PHYSIOLOGIA PLANTARUM 119: 489–493. 2003
Printed in Denmark – all rights reserved

The effect of marine bioactive substances (N PRO) and exogenous cytokinins on nitrate reductase activity in *Arabidopsis thaliana*

Nathalie Durand[a,b], Xavier Briand[a] and Christian Meyer[b,*]

[a]*SECMABIO, BP 65, 22260 Pontrieux,*
[b]*Unité de Nutrition Azotée des Plantes (NAP), INRA Versailles-Grignon, 78026 Versailles Cedex, France*
**Corresponding author, e-mail: meyer@versailles.inra.fr*

Received 13 March 2003; revised 28 April 2003

We investigated the effect of exogenous cytokinins and marine bioactive substances containing seaweed extracts (marketed by the ROULLIER Group under the trade name N PRO^{TM}) on nitrate reductase activity in *Arabidopsis*. Cytokinins, applied either directly in the growth medium or as a foliar spray, did not significantly influence nitrate reductase activity in extracts from in vitro grown *Arabidopsis* plants. Conversely, *Arabidopsis* grown in the presence of or sprayed with N PRO had increased nitrate reductase activity. This stimulatory effect of N PRO was even higher when the plants were grown on low nitrate concentration, suggesting that these marine bioactive substances may be beneficial for plant growth in adverse nutritional conditions.

Introduction

Marine bioactive substances extracted from seaweeds have now been used for several decades to enhance plant growth and productivity. Application of these substances is often performed by foliar spraying and it has been reported that they stimulate, among other processes, seed germination and resistance to various pathogens, and that they induce higher yields and a better nutrient use efficiency (for review see Mooney and van Staden 1986, Jolivet et al. 1991). The stimulatory effect of the marine bioactive substances has often been ascribed to the presence of biologically active cytokinins. But many other organic and inorganic molecules have been found in crude algal extracts used for foliar spray (Verkleij 1992). It has also been reported that the application of these extracts can stimulate nitrogen uptake and metabolism in the treated plants (Jolivet et al. 1991). Nitrate reductase (NR, EC 1.6.6.1) has been shown to be a major controlling step of the plant N-metabolism (Crawford 1995). Indeed NR, which catalyses the first committed step of the nitrate assimilation pathway, i.e. the reduction of nitrate into nitrite, is the subject of many different regulations.

First, it has been shown that the expression of the NR gene is highly regulated at the transcriptional level by many endogenous and environmental factors such as hormones, light, nitrogen source and carbohydrates (for a review see Lillo 1994, Crawford 1995, Meyer and Stitt 2001). For instance nitrate, the NR substrate, activates the transcription of the NR gene (Campbell 1999, Meyer and Stitt 2001). In addition to nitrate, light is an important signal for NR regulation (Lillo 1994). As for other genes, transcriptional regulations probably determine the long-term fluctuations in NR protein level. On the other hand, a reversible post-translational regulation of the NR protein involving protein phosphorylation allows a short-term modulation of the enzyme activity in response to, among others factors, light-dark transitions (Kaiser et al. 1999). Inactivation of NR probably occurs via phosphorylation of a conserved serine residue and subsequent binding of 14-3-3 proteins (Kaiser et al. 1999).

Among the plant hormones, it seems that cytokinin application has the most influence on NR expression. An induction of NR activity by exogenous cytokinins was first described by Borriss (1967) in *Agrostemma githago*. This induction has since also been reported in bean roots (Hänisch Ten Cate and Breteler 1982) and in cucumber (Kuznetsov et al. 1985) among other species

Abbreviations – ABA, abscisic acid; BA, benzyladenine; iP, iso-pentenyladenine; NR, nitrate reductase.

(see Gaudinova 1990 for a review). Furthermore, addition of benzyladenine (BA) to excised chicory roots strongly stimulated NR mRNA, protein and activity (Vuylsteker et al. 1997).

Cytokinins have also been shown to increase NR activity in etiolated plants, or in cell suspension cultures in many species (Banowetz 1992, Lu et al. 1992, Suty et al. 1993). Light is often required in addition to cytokinins for the hormonal enhancement to be effective.

In etiolated barley leaves, it has been shown that cytokinin enhancement of NR expression is in part transcriptional (Lu et al. 1992). The increase in NR activity can be quite substantial in barley where application of cytokinins increases NR activity by 25% in the roots and by 100% in the shoots (Samuelson et al. 1995). In some case, cytokinins may regulate NR mRNA stability through the extent of polyadenylation (Suty et al. 1993). On the other hand, abscisic acid (ABA) can suppress the cytokinin induction of NR mRNA level as well as reduce the NR mRNA accumulation in etiolated barley leaves transferred to light. It seems that the regulatory factor modulating the NR transcription is in fact the BA to ABA ratio (Lu et al. 1992). In *Arabidopsis*, which carries two NR genes, *Nia1* and *Nia2*, it has been shown that the *Nia1* expression is specifically induced by exogenous cytokinins (Yu et al. 1998). This enhancement of the enzymatic activity derived from the *Nia1* gene was observed in both seedlings germinated on cytokinin-containing medium and in soil-grown seedlings sprayed with cytokinins (Yu et al. 1998). This is one of the few examples of NR induction in non-etiolated tissues.

In this work, we have studied the influence of both exogenous synthetic cytokinins and marine bioactive substances (marketed under the trade name N PRO) on NR activity from in vitro grown *Arabidopsis* seedlings. We have thus shown that, in our experimental conditions, exogenous cytokinins have little effects on NR activity whereas the addition of N PRO clearly induces NR activity.

Materials and methods

Plant material and growth conditions

Plants of *A. thaliana* ecotype Columbia were used for the experiments. G5 is a mutant of the same ecotype carrying a complete deletion of the *Nia2* gene (Wilkinson and Crawford 1991). Seeds were surface sterilized and grown in vitro for 3 weeks on solid medium (as described by Estelle and Sommerville 1987) containing nitrate (9 m*M*) as sole nitrogen source with a photoperiod of 16 h light (250 μmol photons $m^{-2} s^{-1}$) at 20°C and 8 h dark at 15°C.

For some experiments, sterile BA, iso-pentenyladenine (iP) or algal marine bioactive substances were added in the growth medium at different concentrations. The marine bioactive substances N PRO was provided by the SECMABIO company (a division of the ROULLIER Group). The cytokinin biological activity for the N PRO preparation was expressed in μg equivalent of BA l^{-1} and was determined by the Amaranthus test (Biddington and Thomas 1973). This cytokinin activity for the N PRO batch number 160197.3, which was used for these experiments, was 220 μg eq. BA l^{-1} (corresponding to a BA concentration of about 1 μ*M*).

NR extraction and activity measurement

Leaves were harvested 2 h after the beginning of the day period from plants grown in vitro and were immediately frozen in liquid nitrogen and stored at − 80°C.

Frozen leaves were ground in liquid nitrogen using a mortar and pestle and extracted at 4°C in 4 ml of the following buffer g^{-1} FW: 50 m*M* KOH-HEPES pH 7.6, 10 m*M* $MgCl_2$, 0.5 m*M* EDTA pH 8.2, 5 μ*M* FAD, 1 μ*M* leupeptine and 2 m*M* β-mercaptoethanol. The mixture was incubated for 15 min on ice, and then centrifuged at 15 000 *g* for 5 min. The supernatant (subsequently referred to as crude extract) was used immediately for NR activity assays as previously described (Pigaglio et al. 1999) with the following modifications: a supernatant volume of 300 μl was used for each assay which was carried out in 50 m*M* KOH-HEPES pH 7.6 containing 1 m*M* EDTA pH 8.2, 5 m*M* potassium nitrate and 0.1 m*M* NADH in a total volume of 1 ml at 30°C for 10 min. The reaction was stopped by the addition of 1 ml of 1% sulfanilamide in 3 *N* HCl followed by 1 ml 0.02% *N*-naphthylethylenediamine dihydrochloride. A blank sample, in which sulfanilamide was added prior to the extract, was used for background control. The OD at 540 nm was measured and enzyme activity was expressed in nmoles NO_2^- formed $min^{-1} mg^{-1}$ soluble proteins.

Soluble proteins were assayed in the crude extract, according to Bradford (1976).

Results

Influence of cytokinins on NR activity

In order to determine the influence of cytokinins on NR activity, *Arabidopsis* plants were grown on solid medium containing increasing concentrations of BA, iP and zeatin (5–500 n*M*) and in the presence or absence of 1% sucrose. It was observed that, as expected, the higher concentrations of cytokinins (100 and 500 n*M*) inhibited plant growth (data not shown). The influence of cytokinins on NR activity was thus only measured between 5 and 100 n*M* for iP as the growth of the seedlings was too slow at 500 n*M*. The presence of BA in the growth medium did not significantly affect NR activity, except maybe at high concentrations of BA, which were found to slightly inhibit NR activity (Fig. 1). The decrease in NR activity may not be a specific response as this was correlated with an overall decrease in plant growth. The effect of iP on NR activity was even less marked (Fig. 2) as was the one of zeatin (data not shown). It was previously reported that BA significantly enhanced NR activity in *Arabidopsis* plantlets grown in vitro and that

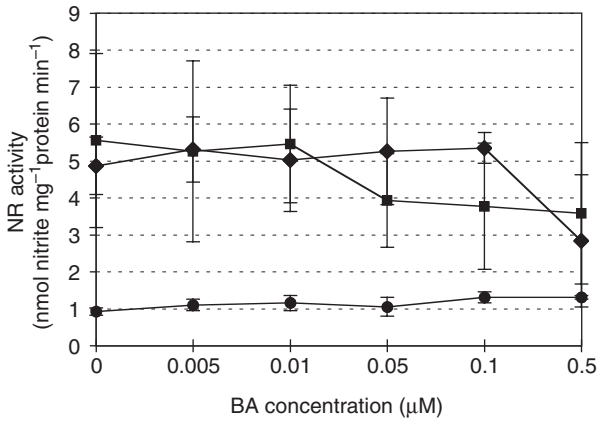

Fig. 1. The effect of exogenous BA addition on NR activity. *Arabidopsis* plants were grown in vitro with increasing concentrations of BA and in the presence (◆) or absence (■) of 1% sucrose. The *Arabidopsis* G5 mutant was grown in the same conditions in the presence of 1% sucrose (●). NR activity was measured in vitro from leaf extracts. Each value represents the mean (±SD) of three independent experiments.

this enhancement was related to an increase in *Nia1* expression (Yu et al. 1998). We did not observe any significant effect of exogenous BA on NR activity in the *Arabidopsis* G5 mutant (Fig. 1). In this mutant, NR activity is exclusively derived from the *Nia1* gene expression (Wilkinson and Crawford 1991). Thus, in our experimental conditions, BA seems to have no effects on NR activity, whether this activity is derived from the *Nia1* gene expression, as in the G5 mutant, or mainly from the *Nia2* gene, as in the wild type. An explanation for the discrepancies observed between the two experiments (Yu et al. 1998 and this work) could be that an environmental factor abolishes the NR activity induction by cytokinins. Indeed, as mentioned in the Introduction,

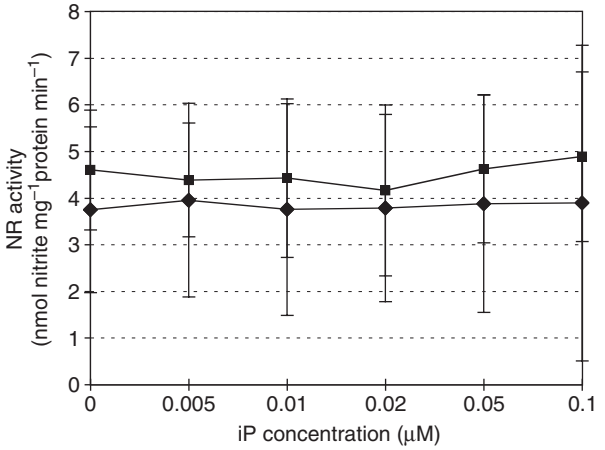

Fig. 2. The effect of exogenous iP addition on NR activity. *Arabidopsis* plants were grown in vitro with increasing concentrations of iP and in the presence (◆) or absence (■) of 1% sucrose. NR activity was measured in vitro from leaf extracts. Each value represents the mean (±SD) of three independent experiments.

it has been shown previously that ABA can suppress the induction of NR activity by cytokinins (Lu et al. 1992). Thus the plants we used could contain high levels of ABA, or of other interacting molecules, as a consequence of our in vitro culture conditions.

Effect of N PRO algal extract in the growth medium

The stimulatory influence of seaweed bioactive substances on the level of NR activity was often reported in the literature (Mooney and van Staden 1986, Jolivet et al. 1991) but these extracts were usually only applied by foliar spraying. We thus wanted to investigate the effect of marine bioactive substances when continuously present in the growth medium of *Arabidopsis* plantlets and in the presence or absence of sucrose. For this purpose, we used the SECMABIO N PRO extract. We first checked that this extract did not contain high concentrations of nitrate, which could then induce NR activity. We found in fact very low nitrate concentrations (between 3 and 5 μ*M*) in the N PRO extracts. As for cytokinins, highly concentrated marine bioactive substances strongly inhibited plant growth (Fig. 3). But, unlike cytokinins, N PRO addition in the growth medium clearly increased NR activity in the absence of sucrose (Fig. 4). In the presence of exogenous sugar, the stimulation of NR activity was less apparent. Indeed, in the absence of sugar, NR activity in the presence of 3.3% N PRO was 2.4 times higher than in the control plants, whereas in the presence of 1% sucrose it was only 1.4 times higher (Fig. 4).

It has been noted previously that marine bioactive substances are more efficient when plant are starved of nutrients (Verkleij 1992). We thus investigated the impact of the marine bioactive substances on NR activity in N-limited and N-sufficient *Arabidopsis* plants growing, respectively, on 1 m*M* and 7 m*M* nitrate with 1.7% (v/v) N PRO. As observed in many plant species (Meyer and Stitt 2001),

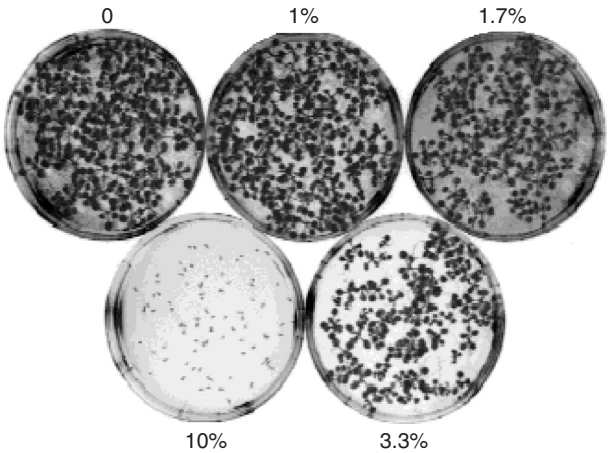

Fig. 3. In vitro growth of *Arabidopsis* plants with increasing concentrations of N PRO extract (in percentage of the growth medium, v/v).

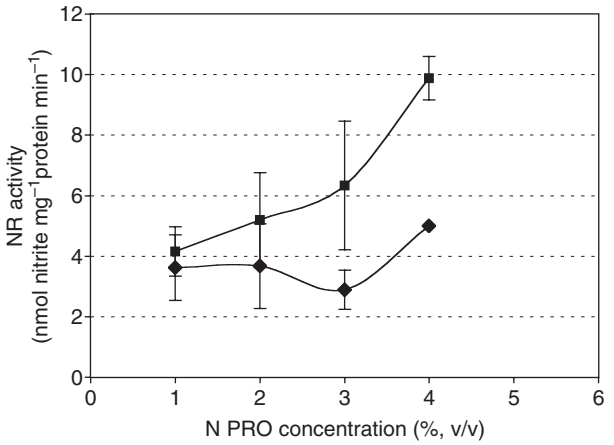

Fig. 4. The effect of N PRO addition on NR activity. *Arabidopsis* plants were grown in vitro with increasing concentrations of the seaweed-containing N PRO extract and in the presence (◆) or absence (■) of 1% sucrose. NR activity was measured in vitro from leaf extracts. Each value represents the mean (±SD) of three independent experiments.

high concentrations of nitrate increased NR activity in *Arabidopsis* but BA seemed to have little or no effect on NR activity (Table 1). As in the experiments described above, the addition of N PRO stimulated NR activity in N-sufficient conditions (Table 1, 2.2-fold increase) but this stimulation was much more apparent in N-limited conditions (Table 1, 9.5-fold increase). Indeed, when plants were growing with N PRO, NR activity was almost the same in both fertilization regimes.

Effect of N PRO spraying

Marine bioactive substances are most often applied by foliar pulverization on crops rather than in the soil. We thus tried to compare the effects of N PRO when added directly to the growth medium or sprayed onto in vitro

Table 1. In vitro NR activity from N-limited (1 m*M* nitrate) and N-sufficient (7 m*M*) *Arabidopsis* control plants and plants growing with BA (0.01 μ*M*) or algal N PRO (1.7% v/v). NR activities are expressed as means (±SD, n = 3).

NR activity (nmol nitrite mg^{-1}protein min^{-1}) Nitrate concentration	1 m*M*	7 m*M*
no addition	0.36±0.18	1.75±0.66
BA	0.39±0.21	1.99±0.76
N PRO	3.43±1.30	3.80±1.38

grown *Arabidopsis* plantlets. For this purpose, 3-week-old *Arabidopsis* plants were directly sprayed in Petri dishes with water (control), N PRO (10%, v/v) or with BA (0.5 μ*M*) and NR activity was measured after 24 and 48 h of culture (Fig. 5). The foliar addition of these compounds had no effects on plant phenotype or growth (data not shown) during this time course. As before, the N PRO enhanced NR activity whereas BA application had little or no effect (Fig. 5). Nevertheless, the increase in NR activity was much more modest in these conditions than when N PRO was added directly to the growth medium. It is likely that these marine bioactive substances are less efficiently taken up through the leaves than through the roots and that their effects are more evident when the plants are in contact with the substances for a longer time. The same effect on NR activity was observed when *Arabidopsis* plants were grown in the greenhouse and subsequently sprayed with diluted marine bioactive substances (N. Durand, X. Briand, C. Meyer, in preparation).

Discussion

We did not see any clear effect of exogenous cytokinins on NR activity, whether they were applied by spraying or added directly in the growth medium. Nevertheless, increasing cytokinin concentrations gradually affected plant growth as expected, showing that they had a

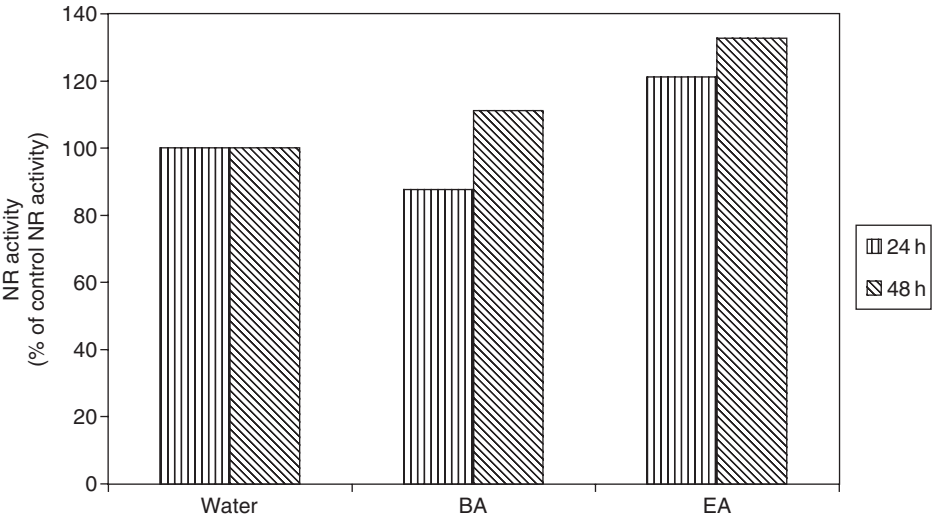

Fig. 5. The effect of BA or N PRO spraying on NR activity. *Arabidopsis* plants were grown in vitro and sprayed with either water (control plants), BA (0.5 μM) or seaweed-containing N PRO extract (EA, 10% v/v). NR activity was then measured in vitro from leaf extracts 24 or 48 h after spraying. NR activities are given as percent of control (plants sprayed with water). Mean NR activity of the control plants was 3.6 nmol nitrite formed mg protein^{-1} min^{-1} after 24 h and 2.3 nmol nitrite formed mg protein^{-1} min^{-1} after 48 h.

biological effect (Gaudinova 1990). Similarly, the addition of marine bioactive substances inhibited plant growth and this effect could be partly due to the presence of cytokinins in these seaweed-containing preparations. But only marine bioactive substances had a clear impact on NR activity. This points to the involvement of other molecules than cytokinins in the stimulation of NR expression by N PRO. Indeed seaweed extracts contain a large number of both organic and inorganic molecules, like macro- and micronutrients, phytohormones, polysaccharides, polyphenols, betaines, etc. (Verkleij 1992, Crouch and van Staden 1993) but nitrate concentration in the N PRO extract is far too low to explain its stimulatory effect on NR activity. The molecules which have mostly attracted attention in seaweed extracts are cytokinins (Mooney and van Staden 1986, Stirk and van Staden 1997) and many authors have ascribed the properties of seaweed extracts to these phytohormones. Therefore information on the role of the other seaweed compounds is hitherto rather scarce. The fact that N PRO produced a higher enhancement of NR activity in limiting nitrogen conditions (low nitrate) suggests that the main influence of this extract would be to induce the nitrate reducing capacities, and thus maybe the overall N nutrition of the plant, in adverse growth conditions. The stimulation of NR activity observed in this study may thus be one of the reasons for the better nitrogen use efficiency observed in seaweed-complemented plants (Jeannin et al. 1991, Verkleij 1992). The fact that exogenously supplied cytokinins had no obvious effects on NR activity does not exclude a participation of these phytohormones in association with other molecules in the complex marine bioactive substances. Our goal is now to identify the molecule(s) of N PRO responsible for the stimulation of NR activity.

Acknowledgements – We thank SECMABIO (Pontrieux) for their financial support throughout this work. ND was also supported by a grant from SECMABIO. We are indebted to Sylvie Salamagne for critical reading of the manuscript and to the members of the laboratory for fruitful discussions and help in setting up these experiments.

References

Banowetz GM (1992) The effect of endogenous cytokinin content on benzyladenine enhanced nitrate reductase induction. Physiol Plant 86: 341–348

Biddington NL, Thomas TH (1973) Chromatography of five cytokinins on an insoluble polyvinylpyrrolidone column. J Chromatogr 75: 122–125

Borriss H (1967) Untersuchungen ueber die Steuerung der Enzymaktivitaet in pflanzichen Embryonen durch Cytokinine. Wiss Z University Rostock, Math-Naturwiss Reihe 16: 629–639

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Campbell WH (1999) Nitrate reductase structure, function and regulation: bridging the gap between biochemistry and physiology. Annu Rev Plant Physiol Plant Mol Biol 50: 277–303

Crawford N (1995) Nitrate: nutrient and signal for plant growth. Plant Cell 7: 859–868

Crouch IJ, van Staden J (1993) Evidence for the presence of plant growth regulators in commercial seaweed products. Plant Growth Regul 13: 21–29

Estelle MA, Sommerville CR (1987) Auxin-resistant mutants of *Arabidopsis thaliana* with an altered morphology. Mol Gen Genet 206: 200–206

Gaudinova A (1990) The effect of cytokinins on nitrate reductase activity. Biol Plant 32: 89–96

Hänisch Ten Cate CH, Breteler H (1982) Effect of plant growth regulators on nitrate utilization by roots of nitrogen-depleted dwarf bean. J Exp Bot 132: 37–46

Jeannin I, Lescure JC, Morot-Gaudry JF (1991) The effects of aqueous seaweed sprays on the growth of maize. Bot Marina 34: 469–473

Jolivet E, de Langlais-Jeannin I, Morot-Gaudry JF (1991) Les extraits d'algues marines: propriétés phytoactives et intérêt agronomique. Année Biologique, Paris, France, pp 109–126

Kaiser WM, Weiner H, Huber SC (1999) Nitrate reductase in higher plants: a case study for transduction of environmental stimuli into control of catalytic activity. Physiol Plant 105: 385–390

Kuznetsov VV, Ulizsch M, Boriseva NN, Preusser E (1985) Effect of pH of the medium on induction of nitrate reductase in isolated cucumber cotyledons and corn cockle embryos: a comparative study. Sov Plant Physiol 31: 521–527

Lillo C (1994) Light regulation of nitrate reductase in green leaves of higher plants. Physiol Plant 62: 89–94

Lu J, Ertl JR, Chen C (1992) Transcriptional regulation of nitrate reductase mRNA levels by cytokinin–abscisic acid interactions in etiolated barley leaves. Plant Physiol 98: 1255–1260

Meyer C, Stitt M (2001) Nitrate reduction and signalling. In: Lea PJ, Morot-Gaudry JF (eds) Plant Nitrogen. Springer-Verlag, Berlin Heidelberg, Germany, pp 37–59

Mooney PA, van Staden J (1986) Algae and cytokinins. J Plant Physiol 123: 1–21

Pigaglio E, Durand N, Meyer C (1999) A conserved acidic motif in the N-terminal domain of nitrate reductase is necessary for the inactivation of the enzyme in the dark by phosphorylation and 14-3-3 binding. Plant Physiol 119: 219–229

Samuelson ME, Campbell WH, Larsson CM (1995) The influence of cytokinins in nitrate regulation of nitrate reductase activity and expression in barley. Physiol Plant 93: 533–539

Stirk WA, van Staden J (1997) Isolation and identification of cytokinins in a new commercial seaweed product made from *Fucus serratus* L. J Appl Phycol 9: 327–330

Suty L, Moureaux T, Leydecker MT, Teyssendier de la Serve B (1993) Cytokinin affects nitrate reductase expression through the modulation of polyadenylation of the nitrate reductase mRNA transcript. Plant Sci 90: 11–19

Verkleij FN (1992) Seaweed extracts in agriculture and horticulture: a review. Biol Agric Hort 8: 309–324

Vuylsteker C, Leleu O, Rambour S (1997) Influence of BAP and NAA on the expression of nitrate reductase in excised chicory roots. J Exp Bot 48: 1079–1085

Wilkinson JQ, Crawford NM (1991) Identification of the *Arabidopsis* CHL3 gene as the nitrate reductase structural gene *Nia2*. Plant Cell 3: 461–471

Yu X, Sukumaran S, Marton L (1998) Differential expression of the *Arabidopsis Nia1* and *Nia2* genes. Plant Physiol 116: 1091–1096

Edited by J. K. Schjørring