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Developmental Stage-Specific and Nitrate-Independent Regulation of Nitrate Reductase Gene Expression in Rapeseed

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cDNA clones for two isogenes of nitrate reductase (NR) have been isolated from rapeseed (*Brassica napus* L.) androgenetic haploid embryos induced by microspore culture. NR mRNA accumulation can be detected by northern hybridization at 14 d after culture initiation when embryos develop to the heart/torpedo-shaped stage. Whole-mount in situ hybridization experiments demonstrate that the mRNA accumulation is developmental stage specific. In addition, even when cultured in media containing no nitrate, embryos accumulated NR mRNA to almost the same level as the control. This indicates the unique regulation of NR in embryogenesis in which NR mRNA transcription is activated in a developmental stage-specific manner that is independent of nitrate induction. In zygotic embryogenesis, a stage-specific accumulation of NR mRNA was also observed. By contrast, the obvious effect of nitrate on NR expression that has been reported in many plant species was also confirmed in rapeseed leaf. Quantitative combined reverse transcription-polymerase chain reaction analysis suggests that the flexible and variable regulation of NR expression, which is organ specific, nitrogen metabolite specific, and developmental stage specific, is caused principally by regulation of one major structural gene.

NR is a key enzyme in the first step of nitrate assimilation in higher plants (reviewed by Pelsy and Caboche, 1992). The first factor identified that regulates NR activity was nitrate (Tang and Wu, 1957), which strongly induces NR mRNA transcription (Crawford et al., 1986; reviewed by Crawford, 1995). Many other factors such as light, phytohormones, and carbon and nitrogen metabolites and their translocation and intracellular compartmentation also appear to be involved in NR expression (reviewed by Caboche and Rouzé, 1990; Warner and Kleinhofs, 1992; Hoff et al., 1994; Lilo, 1994). However, these studies focused mainly on the phenomena observed in vegetative organs such as leaves and roots. There is little information on nitrate assimilation at the molecular level in developing embryos in higher plants, probably because of the difficulty in sampling embryos in early developmental stages.

Recently, we found that Gln is essential for inducing and maintaining androgenetic embryogenesis from isolated microspores in rapeseed (*Brassica napus* L.) (Ohkawa and Maeda, 1992). Further, it was revealed that whereas Gln was the only nitrogen source to be utilized in the first 4 d of the microspore culture, embryos at the multicellular stage could assimilate ammonium salts, and the heart/torpedo-shaped stage embryo at 14 DAP acquired the ability to use nitrate as the sole nitrogen source (Y. Ohkawa, M. Maeda, H. Fukuoka, and T. Ogawa, unpublished data). The NADH:NR activity, examined in 0-, 4-, 8-, and 14-DAP embryos, was detected only in 14-DAP embryos. Since the original culture medium contained nitrate as a nitrogen source, it is possible that NR expression in embryos in the early stages involves nitrate-independent regulatory mechanisms.

Here we report cloning of NR cDNAs and characterization of the embryogenesis-specific regulation of NR genes using a microspore-derived embryogenesis system. In the experiments on NR gene regulation during androgenetic embryogenesis, we found that the NR gene was activated in a developmental-stage-specific and nitrate-independent manner in the course of embryogenesis. Posttranscriptional regulation was found specifically during embryogenesis and not in leaves. The developmental-stage-specific mRNA accumulation was also observed in zygotic embryogenesis. In addition, it was demonstrated using quantitative RT-PCR that one structural gene was the major gene that was nitrate inducible in leaves and that it was stage specifically and nitrate independently activated during embryogenesis.

MATERIALS AND METHODS

Plant Materials and Microspore Culture

Culture methods were according to Keller et al. (1987) with some modifications. Plants of rapeseed (*Brassica napus* L. cv Lisandra) were grown in a growth chamber. Day/night temperatures were 13/8°C, and the photoperiod was 16 h. Inflorescences were harvested when three to six flower buds were blooming. Buds (3–4 mm) were collected, surface sterilized, and squashed in B5–13 medium (Gamborg et al., 1968). Released microspores were filtered through 42-μm nylon mesh and collected by centrifugation at 900 rpm for 3 min. Microspores were washed twice with B5–13 medium and resuspended at a density of 5×10^4 microspores/mL in NLN-13 medium (Lichter, 1982) containing 0.5 mg/L 1-naphthylacetic acid and

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Abbreviations: DAF, days after flowering; DAP, days after plating; NR, nitrate reductase; RT-PCR, combined reverse transcription-PCR.

0.05 mg/L BA. Modified NLN-13 media containing various nitrogen sources were used instead of the original medium in nitrogen-source-replacement experiments (see "Results"). Aliquots of the suspension (10 mL) were plated into 90 × 15 mm Petri dishes. Cultures were subjected to high-temperature treatment at 32.5°C for 4 d and then moved to 25°C. Throughout the culture period, dishes were kept in the dark. For the experiments involving nitrogen-source induction of NR in seedlings, plants were grown under a 16-h photoperiod at 25°C and the first and second true leaves were harvested at the end of the dark period from 30-d-old seedlings. Cotyledons were cut off when the first true leaf began to extend. Plants were grown asceptically on a clean bench to avoid bacterial contamination caused by the high concentrations of amino acids and Suc in the media.

NR cDNA Cloning

Total RNA samples were prepared according to Chomczynski and Sacchi (1987) and purified by lithium precipitation. A rapeseed NR cDNA fragment containing parts of exons 3 and 4 was amplified by RT-PCR using total RNA extracted from androgenetic embryos at the torpedo-shaped stage as the template and a SuperScript preamplification system (GIBCO-BRL). The PCR product was inserted into pGEM-T cloning vector (Promega), and the resulting clone (pBnNR3423) was sequenced to confirm that the clone encoded a sequence that was homologous to those of known NR genes. A λ-Zap cDNA library was constructed using poly(A)$^+$ RNA extracted from torpedo-shaped androgenetic embryos, and 200,000 plaques were screened using pBnNR3423 as a probe for isolating full-length NR cDNA clones. The insert length was checked by PCR, and 14 of the largest clones were selected. The clones were transferred to pBluescript by the in vitro excision procedure and purified by PEG precipitation, and the nucleotide sequences of the 5′ and 3′ ends were partially determined. The deletion series of two clones (pBnNR1405 and pBnNR1412) were constructed using a Kilo-sequence Deletion Kit (Takara Shuzo Co., Shiga, Japan) to determine complete nucleotide sequences in both strands. DNA sequencing was performed by the dideoxynucleotide chain-termination method using a model 373A sequencer (Applied Biosystems).

Northern Hybridization

Twenty micrograms of total RNA were separated by 2.2 mm formaldehyde gel electrophoresis and transferred onto nylon membranes. A digoxigenin-labeled riboprobe was hybridized to the blot. For hybridization and signal detection, a digoxigenin Nucleic Acid Detection Kit (Boeringer Mannheim) was used according to the instructions of the manufacturer.

Quantitative RT-PCR Analysis

An upper primer (5′-ACT CAT CTG GAA CCT CAT-3′) and a lower primer (5′-CCA GGG TGG TCT TTC AAG-3′) were used for RT-PCR. The upper primer was 5′ labeled by fluorescein isothiocyanate, and during the reaction RT-PCR products of each cycle were taken out and digested with the restriction endonucleases *Pvu*II and *Eco*RV. Reaction products were separated by 4.5% PAGE, and the relative amounts of the PCR products derived from the two different NR genes were quantitated using a fluorescent image analyzer (model FI575, Molecular Dynamics, Sunnyvale, CA).

In Vitro NR Enzyme Activity Assay

Embryo or leaf samples were frozen with liquid nitrogen, powdered with a mortar and pestle, and homogenized with protein extraction buffer containing 50 mm sodium phosphate buffer (pH 7.0), 10 mm EDTA, 0.1% Sarcosyl (Fluka), 0.1% Triton X-100, and 0.1 mm 2-mercaptoethanol. After centrifugation, the supernatant was saved and crude protein concentration was determined using the Protein Assay Kit (Bio-Rad). NADH:NR enzyme activity was measured as described by Hageman and Reed (1980).

In Situ Hybridization

In situ hybridization to cross-sections was performed as described by Kouchi and Hata (1993). Whole-mount in situ hybridization was done according to Engler et al. (1994). In all of the experiments, sense probes were used as negative controls to estimate the level of nonspecific signals.

RESULTS

Cloning of NR cDNA Clones

As the first step in our study on regulation of NR expression during androgenetic embryogenesis in rapeseed microspore culture, we tried to obtain NR cDNA clones. It has been reported that the positions of introns in an NR gene are conserved among many higher-plant species (Caboche and Rouzé, 1990). We screened nucleotide sequences that are conserved in exons 3 and 4 among tobacco (Vaucheret et al., 1989), tomato (Daniel-Vedele et al., 1989), Arabidopsis (Wilkinson and Crawford, 1993), and squash (Crawford et al., 1986) and synthesized the corresponding primers (5′-CCC TGC AGC AAG TAC TGG TGT TGG TG-3′ and 5′-CCG GAT CCG TGC CAG CAT TGA T-3′) to amplify a short NR cDNA fragment from rapeseed mRNA. Using genomic DNA as the PCR template, the presence of an intron was confirmed in the genomic sequence between the two PCR primers (data not shown). Thus, we could easily distinguish by size mRNA-derived RT-PCR products from the products derived from genomic DNA contamination. The amplified NR cDNA fragment was inserted into a plasmid vector, and the resulting clone (pBnNR3423) was sequenced. Deduced amino acid sequences obtained from the nucleotide sequence showed high homology to known plant NR sequences (data not shown). Using this clone as a probe, we screened the cDNA library from torpedo-shaped androgenetic embryos to isolate full-length NR cDNA

clones. Forty-six positive clones were isolated from 200,000 plaques, and 14 of them showed sizes that corresponded to nearly full-length inserts (>2.7 kb). These clones were partially sequenced with M13 universal and reverse primers, and we identified two isogenes by examining sequences of putative nontranslated regions. Thus, we completely determined nucleotide sequences of two clones, BnNR1405 (2919 bp) and BnNR1412 (2994 bp), which corresponded to the isogenes NR1 and NR2, respectively. They had open reading frames of the expected lengths for NR protein and were 87% homologous at the nucleotide level in the putative coding region (Fig. 1). The nucleotide sequences and deduced amino acid sequences showed a high degree of homology to the known NR sequences of higher plants (data not shown).

Stage-Specific NR mRNA Accumulation in Androgenetic Embryos

Total RNA samples were extracted from androgenetic embryos at 0, 4, 8, and 14 DAP, and NR mRNA accumulation was examined by northern hybridization with the coding region of NR cDNA clones as probes. Although the two cDNA clones (BnNR1405 and BnNR1412) had such high homology in their sequences that we could not distinguish between them by hybridization experiments, it was found that NR mRNA accumulated only in the 14-DAP embryos (Fig. 2A). This result corresponds with our previous result that NR enzyme activity was detected only in 14-DAP embryos.

The 14-DAP embryos were not homogeneous as to their developmental stages. The most advanced embryos reached the torpedo-shaped stage, but some of them were in the heart-shaped stage and the rest were in the globular stage. Whole-mount in situ hybridization of the NR cDNA probe to 14-DAP embryos showed that embryos in the heart-shaped and torpedo-shaped stages accumulated NR mRNA, whereas no hybridization signal was detected in embryos at the globular stage (Fig. 2B). No nonspecific signal was detected using the sense probe as a negative control (Fig. 2C). This result indicated that NR mRNA accumulation was developmental stage specific and was not related to the culture period.

Developmental Stage and NR mRNA Accumulation in Zygotic Embryogenesis

To determine whether the stage-specific accumulation of NR mRNA was unique to the androgenetic embryogenesis or a general phenomenon in the zygotic embryos, ovules at 10, 13, 17, and 21 DAF were collected and subjected to in situ hybridization with digoxigenin-labeled NR cDNA probes. As shown in Figure 3A, NR mRNA was not detected in embryos at the globular stage. A faint signal could be detected in heart-shaped embryos (Fig. 3C), and an obviously positive signal was observed when embryos reached the torpedo-shaped stage at 17 DAF (Fig. 3E) and the cotyledonary stage at 21 DAF (Fig. 3G). Nonspecific signals were effectively eliminated (Fig. 3, B, D, F, and H). This result indicated that the developmental-stage-specific accumulation of NR mRNA observed during androgenetic embryogenesis was not unique to the development under artificial culture conditions in vitro, but was common to zygotic embryogenesis in vivo.

Effects of Nitrogen Source on NR mRNA Accumulation in Developing Embryos

To identify the relationships between the form of nitrogen source and NR mRNA accumulation, NLN-Q, NLN-NH4E, and NLN-NH4ENO3 media were used instead of NLN for microspore culture. Nitrogen source compositions of the media are summarized in Table I. Since NLN-NH4E and NLN-NH4ENO3 contained no Gln, it was impossible to induce embryogenesis when these media were used at the initiation of the culture. Therefore, in the Gln-deficient treatment with these media, the culture was initiated with NLN and the NLN media were replaced with Gln-deficient media at 4 DAP. No nitrate or Gln carryover from NLN was detected by ion chromatography (data not shown). NR mRNA accumulation was examined at 14 DAP by northern hybridization. To compare the NR regulation in embryos with the regulation in a vegetative organ, seedlings of rapeseed were grown aseptically with the same media and 30-d-old true leaves were harvested for further experiments.

As shown in Figure 4B, the leaves of the seedlings supplied with nitrate (NLN or NLN-NH4ENO3) accumulated high levels of NR mRNA. Only a trace of mRNA accumulation was observed with the samples that were supplied with nitrate-deficient media (NLN-Q or NLN-NH4E). NR enzyme activity correlated with the level of NR mRNA accumulation. These results suggested that in rapeseed, as has previously been reported for many higher-plant species, transcriptional and nitrate-inducible regulation is one of the major mechanisms of NR activity expression in leaves.

On the other hand, as shown in Figure 4A, 14-DAP embryos accumulated high levels of NR mRNA regardless of the type of nitrogen source, which was quite different from the result observed in leaves. Although NLN-Q and NLN-NH4E contained no nitrate, NR mRNA accumulation was detected in the embryos obtained from these nitrate-deficient media at almost the same level as was found in the embryos that developed in the nitrate-containing media. This result indicated that NR mRNA accumulation was induced in a developmental-stage-specific manner rather than in response to nitrate induction. In addition, although embryos obtained from NLN-NH4E contained abundant NR mRNA, an extremely low enzyme activity was detected. This suggested a posttranscriptional suppression of NR enzyme activity.

Estimation of the Number of Structural Genes Involved in Specific Regulation

Sequence analysis of the two cDNA clones BnNR1405 and BnNR1412 revealed some specific restriction sites in each gene. We synthesized a PCR primer pair to amplify

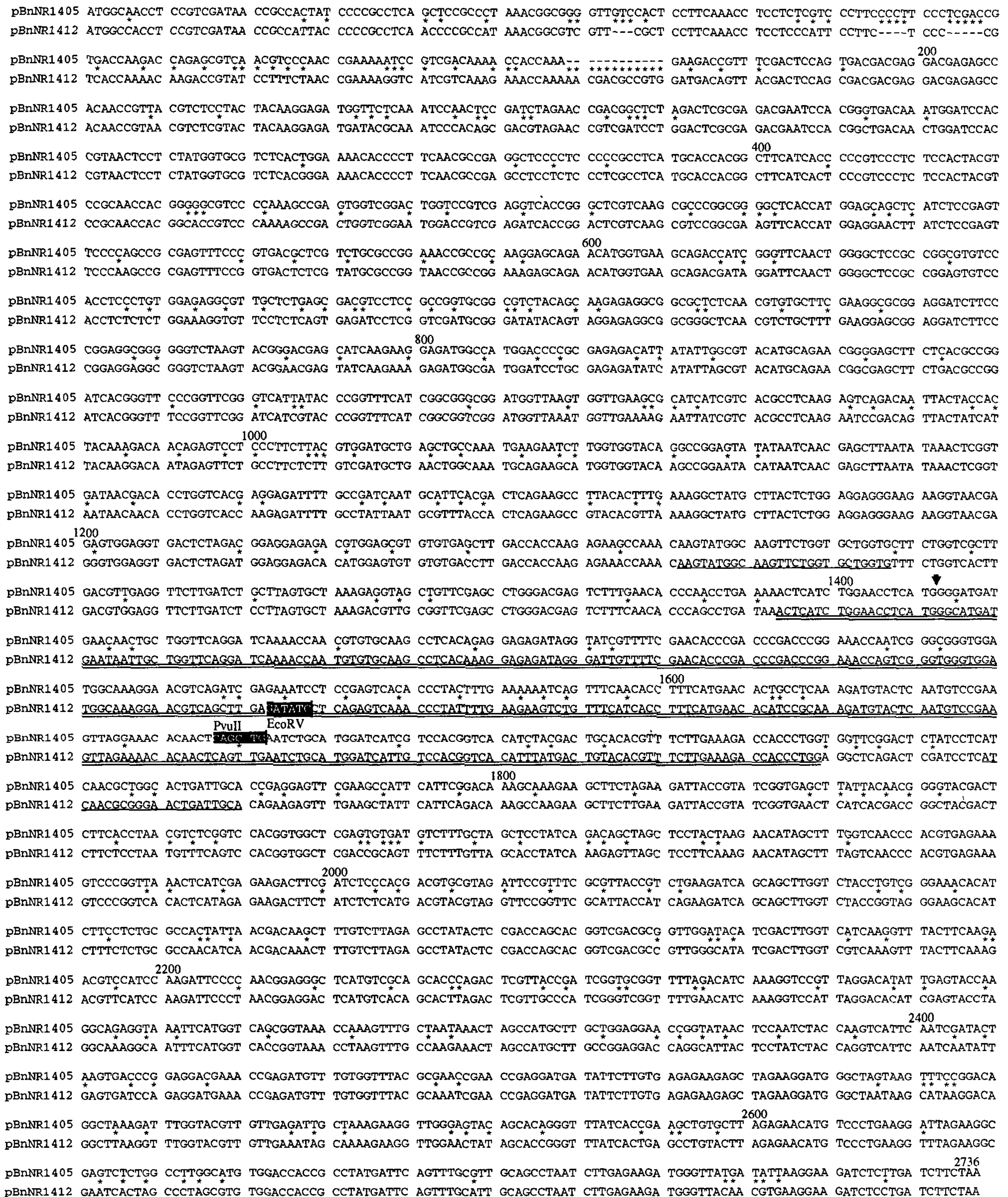

Figure 1. Comparison of nucleotide sequences of rapeseed NR cDNA clones pBnNR1405 and pBnNR1412. The deduced open reading frame sequences of the two NR cDNA clones were aligned using the Genetics Computer Group (Madison, WI) program package. Asterisks indicate differences between the sequences. The sequences corresponding to the sequences conserved among the known NR sequences that were used for RT-PCR primers in order to obtain a rapeseed NR cDNA fragment (cloned as pBnNR3423) are underlined. The conserved position of an intron between exons 3 and 4 among the known NR genes is indicated by an arrowhead. The region used for RT-PCR for estimation of relative amount of mRNA is double-underlined, and unique restriction sites for *Pvu*II and *Eco*RV in each clone are highlighted.

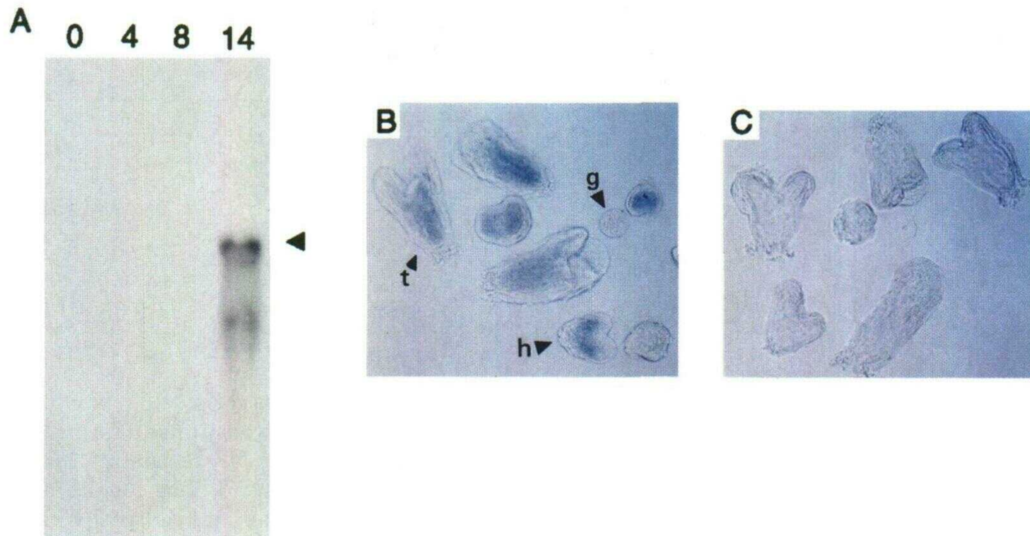

Figure 2. mRNA accumulation in developing androgenetic embryos. A, Northern hybridization of the rapeseed NR cDNA probe to total RNA samples extracted from androgenetic embryos. RNA samples extracted from embryos at 0, 4, 8, and 14 DAP were electrophoresed in lanes indicated by 0, 4, 8, and 14, respectively. NR mRNA is indicated by an arrowhead. B, Whole-mount in situ hybridization of the NR antisense probe to 14-DAP embryos. Developmental stage is indicated as: g, globular; h, heart shaped; t, torpedo shaped. C, Whole-mount in situ hybridization of the NR sense probe as a negative control to 14-DAP embryos.

336-bp NR cDNA fragments (indicated in Fig. 1) in which restriction sites for *Pvu*II (specific to NR1) and *Eco*RV (specific to NR2) were included. The relative amounts of transcript corresponding to the two genes were estimated by quantitative RT-PCR using the same RNA samples as in Figure 4. RT-PCR products of each cycle were digested with *Pvu*II plus *Eco*RV and separated by gel electrophoresis, and the fluorescein isothiocyanate-labeled 260-bp fragment (NR1) and 160-bp fragment (NR2) were quantitated with a fluorescent image analyzer. Figure 5 shows the relative fluorescent intensities of the two fragments at each PCR cycle. The data might not be sufficient for strict quantitation of the transcript because an internal standard of known amount was not included in the experiment. However, preliminary experiments in which mixtures of pBnNR1405 and pBnNR1412 in various ratios were used as PCR templates confirmed that the ratio of the relative amount of PCR products in the logarithmic phase could represent quantitatively the initial ratio of the templates (data not shown). As shown in Figure 5, RT-PCR products derived from NR1 mRNA were always about 10 or more times more abundant than those from NR2 in all samples regardless of organ type and nitrogen source composition. Therefore, the results suggested that NR1, a structural gene corresponding to pBnNR1405, was a common major gene being activated under flexible regulation that was stage specific and nitrate independent through embryogenesis and nitrate inducible in vegetative organs.

DISCUSSION

In past years, isolated microspore culture of rapeseed has been developed to be one of the most efficient systems for obtaining androgenetic embryos in vitro (Keller et al., 1987). Embryogenesis progresses in the same way as in zygotic embryos through globular, heart-shaped, and torpedo-shaped embryos. The resulting cotyledonary haploid embryos can germinate and grow normally to the flowering stage. Embryo-specific accumulations of storage proteins and lipids were also detected in the androgenetic embryos as they were in zygotic embryos (Crouch, 1982; Taylor et al., 1990). In addition, more than 10^4 embryos can easily be obtained from a 10-mL culture. Therefore, microspore-derived embryos can be a suitable material for investigating the early events of embryogenesis.

In the present study, we found that NR mRNA did not accumulate until the embryos reached the heart/torpedo-shaped stage. This corresponded to our previous results that enzyme activity was detected only at 14 DAP, suggesting that the NR expression in embryogenesis was transcriptionally regulated. Transcription of the NR gene has been reported to be induced by nitrate in many higher-plant species (Wray, 1988). Microspore culture of rapeseed was initiated with NLN medium containing 5.5 mM nitrate, which would have been sufficient to induce NR mRNA transcription in vegetative organs, i.e. leaves and roots. Therefore, the restricted accumulation of NR mRNA, which was not seen until the heart/torpedo-shaped stage in androgenetic embryogenesis, might indicate that the development of certain nitrate-responding mechanisms was not completed until the embryos reached this stage. However, the analysis of mRNA accumulation at 14 DAP using media containing various nitrogen sources revealed that NR mRNA accumulation through the embryogenesis process had no relationship to nitrate in the medium. Although Gln was reported to be a factor suppressing NR transcription and

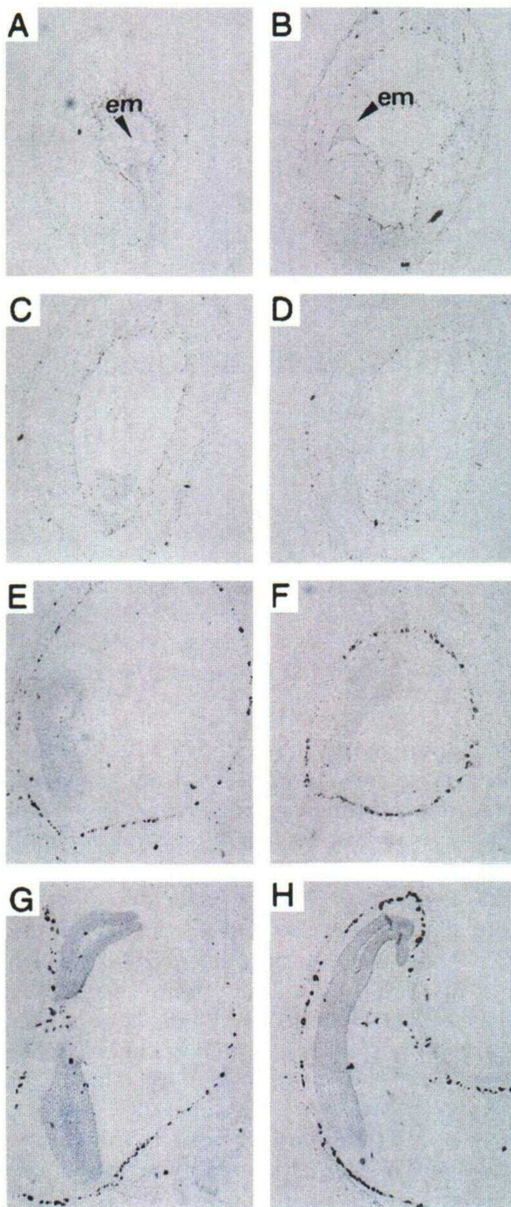

Figure 3. In situ hybridization of antisense (A, C, E, and G) and sense (B, D, F, and H) NR cDNA probes to developing zygotic embryos at 10 DAF (A and B), 13 DAF (C and D), 17 DAF (E and F), and 20 DAF (G and H). In A and B, arrowheads indicate embryos (em).

a cause of circadian fluctuation in NR activity in tobacco leaf (Deng et al., 1990, 1991), the embryos obtained from culture in NLN-Q exhibited almost the same level of NR mRNA accumulation and enzyme activity as did the control. If Gln were limiting the growth of embryos, it might appear to enhance not only NR but also various other enzymes. However, the growth rate of embryos was not significantly affected by the media (NLN, NLN-Q, NLN-NH4E, and NLN-NH4ENO3) from the globular stage onward (Y. Ohkawa, M. Maeda, H. Fukuoka, and T. Ogawa, unpublished data). This suggests that Gln does not enhance whole growth and activity of the embryos at the globular- to torpedo-shaped

Table I. *Nitrogen source compositions of the media used in the experiments*

–, Not present.

Medium	Nitrogen Source				
	NO_3	NH_4	Gln	Glu	Total nitrogen
			mM		
NLN	5.5	–	5.5	–	16.5
NLN-Q	–	–	8.25	–	16.5
NLN-NH4E	–	5.5	–	11.0	16.5
NLN-NH4ENO3	5.5	5.5	–	11.0	22.0

stages. The embryos from NLN-NH4E also accumulated NR mRNA, which was quite different from the results of previous reports on NR regulation in vegetative organs (Crawford et al., 1986). Our results reveal the state of mRNA accumulation and not real transcriptional activity. Further work is required to clarify the relationships among mRNA level, transcriptional activity, and mRNA stability.

Many other factors such as light (Rajasekhar et al., 1988; Melzer et al., 1989), cytokinin (Lu et al., 1990), and carbon metabolites (Vincentz et al., 1993) have been reported to affect transcriptional regulation of NR gene expression. It is possible that some of these could be artificial factors from the culture medium for in vitro-specific regulation of mRNA transcription. The experiment involving in situ hybridization of ovules, however, revealed that the stage-specific accumulation of NR mRNA was common to zygotic embryogenesis in vivo. This suggests the existence of certain genetic mechanism(s) that regulate stage-specific and nitrate-independent transcription, even though the details are not clear. It has been reported in barley that within 4 h after nitrate is supplied, the level of NR mRNA accumulation dramatically increased, whereas almost no in-

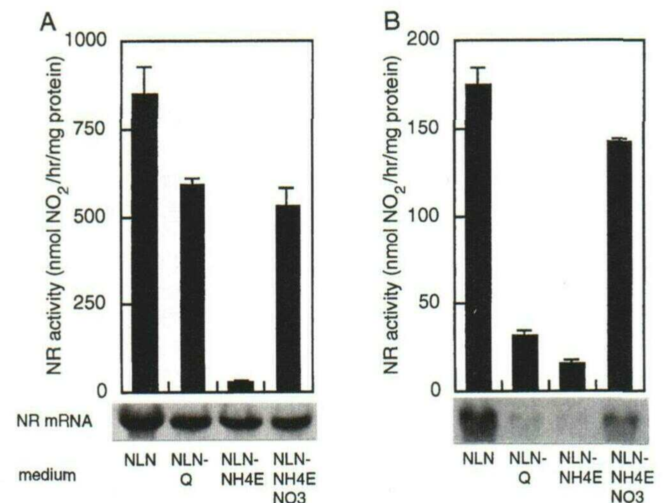

Figure 4. Relationships among nitrogen source, NR mRNA accumulation, and NADH-NR activity in 14-DAP androgenetic embryos (A) and leaves of 30-d-old seedlings (B). Media and northern hybridization signals are indicated below. For NR activity, the means and SE values of three replicates are given.

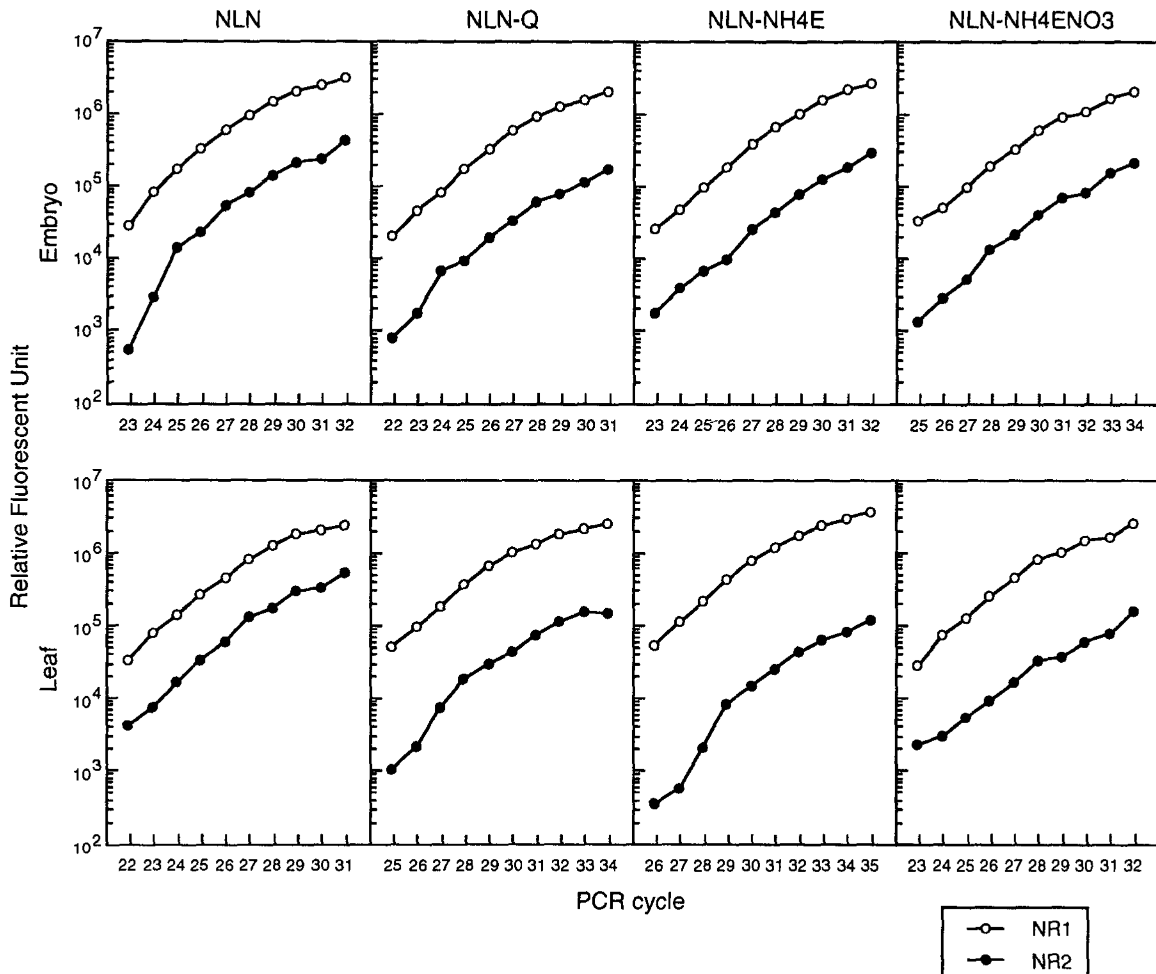

Figure 5. Quantitative RT-PCR for the two NR genes, NR1 (○) and NR2 (●), in 14-DAP embryos and leaves of 30-d-old seedlings grown in the presence of various nitrogen sources. Vertical axes show the relative fluorescence of the products in each PCR cycle. Nitrogen sources are shown at the top.

crease in nitrate concentration was detected in the tissues (Melzer et al., 1989). This observation suggests that NR transcription would not be regulated in parallel with the nitrate concentration in cells, and that a particular signal transduction cascade would be involved. When developing embryos reached the heart/torpedo-shaped stage, factor(s) that reside farther downstream than the nitrate signal may activate NR expression. Kende et al. (1971) reported that BA enhanced NR activity in excised embryos of *Agrostemma githago*. They demonstrated that after nitrate-deficient treatment, NR activity was reinduced by cytokinin but not by nitrate. At the time of reinduction, no nitrate was detected in extracts of the embryos. This result indicates a direct effect of cytokinin on NR expression without nitrate. Schmerder and Borriss (1986) reported that in dark-grown, isolated embryos of *A. githago*, NR activity was strongly induced by cytokinin and ethylene without nitrate. These results suggest that in developing rapeseed embryos, cytokinin might play an important role in nitrate-independent regulation.

Although androgenetic embryos obtained from culture in NLN-NH4E medium, like those obtained from nitrate-containing media, contained abundant NR mRNA, their enzyme activity was extremely low. Further work is required to determine whether the regulation is at the translational or the posttranslational (protein) level. NR regulation in *Chlorella* was found to involve ammonium-dependent inactivation of NR enzyme (Losada et al., 1970), and cyanide was suggested to be involved in the conversion of the enzyme into the inactive form (Solomonson, 1974; Solomonson et al., 1984). There are, however, few reports of ammonium-related posttranscriptional regulation of NR in higher plants. Deng et al. (1991) demonstrated that exogenous ammonium reduced the level of NR mRNA accumulation in tobacco roots that were grown on a relatively low concentration (1 mM) of nitrate. Because C_3 plants release to cells a high level of ammonia produced by photorespiration (Wallsgrove et al., 1983), participation of ammonium in NR regulation would be improbable, especially in leaves (Sakakibara and Sugiyama, 1991). When plants were supplied with ammonium as the sole nitrogen source, the NR mRNA level in leaves was low. The accumulation of NR mRNA in ammonium-treated embryos found in

our work would be an example of the latent relationship between ammonium and NR expression in higher plants.

Cheng et al. (1991) reported on two Arabidopsis NR genes that were regulated differently. Since our northern hybridization could not distinguish isogenes, it is possible to suppose that these complicated regulatory mechanisms were due to the isogenes that are regulated specifically in each situation. Another possibility is that the multifunctional regulatory system directed the expression of one major structural gene. From the analysis of the relative mRNA levels of the two NR genes using quantitative RT-PCR, it was suggested that with each type of nitrogen-source treatment and in both leaves and androgenetic embryos, the mRNA derived from the NR1 gene corresponding to pBnNR1405 accumulated to a much higher level compared to the mRNA derived from NR2 (pBnNR1412). Since rapeseed is amphidiploid, we cannot rule out the possibility of other expressed NR genes. However, all of the isolated 46 positive clones had specific *Eco*RV or *Pvu*II sites and all of the 14 nearly-full-length cDNA clones had 5′ and 3′ nontranslated region nucleotide sequences identical to those of pBnNR1405 or pBnNR1412. Furthermore, in the quantitative RT-PCR experiment, there were no products that were not digested by either *Pvu*II or *Eco*RV. These results suggest that a major portion of NR mRNA originates from NR1 or NR2. Thus, the results suggest that the flexibility and variability of NR activity expression specific to organ, nitrogen metabolite, and developmental stage in each situation was not due to specific isogenes but was caused principally by regulation of one major structural gene, NR1.

Our results demonstrate the unique regulation of NR genes in embryogenesis and that the state of the regulation changes depending on the developmental stage. Furthermore, investigation of the nitrogen assimilatory status must be important for the establishment of in vitro culture systems that provide efficient induction of somatic embryogenesis. The nitrogen source composition would change in the course of differentiation and development.

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