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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[1]

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It has previously been shown that the N-terminal domain of tobacco (*Nicotiana tabacum*) nitrate reductase (NR) is involved in the inactivation of the enzyme by phosphorylation, which occurs in the dark (L. Nussaume, M. Vincentz, C. Meyer, J.P. Boutin, and M. Caboche [1995] Plant Cell 7: 611–621). The activity of a mutant NR protein lacking this N-terminal domain was no longer regulated by light-dark transitions. In this study smaller deletions were performed in the N-terminal domain of tobacco NR that removed protein motifs conserved among higher plant NRs. The resulting truncated NR-coding sequences were then fused to the cauliflower mosaic virus 35S RNA promoter and introduced in NR-deficient mutants of the closely related species *Nicotiana plumbaginifolia*. We found that the deletion of a conserved stretch of acidic residues led to an active NR protein that was more thermosensitive than the wild-type enzyme, but it was relatively insensitive to the inactivation by phosphorylation in the dark. Therefore, the removal of this acidic stretch seems to have the same effects on NR activation state as the deletion of the N-terminal domain. A hypothetical explanation for these observations is that a specific factor that impedes inactivation remains bound to the truncated enzyme. A synthetic peptide derived from this acidic protein motif was also found to be a good substrate for casein kinase II.

Most higher plants obtain the nitrogen metabolites needed for growth and development by taking up and assimilating nitrate. After active transport into the cell, nitrate is reduced to nitrite by NR (EC 1.6.6.1–2), a cytosolic enzyme. Subsequently, nitrite is reduced to ammonium by nitrite reductase, which is localized in the chloroplast.

The expression of the NR gene is highly regulated at the transcriptional level by many endogenous and environmental factors, including hormones, light, nitrogen source, and carbohydrates (for review, see Hoff et al., 1994; Crawford, 1995). These transcriptional regulations probably determine the long-term fluctuations in the NR protein level. On the other hand, a reversible posttranslational regulation of the NR protein involving protein phosphorylation allows short-term modulation of the enzyme activity in response to light-dark transitions, variations in photosynthetic activity, CO_2 level, intracellular pH, or oxygen availability (Kaiser and Förster, 1989; Kaiser and Brendle-Behnisch, 1991; Huber et al., 1992; Kaiser et al., 1992, 1993; MacKintosh, 1992; Kaiser and Huber, 1994a). Inactivation of NR is linked with phosphorylation both in vivo and in vitro.

Proteins involved in the phosphorylation/dephosphorylation mechanism of spinach NR have been purified and characterized, and a two-step regulation model was proposed (Spill and Kaiser, 1994; Glaab and Kaiser, 1995; MacKintosh et al., 1995). According to this model, spinach NR is first phosphorylated on Ser-543, which is conserved among higher-plant NRs (Douglas et al., 1995; Bachmann et al., 1996b) and then becomes inactivated upon binding of a factor called NR inactivator protein, which was recently identified as a mixture of 14-3-3 proteins suggested to interact with the regulatory phosphorylation site of NR (Bachmann et al., 1996a; Moorhead et al., 1996). Moreover, inactivation of the enzyme can be evidenced only when NR activity is measured in the presence of magnesium in the millimolar range. The magnesium ion plays an important role in the mechanism of NR inactivation: it is needed for both the NR phosphorylation step and for the maintenance of NR in its inactive form (Kaiser and Huber, 1994b). After chromatography, three distinct peaks of NR kinase activities were identified in spinach extracts (Bachmann et al., 1996b; Douglas et al., 1997).

Evidence for inactivation of NR from other plant sources by the same mechanism has been obtained for squash (Lillo, 1993), cabbage (Kojima et al., 1995), maize (Huber et al., 1994; Li and Oaks, 1994), barley (Decires et al., 1993), *Nicotiana plumbaginifolia* (Nussaume et al., 1995), pea (Glaab and Kaiser, 1993), and Arabidopsis (Labrie and Crawford, 1994; Su et al., 1996). The precise mechanism of NR inactivation is as yet poorly understood because a complete structural model of the NR molecule is still lacking.

What is known is that NR is a homodimeric enzyme composed of 100- to 115-kD monomers. Each monomer is organized in three main domains housing the three prosthetic groups of NR, MoCo, heme, and FAD. Electrons from NADH travel successively through the FAD, heme,

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Abbreviations: CaMV, cauliflower mosaic virus; CKII, casein kinase II; MoCo, molybdenum cofactor; NR, nitrate reductase.

and MoCo domains before reaching the nitrate molecule for its reduction (for review, see Hoff et al., 1994; Campbell, 1996). These domains are linked by protease-sensitive hinges, and the regulatory phosphorylation site of NR is located on the first hinge, which connects the MoCo and the heme domains (Douglas et al., 1995; Bachmann et al., 1996b). The NR protein sequences are well conserved among higher plants and when compared with fungi or algal NR sequences (apart from the N-terminal region, which varies both in sequence and length; Nussaume et al. [1995]).

This observation prompted us to investigate the possible role of this N-terminal region by expressing in transgenic *N. plumbaginifolia* an NR-coding sequence carrying a 56-amino acid deletion in this region (ΔNR protein, Nussaume et al., 1995). It was found that the ΔNR enzyme was still active and that, apart from a higher thermosensitivity, showed the same enzymatic properties as the wild-type enzyme. However, the ΔNR protein activity was no longer regulated by darkness and phosphorylation; indeed, the ΔNR activation state (the percentage of active enzyme) was higher than in the wild type and was unaffected by light-dark transitions. Moreover, the ΔNR protein was insensitive to MgATP inactivation in vitro. A relationship between NR inactivation and degradation was also confirmed, because the ΔNR protein was more stable in the dark than the complete NR protein (Nussaume et al., 1995).

A similar result was obtained for spinach, in which it was shown that, when the NR protein had lost its first 45 amino acids by proteolysis, the NR activity could no longer be inhibited by 14-3-3 binding, although the degraded enzyme was still phosphorylated (Douglas et al., 1995). These results strongly suggest that the N-terminal region of NR is somehow involved in and is required for the inactivation of the enzyme by phosphorylation. So far, the question of how this N-terminal region of NR participates in the inactivation process remains largely unanswered, mainly because the deletion that was made originally does not allow us to determine which residues are involved in this process.

To determine which segments of the NR N-terminal sequence are necessary for the posttranslational inactivation of the protein, we introduced smaller deletions within the N-terminal region of the tobacco (*Nicotiana tabacum*) NR to express the resulting deleted NR proteins in the closely related species *N. plumbaginifolia*. The deleted NR proteins were expressed in an NR-deficient mutant background. The thermosensitivity, inactivation, and degradation of NR after light-dark transitions were compared in wild-type and transgenic plants expressing the complete NR-coding sequence, the ΔNR protein, or smaller deletions in the N-terminal region.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Plants of *Nicotiana plumbaginifolia* var Viviani (line pbH1D) were used for all experiments. The C1 (Vincentz and Caboche, 1991) transgenic line was obtained by transformation of the *N. plumbaginifolia* E23 *nia* mutant with a full-length coding sequence from the tobacco *Nia2* gene linked to a CaMV 35S RNA promoter. The del8 (Nussaume et al., 1995) transgenic line was obtained in the same way except that the NR-coding sequence carried a 56-amino acid deletion in the N-terminal region. Seeds were sown in vitro on solid B-N medium (Gabard et al., 1987) supplemented with 10 mM KNO_3. Germination was performed under the following photoperiod conditions: 8 h at a light intensity of 75 μE m^{-2} s^{-1} (1 h at 20°C, 6 h at 25°C, and 1 h at 20°C) and 16 h in darkness at 17°C. Plants were grown either in the greenhouse or in a growth cabinet. In the latter case, the photoperiod was 16 h of light (2 h at 20°C, 12 h at 25°C, and 2 h at 20°C; 225 μE m^{-2} s^{-1}) followed by 8 h of darkness at 17°C. Plants in continuous darkness were kept at 17°C. In all cases plants were grown until the rosette stage (about 3 weeks) and watered daily with a nutritive solution (Coïc and Lesaint, 1975).

NR Protein Sequence Analysis

NR protein sequence alignment was performed using the Wisconsin package (Genetics Computer Group, Madison, WI). The structure predictions were made using the PHD neural network (Rost and Sander, 1994) and were performed at the EMBL (Heidelberg, Germany).

Construction of the Chimeric ΔA-NR and ΔS-NR Genes

Standard procedures were used for recombinant DNA manipulations (Maniatis et al., 1982). Enzymes were used according to the supplier's recommendations.

Two internal deletions (ΔA and ΔS, which correspond, respectively, to the deletion of an acidic domain and of a conserved Ser residue in the NR N-terminal region) were introduced in the complete NR-coding sequence corresponding to the tobacco *Nia2* gene (carried by plasmid pCS22, Vincentz and Caboche, 1991) using a site-directed mutagenesis method based on PCR (Chen and Przybyla, 1994). This method uses oligonucleotides whose sequences contain the expected deletion to synthesize megaprimers in a first PCR round. The different steps of the construction are summarized in Figure 1.

First, a 198-bp fragment containing the ΔA deletion (the ΔA megaprimer) was amplified by PCR (Fig. 1A) using the plasmid pCS22 as template with oligonucleotide 26 (5′-ATCGAGCTCTTTTAGAATAATCCA-3′) and oligonucleotide 27 (5′-ATTTGAAGGTACTCATTATCAAGGTAAATGGTGGAA-3′). In parallel, a 110-bp fragment containing the ΔS deletion (the ΔS megaprimer) was obtained using oligonucleotide 26 and oligonucleotide 28 (5′-GTTGCAGCCACGAACCCGGGGCTTGAAAGA-3′). The sequence of primer 26 was derived from the sequence just upstream of the NR-coding sequence in plasmid pCS22, the last base of primer 26 being the first base of the NR-coding sequence. Primer 27 (reverse) corresponds to nucleotides 279 to 297 and 337 to 353 of the tobacco *Nia2* genomic sequence

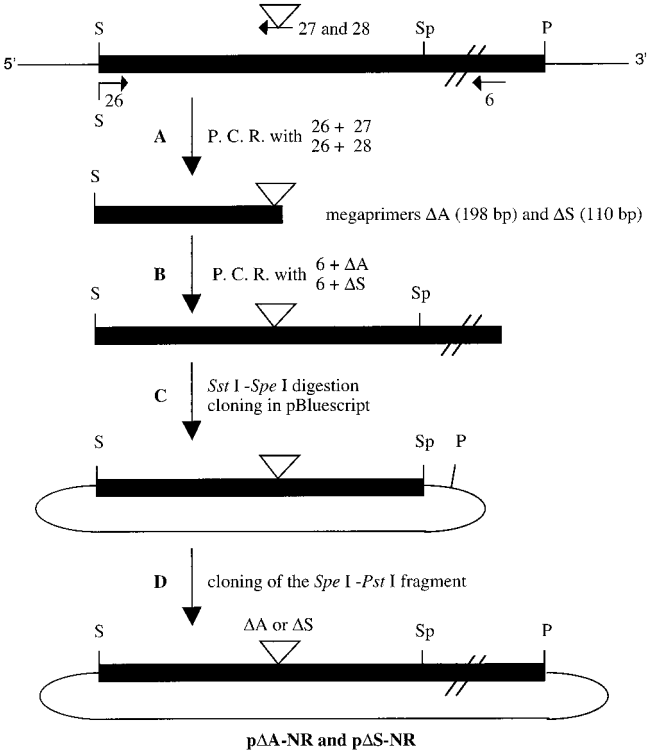

Figure 1. Construction of the plasmid vectors expressing the deleted NR proteins ΔA-NR and ΔS-NR. We first amplified by PCR a 198-bp fragment containing the ΔA deletion and a 110-bp fragment containing the ΔS deletion using, respectively, the primers 26 and 27 or the primers 26 and 28 (A). The amplified fragments called, respectively, megaprimers ΔA and ΔS, were then used as primers for a second PCR with primer 6 (B). The resulting DNA fragments were cloned between the *Sst*I and *Spe*I sites of the plasmid pBluescript (C). These new plasmids were digested by *Spe*I and *Pst*I and an *Spe*I–*Pst*I fragment was then introduced downstream of the deleted sequence to recover the complete NR-coding sequence (D). These constructs were called pΔA-NR and pΔS-NR. The dark bars represent the NR-coding sequence. S, *Sst*I; Sp, *Spe*I; P, *Pst*I.

(Vaucheret et al., 1989), which introduces a 39-bp deletion in the NR-coding sequence. Primer 28 (reverse) corresponds to nucleotides 196 to 210 and 223 to 237 of the tobacco *Nia2* genomic sequence, which introduces a 12-bp deletion.

The ΔA megaprimer was then used for a second PCR round (Fig. 2B) with oligonucleotide 6 (Meyer et al., 1995) using the plasmid pCS22 as a template. This produced a fragment of the NR-coding sequence (exon 1 and the beginning of exon 2) containing the ΔA deletion. A DNA fragment containing the ΔS deletion was synthesized in the same way, using the megaprimer ΔS and oligonucleotide 6 as primers.

The two PCR products were gel purified, digested by *Sst*I and *Spe*I, and cloned in the same sites in plasmid pBluescript (Stratagene). The presence and accuracy of the deletions were verified in the resulting positive clones by DNA sequencing.

The two recombinant plasmids were digested by *Spe*I and *Pst*I, and an *Spe*I–*Pst*I fragment from pCS22 containing the end of the NR-coding sequence was cloned downstream of the previous inserts (Fig. 1D). This reconstituted a complete NR-coding sequence and created the pΔA-NR and pΔS-NR plasmids.

To construct binary vectors expressing the ΔA-NR and the ΔS-NR proteins, the complete ΔA-NR and ΔS-NR chimeric sequences from pΔA-NR and pΔS-NR were isolated as an *Sst*I–*Pst*I fragment, blunt-ended, and cloned into the blunt-ended *Kpn*I site of the binary plant transformation vector pBinDH51 (Vincentz and Caboche, 1991). This put the ΔA- and ΔS-NR-coding sequences under the control of the CaMV 35S RNA promoter and terminator and created the binary plasmids pBΔA-NR and pBΔS-NR.

Plant Transformation and Regeneration

The recombinant vectors pBΔA-NR and pBΔS-NR in the *Escherichia coli* XL1-Blue strain (Bullock et al., 1987) were mobilized into *Agrobacterium tumefaciens* strain LB4404 as described by Bevan (1984). The NR-deficient *nia* mutant E23 of *N. plumbaginifolia*, which is unable to use nitrate as a nitrogen source, was transformed as described previously by infecting leaf discs with an *A. tumefaciens* culture (Vaucheret et al., 1990). The resulting calli were selected on a medium containing 100 mg L^{-1} kanamycin and 10 mM ammonium succinate as the sole nitrogen source and regenerated into plantlets as described previously (Vaucheret et al., 1990). The transformed calli were halved, and each half was placed on a regenerating medium containing either succinate ammonium or 10 mM potassium nitrate as the sole nitrogen source. Regenerated plantlets that were restored for the ability to utilize nitrate for growth were then transferred to the greenhouse and are referred to as primary transformants. Genetic analysis of the progeny obtained from selfing primary transformants (the R_1 generation) was performed on B-N medium containing 100 mg L^{-1} kanamycin and 10 mM potassium nitrate.

Extraction and Analysis of RNA

Total RNAs were extracted from frozen leaf material and analyzed by northern blots as described by Crété et al. (1997). Hybridization was performed using the 1.6-kb *Eco*RI–*Eco*RI cDNA fragment corresponding to the tobacco *Nia2* gene (Vaucheret et al., 1990). Ethidium bromide staining of rRNAs after gel migration was used to ensure homogenous loading of the RNA samples.

NR Extraction and Activity Measurement

Leaves were harvested 2 h after the beginning of the day period from plants grown in the greenhouse. For plants that were kept in the dark, leaves were harvested from two plants under a green light at each sampling time and were immediately frozen in liquid nitrogen and stored at −80°C.

Frozen leaves were ground in liquid nitrogen and extracted in 4 mL g^{-1} fresh weight buffer A (50 mM Hepes-KOH, pH 7.6, 10 mM magnesium chloride, 1 mM DTT, 5 μM FAD, and 1 μM leupeptin). The whole procedure was car-

ried out at 4°C. The mixture was incubated for 15 min on ice, and the homogenate was centrifuged at 12,000*g* for 10 min. The supernatant (crude extract) was either used immediately for NR activity assays or was subjected to further steps of purification.

For ammonium-sulfate precipitation, powdered ammonium sulfate was gradually added to the cool crude extract until 40% saturation at 4°C. The solution was slowly stirred for 1 h at 4°C and centrifuged at 12,000*g* for 30 min. The pellet was dissolved in buffer A in one-tenth of the original volume.

NR activity measurements were carried out in buffer B (50 mM Hepes-KOH, pH 7.6, 10 mM magnesium chloride, 140 μM NADH, and 5 mM potassium nitrate). In some experiments EDTA (15 mM) was added to the reaction buffer. The reaction was initiated by adding 200 to 300 μL of extract and was carried out for 5 to 12 min at 27°C in a total volume of 1 mL. The nitrites formed during the assay were revealed as previously described (Meyer et al., 1995).

The activation state of NR is defined as the ratio of the NR activity measured in the presence of free magnesium ions to the NR activity measured in the presence of EDTA. This activation state is expressed as a percentage and reflects the amount of active NR in an extract.

In Vitro CKII Assays on an Acidic Synthetic Peptide

CKII activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into a synthetic peptide, as detailed by Davies et al. (1989). A recombinant human CKII was obtained from New England Biolabs and was used according to the supplier's recommendation. A synthetic peptide (RRREEET*EEE; New England Biolabs), the substrate for human CKII, was used as a positive control. The synthetic peptide derived from the tobacco acidic domain corresponds to residues 55 to 69 of the NIA2 tobacco protein sequence (SSSEDDDDDDEKNEG, acidic peptide). The labeling reaction (final volume 30 μL) consisted of 100 units of human CKII (0.2 μL), 1 μL of CKII substrate peptide (final concentration 245 μM), or 6 or 12 μL of acidic peptide (300 and 600 μM, respectively); 0.2 mM [γ-^{32}P]ATP (200 μCi/μmol); 20 mM Tris-hydrochloride, pH 7.5; 50 mM potassium chloride; and 10 mM magnesium chloride. After incubation for 30 min at room temperature, a 20-μL aliquot was removed and spotted onto a 2- × 2-cm square of phosphocellulose paper. The papers were then washed in orthophosphoric acid (75 mM), with a final wash in acetone, dried, and placed in 1.5-mL plastic tubes, and the bound radioactivity was measured by direct counting (Cerenkov counts).

RESULTS

Construction of Plant Vectors Expressing NR Proteins Deleted in the N-Terminal Region

The N-terminal extensions of higher-plant NR protein sequences were aligned and compared with an algal sequence from *Volvox carterii* and with a fungal NR sequence from *Ustilago maydis* (Fig. 2). As noted previously (Nussaume et al., 1995), these sequences are poorly conserved even among higher plants. However, in the plant sequences two regions showing more conserved features were identified within the NR N-terminal extensions: a consensus sequence, RXDSPVR, which contained a conserved Ser residue (amino acids 24–30 of the NIA2 tobacco protein sequence) found in most higher-plant sequences (Fig. 2), and an acidic domain rich in Asp and Glu residues (amino acids 53–68 of the NIA2 tobacco protein sequence), which also contained a stretch of conserved Ser residues.

The first motif (RXDSPVR) is absent in the NR N-terminal sequences from Leguminosae (soybean, bean, and lotus) and less conserved in Cruciferae, whereas it is found in the NADH:NR sequences from monocots (rice and *Nia1* in barley). Conversely, an acidic stretch preceded by one to four Ser residues appeared to be present in all higher-plant sequences examined (Fig. 2). One may then wonder whether these protein sequences play any role in the inactivation of NR by phosphorylation, because they are contained in the previous 56-amino acid deletion that was shown to abolish inactivation of NR in the dark. To investigate this, these two motifs were removed from the tobacco NR-coding sequence using a site-directed mutagenesis method based on PCR (Fig. 1). The resulting pΔA-NR construct carries a 13-amino acid deletion (amino acids 54–66 of the NIA2 tobacco protein sequence) corresponding to the acidic domain, whereas in the pΔS-NR construct, the 4 amino acids surrounding the conserved Ser were removed (amino acids 25–28 of the NIA2 tobacco protein sequence). The truncated NR-coding sequences ΔA-NR (2.715 kb) and ΔS-NR (2.742 kb) were placed under the control of the CaMV 35S RNA promoter and terminator in the plant-transformation vector pBinDH51 (Vincentz and Caboche, 1991), which produced the binary plasmids pBΔA-NR and pBΔS-NR.

Complementation of the E23 NR-Deficient Mutant of *N. plumbaginifolia* by the Chimeric Genes ΔA-NR and ΔS-NR

The E23 *nia* mutant of *N. plumbaginifolia* is deficient for NR activity and is unable to grow with nitrate as the sole nitrogen source. Indeed, the NR structural gene is disrupted by an insertion of the *Tnp2* retrotransposon in the first exon (C. Meyer, unpublished results). As a consequence, the E23 mutant does not produce any full-length NR mRNA and is devoid of NR protein. The E23 mutant was transformed with either the pBΔA-NR construct or the pBΔS-NR construct by agroinoculation of leaf discs with *A. tumefaciens*. To avoid any selection pressure on the recovery of transgenic plants expressing a functional NR protein, kanamycin-resistant calli were regenerated on a medium containing ammonium as the nitrogen source. The selected calli were then cut in half and grown on either ammonium or nitrate. Finally, the regenerated plantlets that were able to grow on a medium containing nitrate as the only nitrogen source were retained and then transferred to the greenhouse. Two independent primary transformants (R_0) carrying the ΔA-NR gene (A1 and A2) and four independent

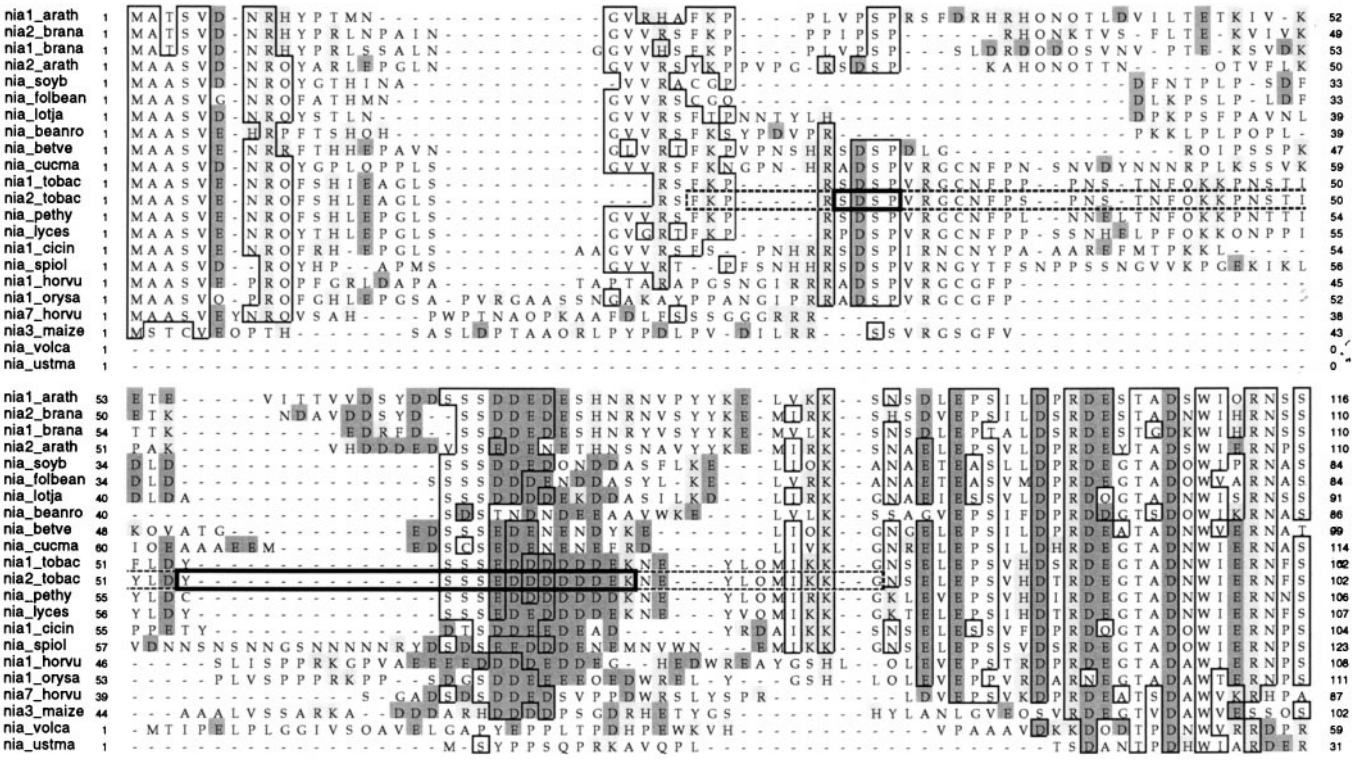

Figure 2. Alignment of the N-terminal domain protein sequences from plant, fungal, and algal NRs. Sequences were first aligned using the PileUp program (Genetics Computer Group), and the resulting alignment was refined by hand. Sequences are all from the SwissProt database, the algal NR sequence (nia__volca) is from *V. carterii*, and the fungal NR sequence (nia__ustma) is from *U. maydis*. Conserved residues are boxed. Acidic residues are shaded dark gray, and basic residues are shaded light gray. The 56-amino acid sequence deleted in the ΔNR protein is indicated (dashed box), as are the sequences deleted in the ΔA-NR and ΔS-NR proteins (bold boxes).

primary transformants carrying the ΔS-NR gene (S2, S3, S4, and S9) were obtained. For each primary transformant, the NR sequence surrounding the introduced deletion was amplified by PCR and verified by DNA sequencing (data not shown).

The transformants were phenotypically similar to the wild type or to the transgenic *N. plumbaginifolia* overexpressing either the NR (C1 plants) or the ΔNR protein (del8 plants). They grew vigorously both in vitro and in the greenhouse with 10 mM nitrate as the sole nitrogen supply.

The selfed progeny (R_1) of the primary transformants were studied for the transmission of the kanamycin-resistance marker and for their ability to grow on nitrate. Three primary transformants (A2, S2, and S4) were found to carry a single functional kanamycin-resistance locus. Three other primary transformants (A1, S3, and S9) showed an aberrant Mendelian segregation (data not shown). The A1, A2, S2, S4, and S9 lines were retained for further studies.

NR activities were measured for each transformant in the R_1 plants and compared with the NR activity of the wild-type, C1, and del8 plants (Fig. 3). All of the transformants had NR activity lower than the wild type. The same range of NR activity was observed for transgenic plants expressing the ΔNR protein (Nussaume et al., 1995).

Thermosensitivity of the ΔA-NR and ΔS-NR Proteins

A difference in thermosensitivity between the NR and ΔNR proteins was previously observed when NR activity was assayed in vitro using ammonium sulfate-precipitated extracts. The activity of the ΔNR protein was found to be more sensitive to temperature than the activity of the wild-type NR (Nussaume et al., 1995).

The thermosensitivity of the ΔA-NR and ΔS-NR proteins was compared with that of NR and ΔNR proteins by measuring NR activity in ammonium sulfate-precipitated extracts (Fig. 4). In the wild type the NR activity was higher at 30°C than at 20°C, whereas after 30 min the NR activity was lower at 35°C than at 20°C. For the ΔNR protein expressed in the del8 transgenic plant, NR activity dramatically decreased after 10 min at 30°C or 35°C. The increase in temperature had the same effect on the ΔS-NR protein and wild-type NR protein activities (Fig. 4). Conversely, the thermosensitivity of the ΔA-NR protein was intermediate between that of the wild type and that of the ΔNR proteins. In the A1 and A2 transgenic plant extracts the nitrite accumulation measured when the enzyme was incubated at 30°C was lower than that measured at 20°C, but higher than that in del8 extracts at the same temperature (Fig. 4). At 35°C the nitrite accumulation was comparable

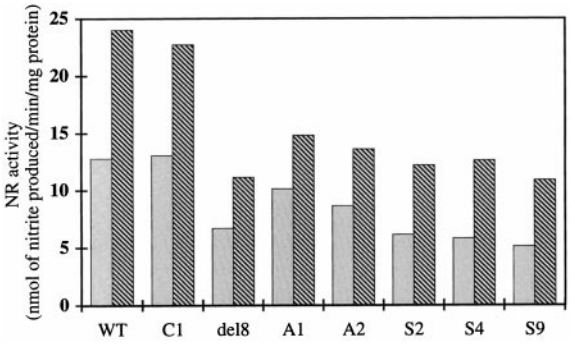

Figure 3. NR activities in wild-type (WT) and transgenic *N. plumbaginifolia* plants ectopically expressing NR. Plants were grown in the greenhouse and harvested at the beginning of the light period. Results are from two independent experiments. C1 and del8, Transgenic plants expressing, respectively, a wild-type NR protein (35S-NR) and the ΔNR protein (35S-ΔNR). A1 and A2, Transgenic plants expressing the ΔA-NR protein. S2, S4, and S9, Transgenic plants expressing the ΔS-NR protein.

in A1, A2, and del8 plant extracts. These results suggest that the ΔS-NR protein is as stable as the wild-type NR, whereas the ΔA-NR protein is more thermosensitive (although less so than the ΔNR protein).

Reversible Inactivation of NR by Darkness

Spinach NR is known to be inactivated in the dark, and this inactivation was best revealed when NR activity was assayed in a magnesium-containing buffer. The inactivation was reversible in vitro by adding EDTA to the reaction buffer (Kaiser and Brendle-Behnisch, 1991). Therefore, the NR activation state, or percentage of active NR, can be estimated by calculating the ratio between NR activity assayed without EDTA (which corresponds to the activity of the dephosphorylated NR in the extract) and NR activity assayed with EDTA (reactivated NR). It was previously shown that the enzyme from *N. plumbaginifolia* was also inactivated in the dark, but at that time the kinetics of NR inactivation had not been investigated (Nussaume et al., 1995).

Figure 5 shows the inactivation time course of NR in wild-type *N. plumbaginifolia* plants exposed to darkness. The light-dark transition induces a decrease in NR activity of about 65%. The half-time of the inactivation reaction was about 15 min, which is in agreement with previous results in spinach leaves (Huber et al., 1992; Kaiser et al., 1992). Until 30 min of darkness this inactivation is fully reversible by EDTA. After that point NR inactivation becomes irreversible, probably because of the degradation of the protein. Such results are in agreement with previous reports of species such as spinach (Huber et al., 1992; Kaiser et al., 1992; MacKintosh, 1992), barley (Decires et al., 1993), cabbage (Kojima et al., 1995), maize (Li and Oaks, 1994), pea (Glaab and Kaiser, 1993), and squash (Lillo, 1993).

Effect of Light on the Expression of the ΔA-NR and ΔS-NR Chimeric Genes and on the Activity of the ΔA-NR- and ΔS-NR-Deleted Proteins

The posttranscriptional regulation by light of the expression of a 35S-NR gene was demonstrated previously (Vincentz and Caboche, 1991). To study the effect of light on the expression of the ΔA-NR and ΔS-NR genes, wild-type and control transgenic plants (C1 and del8) were kept in the dark for 72 h, along with A1, A2, S2, S4, and S9 transgenic plants (Fig. 6A).

In wild-type plants the NR mRNA level was lower in the light than in the transgenic plants and decreased to undetectable levels after 72 h of darkness (Fig. 6A). In the transgenic plants C1, del8, A1, A2, S2, S4, and S9, in which the transcription of the NR gene is under the control of the CaMV 35S promoter, the NR mRNA accumulation was unaffected by the dark treatment (Fig. 6B), as was observed previously for the C1 and del8 transgenic plants (Vincentz and Caboche, 1991; Nussaume et al., 1995). In the transgenic plants the NR mRNA remained constitutively overexpressed throughout the dark treatment (data not shown). Since the transcriptional regulation by light of NR expres-

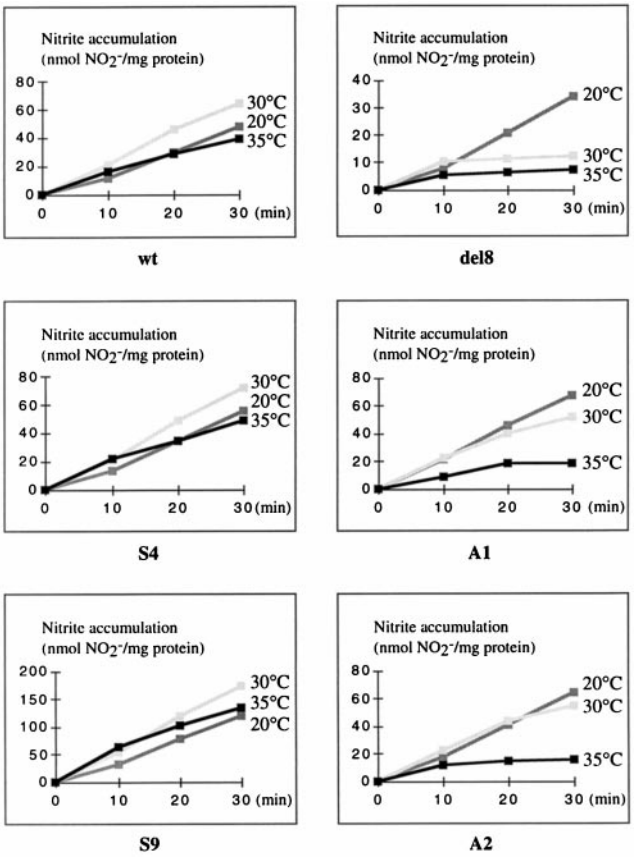

Figure 4. Thermosensitivity of the ΔA-NR and ΔS-NR proteins. NR activity (nitrite accumulation) was assayed in vitro at different temperatures (20°C, 30°C, and 35°C) on ammonium sulfate-precipitated extracts obtained from the wild-type (wt) or from different transgenic lines. Abbreviations of the genotypes of the transgenic lines are as defined in the legend of Figure 3.

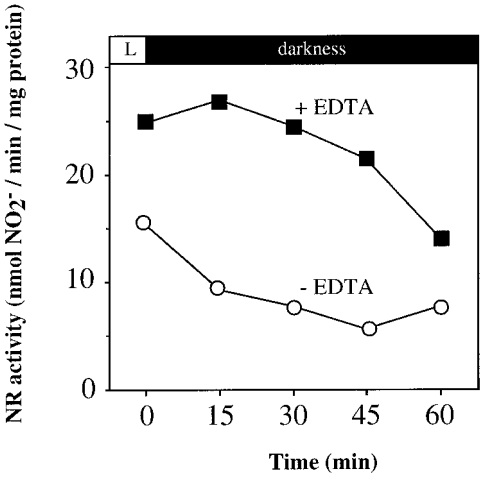

Figure 5. Inactivation kinetics of extractable NR from *N. plumbaginifolia* leaves in response to a light-dark transition. Plants grown in the greenhouse with light (L) were put in a dark room (t = 0) and kept in darkness for various times. Leaves were harvested from two different plants and NR was extracted in a magnesium-containing buffer. NR activity was measured in the crude extracts with 15 mM EDTA (■) or without EDTA (○).

sion was absent in these transgenic plants, the modulation of NR expression by light should only be the result of posttranscriptional regulation.

To study the effect of darkness on the activity of the deleted proteins ΔA-NR and ΔS-NR, leaf samples were harvested from the same plants at the beginning of the light period and at various times after the onset of darkness (30 min and 2, 4, 6, 24, 48, and 72 h). The effect of darkness on the activation state of the NR proteins expressed in wild-type and transgenic plants was investigated first (Fig. 7). In illuminated leaves about 40% to 50% of NR was active in the wild-type, C1, and S plants, whereas the amount of active NR was higher in the del8 and A plants (approximately 60%, Fig. 7). Upon transfer of the plants to darkness, NR was further inactivated in the wild-type, C1, and S plants (approximately 25% of active NR after 30 min), and this low activation state remained more or less constant during the dark period (Fig. 7). On the contrary, the activation state of NR in the del and A plants seemed to be unaffected by darkness (approximately 60%, Fig. 7). The results with the C1 and del8 plants agree with those previously obtained by Nussaume et al. (1995). Taken together, these results suggest that, although the ΔS-NR protein behaves like the wild-type protein in response to a light-dark transition, the deletion of the acidic motif (ΔA-NR) abolishes the NR inactivation by darkness and, therefore, has the same effect as the 56-amino acid deletion of the ΔNR protein.

We have also represented the maximal NR activity (assayed in the presence of EDTA) measured in the above experiment, which should reflect the total amount of functional NR protein (Fig. 8). After 30 min of darkness the maximal NR activity for all genotypes tested showed little change compared with the maximal NR activity measured in the light (data not shown). Conversely, after 2 h of darkness the maximal NR activity decreased in the plants to about 25% to 35% of the NR activity measured in the illuminated leaves (Fig. 8), which indicates a probable degradation or irreversible inactivation of the NR protein. The NR activity then decreased rapidly in the wild-type, C1, and S plants and was almost undetectable after 48 h in the dark. In the del8 and A plants, the maximal NR activity also decreased but less rapidly (Fig. 8). After 4 h in the dark the NR activity measured with EDTA was always 3- to 4-times higher in the del8 and A plants than in the wild-type, C1, or S plants. Again, these results suggest that the characteristics of the ΔA-NR protein are similar to those of the ΔNR protein and that the ΔA-NR protein, although finally degraded after an extended period of darkness, was less sensitive to degradation than the wild-type NR protein or the ΔS-NR protein.

Phosphorylation of the NR N-Terminal Acidic Cluster by CKII

The 13-amino acid sequence that was deleted in the ΔA-NR protein contains three consecutive Ser residues followed by one Glu and six Asp residues (Fig. 2). Such a sequence is reminiscent of a consensus CKII phosphorylation site (S/TXXE/DX; Pearson and Kemp, 1991). The consensus sequence for CKII phosphorylation includes a cluster of acidic residues on the C-terminal side of the target Ser or Thr, with Asp successfully replacing Glu (Pearson and Kemp, 1991). A synthetic peptide was derived from the deleted protein sequence in the ΔA-NR protein and then used as a substrate for a kinase assay with human CKII (Table I). It appears that this peptide (amino acids 55–69 of the NIA2 tobacco protein sequence) can be efficiently phosphorylated in vitro by human CKII.

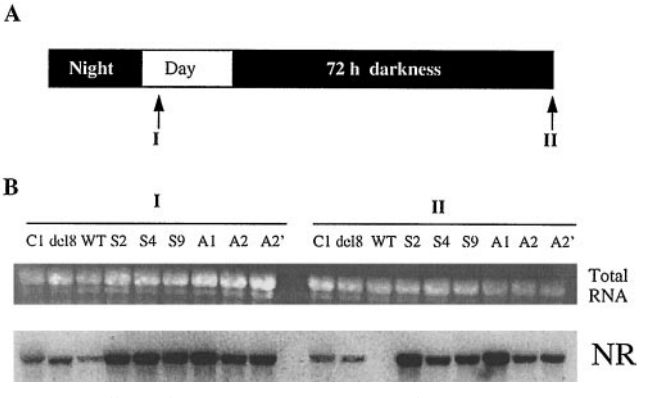

Figure 6. Effect of light on the expression of the ΔA-NR and ΔS-NR chimeric genes. A, Plants grown in the greenhouse were placed in the dark and leaves were harvested at the beginning of a normal day/night cycle (I) and after 72 h of darkness (II). B, Northern analysis of total RNA (5 μg) using as a probe the 1.6-kb *Eco*RI fragment of the tobacco Nia2 NR cDNA. Ethidium bromide staining of rRNA (total RNA) is shown as a control for homogenous loading. Abbreviations of the genotypes of the transgenic lines are as defined in the legend to Figure 3 (A2′ is another sample from the A2 line).

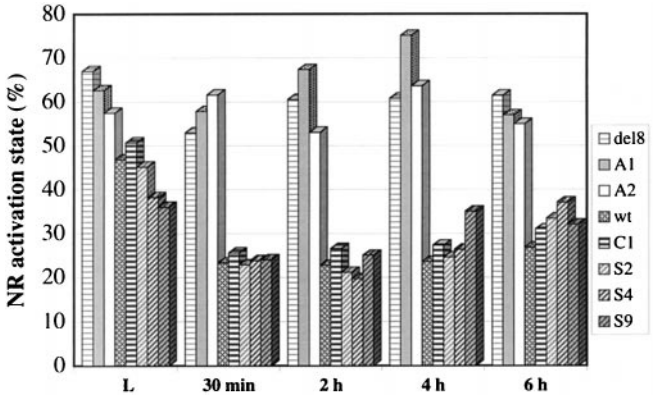

Figure 7. NR activation state in leaves of wild-type and transgenic *N. plumbaginifolia* plants during prolonged darkness. Plants were grown in the greenhouse. Leaves were first collected at the beginning of a normal day/night cycle (L), the plants were subsequently placed in the dark, and leaves were harvested at the indicated times from four different plants of the same genotype. NR activity was assayed in crude extracts for 10 min with or without EDTA (15 mM). The NR activation state is the percentage of active NR, which corresponds to the ratio between NR activity assayed without EDTA and NR activity assayed with EDTA. Abbreviations of the genotypes of the transgenic lines are as defined in the legend to Figure 3.

DISCUSSION

Complementation of NR Deficiency by the ΔA-NR- and ΔS-NR-Deleted Genes

The transformation of the *nia* E23 mutant of *N. plumbaginifolia* with the ΔA-NR and ΔS-NR deleted genes allowed wild-type NR activity and a wild-type phenotype to be restored. These observations confirm previous results obtained with transgenic E23 plants expressing either the full-length tobacco NR cDNA (C1 plants, Vincentz and Caboche, 1991) or the truncated ΔNR protein (del plants, Nussaume et al., 1995) under the control of the CaMV 35S RNA promoter. However, the A and S transformants did not seem to be less fertile than the wild type, unlike the del transformants (Nussaume et al., 1995). The 4 (ΔS deletion) or 13 (ΔA deletion) amino acids that were removed from the tobacco NR sequence were not required for the functionality of the protein. Such a result was expected, because these two deletions are included in a larger region of 56 amino acids that was previously shown to have no effect (except on thermosensitivity) on the functionality of the ΔNR protein (Nussaume et al., 1995).

Role of the Two Deleted Regions in the in Vitro Stability of the NR Protein

The ΔNR protein was previously found to be less stable than the NR protein in vitro when the NADH:NR activity was assayed on an ammonium sulfate-precipitated extract, whereas no difference was detected when assayed on a crude extract (Nussaume et al., 1995). Since the NADH:Cyt *c* reductase activity was not affected in the ΔNR protein, it was concluded that this instability concerned only the MoCo domain. We then postulated that the 56-amino acid deletion might alter MoCo binding and that the instability of the ΔNR protein observed after precipitation could be due to the removal of plant factors ensuring MoCo binding or stabilization (Nussaume et al., 1995).

The ΔA-NR and ΔS-NR proteins were found to behave differently in response to temperature by exhibiting different thermosensitivity profiles (Fig. 4). The ΔS-NR protein was as stable as the wild-type NR, whereas the ΔA-NR was more thermosensitive (although less so than the ΔNR protein). These results suggest that the sequence deleted in the ΔA-NR protein was the structural feature involved in the loss of MoCo and, therefore, in the thermosensitivity of the protein. The fact that the ΔA-NR protein was more stable than the ΔNR protein could then be explained by a larger destabilization of the ΔNR protein due to the size of the deletion. In both the ΔA-NR and the ΔNR proteins, the thermosensitivity of the NR protein was linked to a higher activation state of the enzyme and to the insensitivity to dark inactivation in *N. plumbaginifolia* (compare Figs. 4 and 7). The actual relationship between the NR N-terminal acidic motif and the stability of MoCo binding is as yet undetermined, since structural data concerning the MoCo domain are relatively scarce.

The ΔS-NR Protein Exhibits the Same Properties as the Tobacco Wild-Type NR in *N. plumbaginifolia*

The alignment of the NR N-terminal domain protein sequences revealed two main conserved motifs among higher plants (Fig. 2). One of these motifs (RXDSP) was centered around Ser-27 in the tobacco NIA2 sequence and was therefore included in the sequence that was removed in the ΔNR protein. Since several Ser residues were found to be phosphorylated in spinach (Huber et al., 1992), Arabidopsis (Labrie and Crawford, 1994), and maize (Huber et

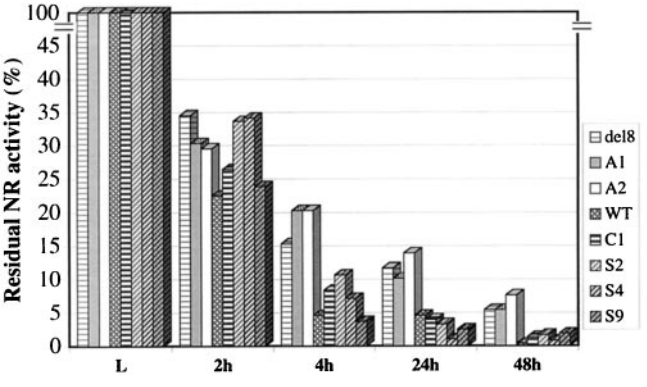

Figure 8. NR total activity in leaves of wild-type and transgenic *N. plumbaginifolia* plants during prolonged darkness. Leaves were harvested as described in the legend to Figure 7. NR activity was then assayed in the crude extracts for 10 min in the presence of 15 mM EDTA. The NR activities shown correspond to the NR activities measured with EDTA in the experiment described in Figure 7. NR activity is given as a percentage of the NR activity measured in the light (L, 100%). Values for specific NR activities in the light (in nanomoles of nitrite produced per minute per milligram of protein) were 24 for wild type, 23 for C1, 11 for del8, 15 for A1, 14 for A2, 12 for S2, 13 for S4, and 11 for S9.

Table I. *Phosphorylation by CKII of a synthetic peptide derived from the tobacco NR N-terminal acidic cluster*

The in vitro phosphorylation by human CKII of a substrate peptide and of the peptide derived from the sequence of the tobacco NR N-terminal acidic cluster (acidic peptide) were compared. For details, see "Materials and Methods." The possible phosphorylation sites for CKII are in bold type and are underlined.

Peptide	Sequence	Concentration	Labeling
		μM	cpm
None	—	—	807
CKII substrate	RRREEE**T**EEE	245	61,911
Acidic	**SSS**EDDDDDDEKNEG	300	12,529
	SSSEDDDDDDEKNEG	600	24,613

al., 1994) NR, we questioned whether this conserved Ser was involved in the NR-inactivation process by introducing a four-amino acid deletion (residues 25 to 28 of the NIA2 tobacco protein sequence) into the tobacco NR-coding sequence and expressing the resulting ΔS-NR protein in transgenic *N. plumbaginifolia* plants. The ΔS-NR protein was found to be inactivated and degraded in the dark to the same extent as the wild-type NR protein.

This suggests that the conserved RXDSP motif in the NR N-terminal domain is not directly involved in the inactivation of the enzyme by phosphorylation, which is in agreement with this motif being absent in the NR N-terminal sequences from Leguminosae (Fig. 2). Nevertheless, the modulation of the ΔS-NR activity has been investigated only in response to light modifications; therefore, this motif could be involved in the regulation of NR activity in response to other external or internal changes.

Identification of a 13-Amino Acid Sequence Involved in the Posttranslational Inactivation of NR

The second conserved motif that was identified in the NR N-terminal domain was a stretch of acidic amino acids preceded by several Ser residues (Fig. 2). An NR-coding sequence deleted from this motif was then obtained and expressed in transgenic *N. plumbaginifolia* plants under the control of the 35S promoter. It was found that the ΔA-NR activation state was higher than for the wild-type NR protein and was unaffected by the light-dark transition, even after prolonged darkness. This suggests that, like the ΔNR protein (Nussaume et al., 1995), ΔA-NR is less sensitive to inactivation by phosphorylation. Therefore, the absence of the above motif in the ΔNR protein is probably the reason for the loss of posttranslational regulation. This acidic motif seems to be necessary for the inactivation of NR but may not be sufficient. A spinach NR that had been partially proteolyzed at the N terminus could not be completely inactivated by phosphorylation and 14-3-3 binding, even though it still contained the N-terminal acidic motif (Douglas et al., 1995).

After an extended period of darkness, the ΔA-NR and ΔNR activities measured in the presence of EDTA were found to disappear less quickly than wild-type NR activity. These activities should reflect the total amount of reactivatable NR protein. This suggests that the ΔA-NR and ΔNR proteins are more stable than the wild-type NR in the dark. Indeed, 48 h of darkness led to a 10- to 12-fold decrease in ΔNR and ΔA-NR total activity, whereas the decrease was about 50- to 100-fold in wild-type NR. After 72 h of darkness the ΔNR and ΔA-NR activities were still higher than for the wild-type NR (data not shown). The finding that the ΔNR and ΔA-NR proteins were still present after extended periods of darkness is in agreement with our previous results (Nussaume et al., 1995). However, the total amount of NR protein measured in the dark was lower in the present study, possibly because of the methods that were used to measure the amount of NR protein. In the present study, NR activity was assayed as NADH:NR activity after reactivation by EDTA. We now believe that this assay is more representative of the amount of functional NR in the plant. In a previous study, Nussaume et al. (1995) determined the amount of NR protein by using an ELISA or a partial NR activity measurement (NADH:Cyt *c* reductase activity), in which the NR protein can be recognized by the antibody or exhibit a partial activity even if partially degraded. These results point to a close relationship between NR inactivation by phosphorylation and degradation, as has been suggested in previous reports (Nussaume et al., 1995; Kaiser and Huber, 1997).

It has been shown that NR is phosphorylated on a conserved Ser residue located in the hinge separating the MoCo and the heme domain (Douglas et al., 1995; Bachmann et al., 1996b), and it has been suggested that the inactivating 14-3-3 proteins bind this phosphorylated Ser. The question that remains is the actual role of the acidic motif within the N terminus. Secondary structure prediction by the PHD neural network (Rost and Sander, 1994), which is based on multiple alignment, suggested with a high probability that the whole N-terminal domain forms an exposed loop ending with a short α-helix beginning right after the acidic stretch. The sequence that was removed in the ΔA-NR protein is somewhat reminiscent of a sequence rich in P, E/D, S, and T residues (the "PEST" motif; Rechsteiner and Rogers, 1996), which has been shown to serve as a proteolytic signal. For example, it was proposed that phytochrome is rapidly degraded upon light absorption because of the exposure of a potential PEST sequence (Rechsteiner and Rogers, 1996).

It has also been shown that constitutive phosphorylation by CKII of IκBα, a cytoplasmic protein sequestering the animal transcription factor NF-κB, is required for its degradation (Lin et al., 1996; Schwarz et al., 1996). Interestingly, phosphorylation by CKII is located in a C-terminal PEST motif that is required for degradation of the IκBα protein. Therefore, it seems that phosphorylation by CKII of PEST sequences, which often contain consensus sites for CKII phosphorylation, could be required for their role in proteolysis. The fact that a peptide derived from the NR N-terminal acidic motif is a good substrate for CKII further supports the involvement of this sequence in NR degradation. Indeed, the ΔA-NR and ΔNR proteins, in which the acidic motif is absent, seem to be more stable than the wild-type enzyme. As a hypothetical model for NR degradation, we propose that upon phosphorylation of the regulatory Ser residue in the first hinge domain the NR

N-terminal acidic motif phosphorylated by CKII could serve as a signal for NR proteolysis.

This model does not solve the problem of the role of the acidic motif in modulating NR inactivation in the dark. It was previously shown that the ΔNR protein could be inactivated by yeast 14-3-3 proteins when purified from dark-exposed leaves of del plants in the presence of phosphatase inhibitors (Lillo et al., 1997). When purified in the absence of phosphatase inhibitors the ΔNR protein was less inhibited, suggesting that the deletion of the N-terminal domain does not hinder phosphorylation of the ΔNR enzyme in planta or its inactivation in vitro by yeast 14-3-3 proteins. This is true only when the ΔNR protein is purified, because this inactivation was not observed in crude extracts (Lillo et al., 1997).

We propose the following hypothetical model to explain our observations: A putative NR activation factor binds to the dephosphorylated form of NR. Upon phosphorylation of the regulatory Ser residue, the acidic motif itself or the whole N-terminal domain triggers the release of this factor by becoming exposed to the outside. This would then allow binding of 14-3-3 proteins and subsequent inactivation of the NR enzyme as well as degradation of the protein by CKII phosphorylation. This model could explain the previous set of data: In ΔNR or ΔA-NR proteins expressed in *N. plumbaginifolia*, the NR activation factor would remain bound to NR, and therefore the binding of 14-3-3 proteins would either not take place or would be very reduced (Nussaume et al., 1995; this work). Upon purification of NR the putative NR activation factor would be lost and inhibition of the ΔNR protein could take place (Lillo et al., 1997). However, we do not know yet whether such an activating factor exists. Experiments are currently under way to characterize the putative NR activation factor and to confirm the above hypothetical model. The in vivo phosphorylation of NR by CKII also remains to be confirmed, as well as its role in the NR protein degradation observed upon inactivation.

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