonucleotide HK38 were used in PCR amplification to analyze the presence of a genomic clone corresponding to Agnr2. PCR amplification of lambda phage DNA isolated from either the amplified or unampified genomic libraries indicated that clones for Agnr2 were not present in the libraries. PCR amplification of genomic DNA indicated the

presence of an intron estimated to be 650 bp between HK52 and HK38, likely corresponding to I3 (data not shown; Figure 5.2).

Analysis of A. githago genomic DNA. Total genomic DNA from *A. githago* was digested with restriction enzymes and subjected to Southern blot analysis. Figure 5.4 shows a Southern blot hybridized with the random-primed labelled insert of Agpcr1 (Materials and Methods, Chapter 3). One to three hybridization bands are seen with each digest. This suggests that there are probably no other unidentified NR genes in *A. githago.*

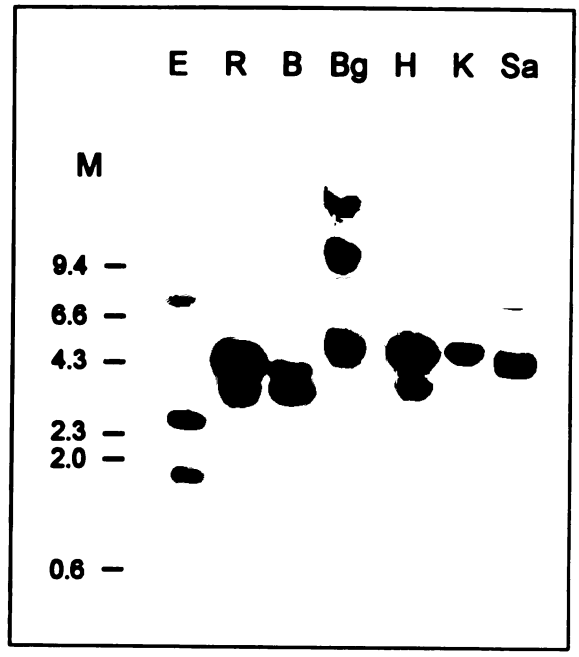

Figure 5.4. Southern blot analysis of *A. githago* genomic DNA.

Genomic DNA (approximately 12 µg) was digested with the following restriction enzymes: *Eco*RI (E), *Eco*RV (R), *Bam*HI (B), *Bgl*II (Bg), *Hind*III (H), *Kpn*I (K), and *Sac*I (Sa). DNA was hybridized with the 495-bp insert of Agpcr1 (Chapter 3). M=molecular weight markers, bp.

DISCUSSION

Genomic clones corresponding to Agnr1 and Agnr3 have been isolated from *A. githago*, but no clones for Agnr2 were found in the genomic libraries. The organization of the genes appears to be similar to other NR genes. λAgnr3 contains three introns at the conserved positions of other NR genes. λAgnr1 contains intron 3 and likely also contains intron 1 and/or intron 2. Although no genomic clone corresponding to Agnr2 was found, PCR amplification indicated that a 650-bp intron lies between HK52 and HK38, likely corresponding to intron 3. Of the NR genes sequenced, the size of intron 1 varies from 74 bp in tomato (Daniel-Vedele *et al.*, 1989) to 854 bp in bean (Jensen *et al.*, 1994), but is not present in one of the two barley genes (Schnorr *et al.*, 1991). Intron 2 varies from 96 bp in *Arabidopsis thaliana* (Wilkinson and Crawford, 1991) to 1954 bp in rice (Choi *et al.*, 1989), and is present in all higher plant genes isolated to date. Intron 3 varies from 85 bp in rice (Choi *et al.*, 1989) to 1646 bp in bean (Jensen *et al.*, 1994), but is not present in either of the barley genes (Schnorr *et al.*, 1991; Miyazaki *et al.*, 1991). All three intron sizes in λAgnr3 and intron 3 of λAgnr1 lie within these ranges. It is not known whether there are other introns present.

Because NR has three defined functional domains, it is a good example of evolution by gene fusion (Rouzé and Caboche, 1992). One hypothesis claims that this results from exon shuffling. Exons encoded functional domains, and the exons rather than the whole genes were the evolutionary units. Exon shuffling among various sites in the genome would form functionally complex

proteins (Campbell and Kinghorn, 1990). The three functional domains of NR share homology with domains of other eukaryotic proteins utilizing these cofactors (Figure 3.1). This observation supports a role for exon shuffling as a mechanism in the evolution of NR.

The conservation of intron positioning in NR has been used for (Campbell and Kinghorn, 1990) and against (Rouzé and Caboche, 1992) the hypothesis of exon shuffling. If a multifunctional enzyme arose by exon shuffling, the introns might be expected to lie between the functional domains. Although the positions of the first three introns in NR are conserved, they all lie within the molybdenum binding domain and not between binding domains. This might suggest that NR did not evolve through exon shuffling.

In fungi, the *Aspergillus nidulans* NR gene contains 6 introns (Johnstone *et al.,* 1990) while *Neurospora crassa* contains only the last one (Okamoto *et al.*, 1991). Five of these introns are in the molybdenum binding domain and the sixth intron is located in the heme binding domain, but none correspond to the introns found in higher plants. In algae, the *Volvox carteri* NR gene contains 10 introns, only two of which correspond to higher-plant introns; one separates the heme and FAD binding domains (Gruber *et al.*, 1992). Campbell and Kinghorn (1990) suggest that the primordial gene for NR must have contained all of these introns plus additional ones for dividing the functional domains. Many of the introns would have been lost during evolution of the NR genes. A fourth intron in higher plants, thus far only found in bean (Jensen *et al.*, 1994), is the first example of an intron which divides the functional domains of higher-plant NR.

Thus, exon shuffling may have been fundamental in the evolution of the NR gene.

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CHAPTER 6

Summary and Discussion

SUMMARY

The regulation of NR by cytokinin and nitrate in *A. githago* embryos was examined. Cytokinin and nitrate increased both NR activity (4 to 6 times control) and Agnr1 and Agnr3 mRNA levels (8 to 10 times control) transiently in a time-dependent manner, with the increases in mRNA levels preceding the increases in NR activity (summarized in Figure 6.1). Nitrate increased both NR activity (peak at 6-12 h) and NR mRNA levels (peak at 2 to 4 h) sooner than did cytokinin (NR activity peak at 12 to 18 h; mRNA levels peak at 4 to 6 h). Nitrate-regulated NR activity and NR mRNA levels decreased sooner than did cytokinin-regulated NR activity or NR mRNA levels. Cytokinin and nitrate effects on NR activity were often additive, but sometimes an additive effect was not observed, particularly after 12 h of treatment. Cytokinin and nitrate effects on Agnr1 and Agnr3 mRNA levels were not additive. Ammonia had no effect on NR activity, either by itself or in comination with cytokinin or nitrate. Ethylene alone increased NR activity, but in combination with nitrate had no effect and in combination with cytokinin decreased NR activity.

There were low levels of NR activity in embryos during imbibition, while there were high levels of the three NR mRNAs. Within 2 h of embryo isolation

Figure 6.1. Summary of cytokinin and nitrate regulation of NR activity and NR mRNA levels.

Relative levels of NR activity and Agnr1 or Agnr2 mRNAs are summarized. Basal levels in the controls were set at 1. Agnr2 mRNA levels are regulated by nitrate but not cytokinin. Agnr3 mRNA levels were similar to Agnr1 mRNA levels.

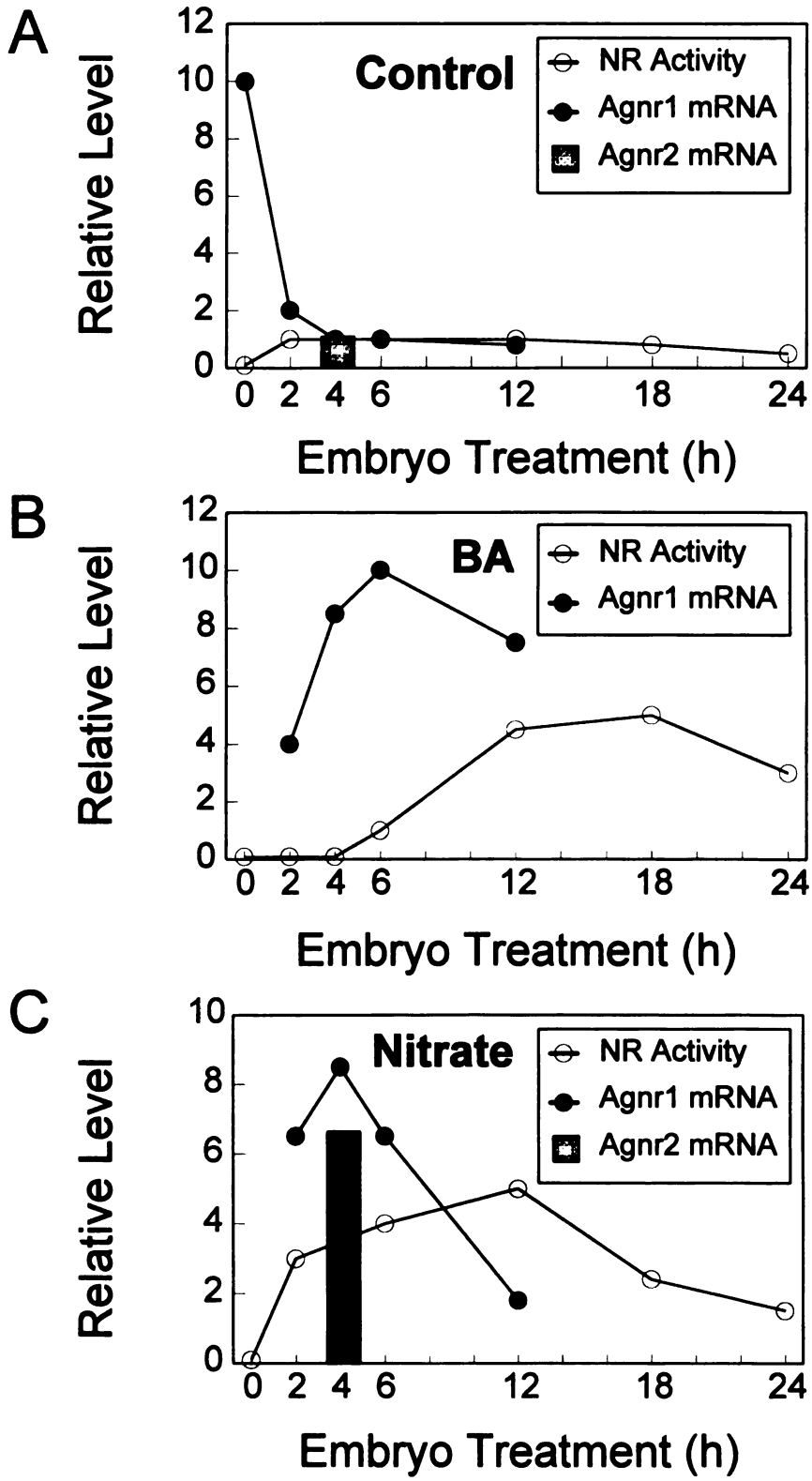

Figure 6.1. Summary of cytokinin and nitrate regulation of NR activity and NR mRNA levels.

and incubation on water, the high levels of the NR mRNAs had decreased dramatically, while there was little change in NR activity. Embryos incubated in water showed a transient increase of the low level of NR activity, while NR mRNA levels decreased after 2 h of treatment. NR activity in embryos isolated from seeds imbibed for various times was temperature dependent, as was the NR activity in embryos incubated on water.

Cytokinin increased NR activity primarily in the cotyledons, but increased Agnr1 and Agnr3 mRNA levels in both roots and cotyledons. Nitrate increased NR activity and Agnr1 and Agnr3 mRNA levels in both roots and cotyledons. The increases in NR mRNA levels by both cytokinin and nitrate were observed sooner in the cotyledons than in the roots. Nitrate also increased Agnr2 mRNA levels, but primarily in the roots. Agnr2 mRNA levels were much less abundant than either Agnr1 or Agnr3 mRNA levels. The decrease in nitrate-regulated NR activity and NR mRNAs over time was initially due to loss of NR activity and NR mRNA levels in the cotyledons.

Three NR cDNA clones all hybridized to a 3.5-kb mRNA and were highly homologous to each other at both the nucleotide and amino acid levels. Agnr1 and Agnr3 were at least in part transcriptionally regulated by nitrate but not by cytokinin. The nitrate regulation of Agnr1 and Agnr3 likely also involves post-transcriptional regulation. There was also transcriptional regulation of these genes during imbibition. It is not known how Agnr2 is regulated.

Genomic clones containing Agnr1 and Agnr3 were isolated, and both appear to contain the promoter sequences. Three introns, corresponding to

conserved intron positions for NR genes, were identified in λAgnr3. Intron 3 was identified in λAgnr1; one or more other introns were also present, but their positions were not identified.

Physiological significance of cytokinin on NR

Because the study of cytokinin regulation of NR in *A. githago* has involved the application of cytokinins, the question often arises whether endogenous cytokinins regulate NR. While this has not been determined, a number of observations suggest that endogenous cytokinins might play a role in the regulation of NR.

In a number of plants, cytokinins synthesized in the embryonic axis are translocated to the cotyledons where they regulate amyolytic or proteolytic enzymes (for a review, see Letham, 1994). Cytokinin increased NR activity in the cotyledons (Chapter 3) and increased NR mRNA levels in both roots and cotyledons (Chapter 4). It has also been reported that there are endogenous cytokinins in *A. githago* embryos whose levels vary during imbibition and germination (Borriss, 1977, and references within). Therefore, endogenous cytokinins are present, and their concentrations vary in *A. githago* embryos. It is possible that these cytokinins play a role in regulating NR as other enzymes have been shown to be regulated by endogenous cytokinins in the cotyledons.

Banowetz (1992) showed that cytokinin regulation of NR activity in wheat was correlated with endogenous cytokinin levels. When endogenous cytokinin levels were low, exogenous cytokinins increased NR activity to a

greater extent than when endogenous cytokinin levels were high. A similar situation in *A. githago* could explain some results. Dependent on the growth conditions of plants, seeds may contain or have the ability to synthesize differing amounts of cytokinins and/or contain differing amounts of nitrate. These factors could affect the levels of NR activity in embryos incubated in water as well as the ability of the embryos to increase NR activity. Both of these factors were found to vary with different seed lots and with the age of the seeds. Schmerder and Borriss (1986) showed that the changes in NR activity in *A. githago* embryos incubated on water were likely due to endogenous cytokinins and not endogenous nitrate. Therefore, endogenous cytokinins may regulate NR activity in water-treated embryos and applied cytokinins increase the response.

Cytokinins increase NR activity in *A. githago* embryos only during a limited phase of seed germination and growth, whereas nitrate increases NR activity in seedlings that are at least 5 d old (Kende *et al.*, 1974). This suggests a more specific role for cytokinins in regulating NR activity during seed germination and early plant development. Because of the increased growth during seed imbibition and germination, a higher demand on protein synthesis can be expected. Cytokinins and nitrate may regulate NR to help meet this demand. Perhaps cytokinins regulate NR only during a limited time because this is a crucial developmental period, and/or cytokinins already present to regulate other cell functions such as cell division are utilized to regulate NR as well.

DISCUSSION

This project has accomplished many of its initially established goals. However, the system lends itself to further analysis. The following is a brief discussion of future experiments that would help in the understanding of the mechanism(s) by which cytokinin regulates gene expression.

The first objective in continuing this project would be further analysis of the genomic clones. The complete sequences of the Agnr1 and Agnr3 genes should be determined, including promoter and 3'-untranslated sequences. Since nuclear runoff trancription analysis showed post-transcriptional regulation of NR by cytokinin, the 3'-untranslated sequences should be examined for their ability to regulate reporter gene expression in response to cytokinin in transgenic plants such as *Arabidopsis thaliana*. If cytokinin regulation of the reporter gene is controlled by the 3'-untranslated sequence, further analysis could determine *cis* and *trans* elements involved with this regulation.

Although nuclear runoff transcription analysis did not show transcriptional regulation of NR by cytokinins, it cannot exclude that some transcriptional regulation does occur. Promoter analysis of Agnr1 and Agnr3 would determine if there is any transcriptional regulation by cytokinins. If transcriptional regulation is present, the promoter could be used to isolate *cis* and *trans* factors involved with that regulation. The use of reporter genes for the analysis of transcriptional regulation of nitrate reductase has not been successful in transient expression assays in protoplasts (*i.e.* Vaucheret *et al.*, 1992). Therefore, stable transformation methods should be used, *i.e.*

transformations into the *Arabidopsis thaliana* mutant carrying mutations in both NR genes (Wilkinson and Crawford, 1993).

There have been no published results on the mRNA localization of NR. *In situ* hybridizations could give information on the localization of the NR mRNAs in *A. githago* embryos. These results might also provide a better understanding of the physiological significance of cytokinin regulation of NR. *In situ* hybridization experiments in *A. githago* are being done (Wilfried Peters, personal communication). It would be interesting to see if a reporter gene linked to the 3'-untranslated sequence of NR is not only cytokinin regulated, but tissue specifically regulated as well.

The large differences between NR mRNA levels and NR activity in isolated embryos not incubated on water suggest that there may be regulation at the protein level. The determination of NR protein levels in *A. githago* embryos would give a better understanding of cytokinin and nitrate regulation of NR. Several monoclonal and polyclonal antibodies to NR have been raised, but all have drawbacks. The most likely candidate for analysis in *A. githago* is a spinach monoclonal antibody (Sueyoshi *et al.*, 1988). The *A. githago* NR genes show the most sequence identity to the spinach sequence (Prosser and Lazarus, 1990) and, therefore, the spinach antibody may most likely cross-react with *A. githago* NR. This antibody will also cross-react with NR proteins on Westerns, unlike some of the other antibodies available. However, all of the NR antibodies that cross-react with proteins on Westerns require at least partial protein purification or many non-NR proteins will also cross-react (Wilbur

Campbell, personal communication).

Protein kinases and phosphatases have been shown to be involved in cytokinin regulation of gene expression, but with differing effects. Dominov *et al.* (1992) showed that the enhancement of *pLS216* mRNA levels by cytokinin is blocked by the protein kinase inhibitor staurosporine and stimulated by the protein phosphatase inhibitor okadaic acid (a specific inhibitor of type 1 and type2A protein phosphatases). In contrast, Crowell (1994) showed that the enhancement of *cim1* mRNA level is stimulated by staurosporine and blocked by okadaic acid. Cytokinin enhancement of *SrEnod2* mRNA levels was inhibited by both staurosporine and okadaic acid (David Silver, personal communication). *pSL216* is at least partially transcriptionally regulated by cytokinin (Dominov *et al.*, 1992), *SrEnod2* is post-transcriptionally regulated by cytokinin (David Silver, personal communication), and the regulation of *cim1* is unknown. Thus, it appears that cytokinin regulation of gene expression involves protein kinases and phosphatases but that different mechanisms underly these processes. Inactivation of NR in the dark has been shown to involve a rapid and reversible protein phosphorylation in spinach (Huber et al., 1992), *A. thaliana* (LaBrie and Crawford, 1994), and maize (Huber *et al.*, 1994). In spinach, a calcium-dependent protein kinase required for activation of NR has been partially purified (Bachmann *et al.*, 1995), and okadaic acid prevented the dephosphorylation and inactivation of NR (Huber *et al.*, 1992). Therefore, inhibitor studies could reveal the involvement of protein kinases and/or phosphatases in cytokinin regulation of NR. Cycloheximide, an inhibitor of protein synthesis, could also be used to

determine if the cytokinin-regulated increase in NR mRNA levels requires protein synthesis.

Concluding Remarks

Cytokinin studies fell behind studies of other plant hormones for many years. Recently, much information has been gathered on the effects of cytokinin in plants at the molecular level. However, many cytokinin studies examined gene expression over long periods of time (*e.g.* days). Thus, these cytokinin-stimulated genes are not likely to represent the primary molecular responses to cytokinins but rather the results of a series of signalling activities. The goal of this project was to examine the regulation of NR by cytokinins in *A. githago*, a fast and specific response. The ultimate goal is to better understand the mechanism(s) by which cytokinins regulate gene expression. This work will contribute to the knowledge of cytokinin responses and lay a foundation for future studies on cytokinin signal transduction. Future studies examining cytokinin regulation of gene expression of different genes in different plants are likely to reveal many of the molecular events that mediate the effects of cytokinin on gene expression and, thus, on the growth and development of plants.

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APPENDIX

APPENDIX

Nomenclature

OLIGONUCLEOTIDES

HK30 5′-AACCARCARTTRTTCATCAT-3′ : 20-mer; redundancy = 8; antisense; in the molybdenum binding domain; 562-581; used as an internal probe in initial screening of NR clones.

HK34 5′-TCRAAYTCYTCNGTRCARTC-3′ : 20-mer; redundancy = 128; antisense; in the heme binding domain; 910-929; used in PCR for obtaining NR clones.

HK35 5′-TGGTGYTGGTGYTTYTGG-3′ : 18-mer; redundancy = 8; sense; in the molybdenum binding domain; 433-450; used in PCR for obtaining NR clones.

HK38 5′-ACGCGTGGCTAACTGCGTCC-3′ : 20-mer; antisense; in the hinge 1 region specific to Agnr2; 705-724; used to identify clones, and in RT-PCR analysis.

HK39 5′-GGTTATTGCCACCTGGCACA-3′ : 20-mer; antisense; in the hinge 1 region specific to Agnr1; 705-724; used to identify clones, and in RT-PCR analysis.

HK40 5′-TGTGTTCGAGCATCCGAC-3′ : 18-mer; sense; in the molybdenum binding domain, complementary to Agnr1, Agnr2, and Agnr3; 630-647; used as an internal probe in RT-PCR analysis.

HK52 5′-TGGTGTTTTTGGTCATTAGAAGT-3′ : 23-mer; sense; in the molybdenum binding domain; complementary to Agnr1, Agnr 2, and Agnr3; 439-461; used for PCR amplification from genomic clones.

HK76 5′-AGTTATTGCCACCCGGCACT-3′ : 20-mer; antisense; in the hinge 1 region specific to Agnr3; 705-724; used to identify clones, and in RT-PCR analysis.

PCR Clones

Agpcr1 a 497-bp clone in the *Eco*RI site of pCRII generated by PCR amplification with HK35 and HK34; corresponds to Agnr1; encodes part of the molybdenum binding domain, the hinge 1 region, and part of the heme binding domain; 433-929.

Agpcr2 a 497-bp clone in the *Eco*RI site of pCRII generated by PCR amplification with HK35 and HK34; corresponds to Agnr2; encodes part of the molybdenum binding domain, the hinge 1 region, and part of the heme binding domain; 433-929.

Agpcr3 a 497-bp clone in the *Eco*RI site of pCRII generated by PCR amplification with HK35 and HK34; corresponds to Agnr3; encodes part of the molybdenum binding domain, the hinge 1 region, and part of the heme binding domain; 433-929.

NR cDNA Clones

Agnr1 *i.e.* Agnr125; a 2046-bp clone the *Eco*RI site of pBluescript SK^-; encodes part of the molybdenum binding domain, the hinge 1 region, the heme binding domain, the hinge 2 region, the FAD binding domain, and 156 bp of 3′ untranslated sequence.

Agnr2 *i.e.*Agnr223; a 1502-bp clone in the *Eco*RI site of pBluescript SK^-; encodes part of the molybdenum binding domain, the hinge 1 region, the heme binding domain, the hinge 2 region, the FAD binding domain, and 36 bp of 3′ untranslated sequence.

Agnr3 *i.e.* Agnr321; a 1729-bp clone in the *Eco*RI site of pBluescript SK^-; encodes part of the molybdenum binding domain, the hinge 1 region, the heme binding domain, the hinge 2 region, the FAD binding domain, and 234 bp of 3′ untranslated sequence.