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Differential Expression of the Arabidopsis *Nia1* and *Nia2* Genes[1]

Cytokinin-Induced Nitrate Reductase Activity Is Correlated With Increased *Nia1* Transcription and mRNA Levels

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Nitrate reductase (NR) activity increased up to 14-fold in response to treatment of *Arabidopsis thaliana* seedlings with the cytokinin benzyladenine. NR induction was observed in seedlings germinated directly on cytokinin-containing medium, seedlings transferred to cytokinin medium, and seedlings grown in soil in which cytokinin was applied directly to the leaves. About the same level of induction was seen in both wild-type and *Nia2*-deletion mutants, indicating that increased NR activity is related to the expression of the minor NR gene, *Nia1*. The steady-state *Nia1* mRNA level was increased severalfold in both wild-type and mutant seedlings after benzyladenine treatment. Transcript levels of the *Nia2* gene, which is responsible for 90% of the NR activity in developing wild-type seedlings, did not show any changes upon cytokinin treatment. Nuclear run-on assays demonstrated that *Nia1* gene transcription increased dramatically after cytokinin treatment.

NR (EC 1.6.6.1) is the first enzyme of the nitrate assimilation pathway in higher plants. It reduces the major plant N source, NO_3^-, into NO_2^-, which then is further reduced to NH_3 by NO_2^- reductase. This vital, energy-consuming process is tightly regulated and responsive to various factors, including NO_3^-, NH_3, light, diurnal rhythms, plastid factors, photosynthesis status, and phytohormones (for reviews, see Redinbaugh and Campbell, 1991; Pelsy and Caboche, 1992; Crawford, 1995).

The major phytohormone that affects NR levels is cytokinin. Cytokinin induction of NR activity was first described in *Agrostemma githago* embryos (Borriss, 1967; Kende et al., 1971). Since then, similar effects have been observed in many other species (for review, see Gaudinova, 1990). Cytokinin stimulated NR activity in haploid *Nicotiana plumbaginifolia* (tobacco) cultures and was used to increase the efficiency of ClO_3^- selection for NR-deficient mutants (Márton et al., 1982a). Among the different hormones tested, only cytokinins stimulated NR activity in etiolated corn leaves (Rao et al., 1984). Banowetz (1992) found that application of BA to shoots enhanced the NO_3^- induction of NR in etiolated wheat seedlings in a concentration-dependent manner, and root-applied BA enhanced NO_3^- induction in both etiolated and light-grown seedlings.

Early results showed that cytokinin induction of NR activity involves de novo synthesis of NR (Rao et al., 1984). In split-root cultures of barley (*Hordeum vulgare* L.), external application of various cytokinins resulted in up to a 25% increase in NR mRNA in roots and up to a 100% increase in shoots of barley (Samuelson et al., 1995). In nuclear run-on assays, the addition of BA partially reversed the ABA suppression of NR gene transcription in barley (Lu et al., 1992). Protein-synthesis inhibitors depressed BA enhancement of NR activity but did not inhibit BA-enhanced NR transcription. However, posttranscriptional and translational regulation of NR activity may also occur. Using a tobacco cell-suspension culture, Suty et al. (1993) found that cytokinin exerted a specific effect on NR mRNA accumulation through modulation of polyadenylation.

Arabidopsis thaliana has two NR genes, *Nia1* and *Nia2* (Cheng et al., 1986, 1988; Wilkinson and Crawford, 1993). *Nia2* is responsible for 90% of the total NR activity in seedlings, whereas *Nia1* accounts for the remaining 10% (Wilkinson and Crawford, 1991). However, in the *Nia2*-deletion mutant G5, in which the entire *Nia2* gene is missing, *Nia1* activity alone permits normal growth on NO_3^- (Wilkinson and Crawford, 1993). The tissue-specific expression of the two Arabidopsis NR genes and their regulation in response to NO_3^-, light, and circadian rhythm were investigated by Cheng et al. (1991). The basal levels of expression of the two genes in the absence of NO_3^- are quite different. In addition, these genes exhibit differences in the kinetics of their induction by light. In subsequent work with reporter gene fusions (Chen et al., 1992; Lin and Cheng, 1997), Suc was found to induce the transcription of the *Nia1* gene, and the NO_3^--responsive regions in both NR gene promoters were identified.

Recently, we observed unexpectedly high ClO_3^- sensitivity/NR activity in cultures initiated from *Nia2*-deficient Arabidopsis seedlings grown on high-concentration cyto-

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Abbreviation: NR, nitrate reductase.

kinin medium. Increased in vivo NR activity was also detected when cytokinin was applied directly onto the leaves of soil-grown seedlings. As shown here, the induction of NR activity could only be explained by a 10-fold elevation in *Nia1* gene expression. An increased contribution of *Nia1* to the total NR activity was also observed in wild-type plants grown on high-cytokinin medium. These results indicated that the two NR genes are differentially regulated by cytokinin. The increased NR activity was accompanied by a simultaneous and specific increase in *Nia1* transcript level under standard culture conditions (light, Suc, and NO_3^-). Nuclear run-on assays demonstrated that this *Nia1* induction correlated with an increase in *Nia1* transcription.

MATERIALS AND METHODS

Plant Material and Growth Conditions

G5, a γ-irradiation-induced *Nia2*-deletion mutant of *Arabidopsis thaliana* (L.) Heynh ecotype Columbia, and G′4–3, a *Nia1/Nia2* double mutant derived from G5, were kindly provided by Dr. N.M. Crawford (Wilkinson and Crawford, 1991, 1993). Arabidopsis seed-germination and tissue-culture conditions were described previously (Czakó et al., 1993). Plants were cultured on basal Murashige-Skoog medium containing Murashige-Skoog salts, 3% Suc, 0.7% agar, 100 mg L^{-1} myo-inositol, and 10 mg L^{-1} thiamine HCl (Márton and Browse, 1991).

NR Activity Assay

In vivo NR activity was assayed by the protocol of Márton et al. (1982b). NR activity was measured in a spectrophotometric assay by determining the amount of NO_2^- released from the tissue. Seedlings were weighed and transferred into 1 mL of reaction buffer (40 mM KNO_3, 0.08 M Na_2HPO_4, 0.02 M NaH_2PO_4, and 4% [v/v] *n*-propanol, pH 7.5) and incubated in the dark for 2 h. The reaction was stopped by the addition of 200 μL of 1% sulfanilamide (dissolved in 3 N HCl) and 200 μL of 0.05% *N*-(1-napthyl)ethylenediamine hydrochloride. The concentration of NO_2^- was determined by the A_{540} of the solution. If the absorbance was higher than 0.5, the reaction solution was diluted 10-fold with reaction buffer and sulfanilamide.

The in vitro NR activity assay was adapted from the protocol of Samuelson and Larsson (1993). Arabidopsis seedlings (2 g fresh weight) were homogenized in 3 mL of extraction buffer (250 mM Tris, pH 8.0, 1 mM EDTA, 1 μM Na_2MoO_4, 5 μM FAD, 3 mM DTT, 1% [w/v] BSA, 12 mM β-mercaptoethanol, and 250 μM PMSF). The homogenate was centrifuged at 10,000*g* for 10 min. One-hundred-fifty microliters of the supernatant was added to 850 μL of reaction buffer (40 mM $NaNO_3$, 0.08 M Na_2HPO_4, 0.02 M NaH_2PO_4, pH 7.5, and 0.2 mM NADH) and incubated at room temperature for 2 h. Sulfanilamide and *N*-(1-napthyl)ethylenediamine hydrochloride were added, and NO_2^- concentration was measured as an in vivo assay.

Gel-Blot Hybridization

RNA isolation was performed as described previously (Czakó et al., 1995). Northern hybridizations were performed according to standard protocols (Sambrook et al., 1989). A 3.1-kb *Xho*I fragment from plasmid pAt60 was used as the *Nia2*-specific probe (Wilkinson and Crawford, 1991). A 1.8-kb *Eco*RI-*Hin*dIII fragment from pALCO74 was used as the *Nia1*-specific probe (Wilkinson and Crawford, 1993). An 801-bp *Eco*RI fragment from pCG22 was the cyclophilin probe, which was used as an internal control (Lippuner et al., 1994). A 1-kb *Eco*RI/*Hin*dIII fragment of a cDNA clone pCDMC13 was used as the AtDMC1 probe (Klimyuk and Jones, 1997). The probes were labeled using the Megaprime DNA-labeling kit from Amersham. Zeta-probe GT-blotting membranes were obtained from Bio-Rad. PhosphorImager (Storm 860) and image-analyzing software (Image Quant) were obtained from Molecular Dynamics (Sunnyvale, CA).

Nuclei Isolation and Run-on Assay

We adapted a nuclei isolation and run-on assay protocol from Brusslan et al. (1993). Seven grams of 12-d-old seedling leaves, harvested on ice, was ground for 6 min using a mortar and pestle in 40 mL of ice-cold modified Honda buffer (0.44 M Suc, 25 mM Tris, pH 8.5, 10 mM $MgCl_2$, 10 mM spermine, 2.5% [w/v] Ficoll 400, 4% dextran 40, 0.5% [v/v] Triton X-100, and 10 mM β-mercaptoethanol), and the homogenate was filtered through four layers of cheesecloth and one layer of Miracloth (Calbiochem). The filtrate was centrifuged at 4°C and 4500 rpm for 7 min, and the pellet was washed once with 5 mL of the same buffer minus spermine and once in a washing buffer containing 50 mM Tris, pH 8.5, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 20% (v/v) glycerol. The pellet was then resuspended in 200 μL of the same washing buffer.

For in vitro transcription, 50 μL of nuclei (5×10^7) was used in a 100-μL reaction with 100 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 500 μM ATP, GTP, and CTP, 30 μM UTP, 20 μL of 111 TBq/mmol (200 μCi) [α-^{32}P]UTP (ICN), 0.1 mM phosphocreatine, 1 mg of phosphocreatine kinase, and 80 units of RNasin (Promega). The mixture was incubated at 30°C for 30 min. The reaction was stopped by the addition of equal volumes of run-on stop buffer (2% SDS, 10 mM EDTA, 20 mM Tris, pH 7.4, and 200 μg/mL proteinase K), incubated at 42°C for 30 min, and purified by phenol-chloroform; 100 μg of yeast tRNA was added before ethanol precipitation. The RNA pellet was resuspended in 100 μL of H_2O and hybridized to DNA blots. The blots were prepared by digestion of 10 μg of plasmid DNAs, separation on 0.9% agarose gels, and transfer to Zeta-probe nylon membranes by capillary transfer.

RESULTS AND DISCUSSION

The Induction of NR Activity by Cytokinin

In previous experiments we found that the NR activity was several times higher in Arabidopsis tissue cultures

grown on high-concentration cytokinin-containing (regeneration) medium than in plantlets grown on hormone-free medium (data not shown). To investigate cytokinin induction of NR activity, seedlings of three *A. thaliana* ecotypes, Columbia, RLD, and Landsberg erecta, were tested for NR activity on Murashige-Skoog basal medium that provided an inductive amount of NO_3^-. Three hormone combinations were applied: 1 mg L^{-1} BA, 1 mg L^{-1} BA plus 0.1 mg L^{-1} NAA, and 0.1 mg L^{-1} NAA. The seedlings were subjected to two types of treatments. In treatment 1, seeds were germinated directly on hormone-containing medium. In treatment 2, 5-d-old seedlings germinated on hormone-free medium were transferred onto hormone-containing or hormone-free medium. The in vivo NR activity was measured 5 and 12 d after hormone treatment.

The induction of NR activity correlated with cytokinin treatment in all three wild-type ecotypes (Table I). NR activity in RLD and Landsberg erecta ecotypes was induced 3- to 14-fold on 1 mg L^{-1} BA-containing medium. This extent of induction was observed after 5 and 12 d in the two hormone treatments (treatments 1 and 2). The induction of NR activity in the Columbia ecotype was less dramatic than the other two ecotypes in this experiment, but all of the BA-treated seedlings showed higher NR activity. Treatment with 1 mg L^{-1} BA plus 0.1 mg L^{-1} NAA resulted in approximately the same increase in NR activity as BA treatment alone. There were no reports about auxin effects on NR activity in previous literature. In our experiments we observed a transient and moderate increase in NR activity on 0.1 mg L^{-1} NAA medium. This increase was seen only in seedlings treated with NAA for 5 d; it completely disappeared after 12 d, and the magnitude of increase was significantly less than that for BA-treated seedlings (an average 2.2-fold increase with NAA versus 5.6-fold with BA, Table I).

To distinguish the response of each NR gene to cytokinin, mutants carrying a major deletion in the *Nia2* gene were subjected to the same hormone treatment as described for the wild type. G5 in the *A. thaliana* Columbia ecotype (Wilkinson and Crawford, 1991) has a deletion of at least 25 kb at the *chl3–5* locus, including the entire *Nia2* gene. The other mutant, G′4–3, is a double mutant derived from G5 (Wilkinson and Crawford, 1993). In addition to the deletion at the *chl3–5* locus, G′4–3 also carries a point mutation in the coding region of *Nia1*, rendering its NR activity 10% that of the G5 mutant. This residual NR activity (about 1% of the wild type) is sufficient to allow some growth in soil. On NH_4^+/NO_3^--based synthetic medium in sterile cultures, the G5 and G′4–3 mutants showed about 44 and 22% of the wild-type NR activity, respectively (Tables I and II), indicating that the contribution of *Nia1* gene to NR activity is much higher than that reported for soil-grown seedlings (Wilkinson and Crawford, 1993).

NR activity in both mutants was induced more than 2.6-fold by BA and BA plus NAA treatment in seeds germinated directly on hormone-containing medium or in seedlings transferred to hormone medium after germination (Table II). In G5, all of the seedlings transferred to BA medium and the 5-d-old seedlings germinated directly on BA medium had a more than 4-fold increase in NR activity. The G′4–3 double mutant showed even higher induction by cytokinin, with seven of eight different treatments (Table II, 5 and 12 d, treatments 1 and 2, BA and BA plus NAA) and exhibited a more than 6-fold increase in NR activity. Again, this induction was not observed on me-

Table I. *In vivo NR activity from hormone-treated wild-type seedlings*

The average NR activity of five seedlings from each treatment are expressed as means ± SE ($n = 5$).

Medium	NR Activity			
	Treatment 1		Treatment 2	
	5 d	12 d	5 d	12 d
	nM NO_2^- mg^{-1} seedlings			
Columbia wild type				
ϕ[a]	610 ± 167	1608 ± 242	1785 ± 271	1025 ± 232
NAA[b]	928 ± 290	1485 ± 477	1950 ± 155	813 ± 135
BA[c]	1700 ± 387	1903 ± 290	3304 ± 382	2438 ± 341
BA + NAA[d]	1904 ± 616	2194 ± 660	3482 ± 257	3150 ± 355
RLD wild type				
ϕ	422 ± 132	905 ± 233	896 ± 187	855 ± 166
NAA	1182 ± 380	1519 ± 240	2023 ± 167	503 ± 67
BA	1470 ± 345	5501 ± 787	4478 ± 800	4991 ± 788
BA + NAA	5993 ± 1426	4012 ± 665	5799 ± 986	3290 ± 896
Landsberg erecta				
ϕ	498 ± 86	927 ± 128	1102 ± 290	523 ± 90
NAA	1919 ± 321	861 ± 120	1722 ± 251	442 ± 104
BA	3027 ± 689	4054 ± 585	8213 ± 1029	4168 ± 824
BA + NAA	5110 ± 819	3318 ± 373	5566 ± 1336	4083 ± 1034

[a] ϕ, Murashige-Skoog basal medium without any phytohormone. [b]NAA, Murashige-Skoog basal medium plus 0.1 mg L^{-1} NAA. [c]BA, Contained 1 mg L^{-1} BA. [d]BA + NAA, Contained 1 mg L^{-1} BA plus 0.1 mg L^{-1} NAA.

Table II. *NR activity from hormone-treated mutant seedlings*

The average NR activity of five seedlings from each treatment are expressed as means ± SE ($n = 5$).

Medium[a]	NR Activity			
	Treatment 1		Treatment 2	
	5 d	12 d	5 d	12 d
	nM NO_2^- mg^{-1} seedlings			
G5 mutant				
ϕ	270 ± 38	589 ± 214	380 ± 54	366 ± 62
NAA	274 ± 87	669 ± 193	530 ± 50	377 ± 55
BA	1179 ± 301	1526 ± 360	1629 ± 242	1652 ± 244
BA + NAA	1137 ± 296	1682 ± 105	1790 ± 440	2091 ± 433
G′ 4–3 mutant				
ϕ	136 ± 49	395 ± 72	293 ± 59	273 ± 26
NAA	145 ± 30	506 ± 78	781 ± 115	531 ± 31
BA	994 ± 277	2518 ± 499	2067 ± 112	1768 ± 135
BA + NAA	855 ± 182	1626 ± 295	1735 ± 191	1925 ± 301

[a] See Table I for explanation of media.

dium containing NAA only. Thus, the effect of cytokinin on induction of *Nia1* activity is clear.

Additional experiments demonstrated that in vitro NR activity was also increased similarly by cytokinin treatment to the above in vivo NR activity. Twelve-day-old Columbia wild-type seedlings showed a 2.3-fold increase in in vitro NR activity when treated with 1 mg L^{-1} BA (460.5 ± 26.5 versus 1066.5 ± 49.5 nM NO_2^- h^{-1} mg^{-1} tissue in BA-treated seedlings; 16.4 μg of proteins were extracted out of 1 mg of tissue). The G5 mutant showed a 4.5-fold increase in in vitro NR activity following the same treatment (117.1 ± 4.5 versus 523.2 ± 76.2 nM NO_2^- h^{-1} mg^{-1} tissue in BA-treated seedlings). The in vitro results suggest that any indirect effects on NR activity, such as cofactor availability or changes in overall posttranscriptional regulation, do not account significantly for the increased NR activities.

We also examined the NR activity of soil-grown seedlings with and without cytokinin treatment. About 100 Arabidopsis seedlings grown in a 4-inch (diameter) pot for 10 d were sprayed with 2 mL of a 1 mg L^{-1} BA solution. Both the Columbia wild type and the G5 mutant demonstrated higher NR activity when assayed after 48 h. The in vivo NR activity was 1.7-fold higher in BA-treated Columbia seedlings (98.1 ± 14.5 versus 166.6 ± 61.0 nM NO_2^- mg^{-1}) and 4.5-fold higher in BA-treated G5 seedlings (20.2 ± 17.2 versus 91.2 ± 37.6 nM NO_2^- mg^{-1}). This result shows that cytokinin induction occurs not only under sterile culture conditions but also in soil-grown seedlings given an external application of cytokinin.

When the seedlings were germinated on different concentrations of BA medium, we observed that the minimum concentration of hormone needed to induce a significant increase of in vivo NR activity was 0.05 mg L^{-1} (1.3×10^{-7} M, $P < 0.05$). This result is comparable to previous reports using other plant species (Kende et al., 1971; Banowetz, 1992).

To estimate the contribution of the major NR gene *Nia2* to the increased NR activity in cytokinin-treated wild-type plants, the NR activity of the *Nia2*-deletion mutant G5, which expresses only the *Nia1* gene (Columbia background), was subtracted from the NR activity of the Columbia wild type of the same treatment. For example, in the 5-d-old seedlings germinated directly on 1 mg L^{-1} BA medium (Tables I and II, treatment 1), the total NR activity (Columbia) changed from 610 to 1700 nM NO_2^- mg^{-1} seedlings (up by 1090 nM NO_2^- mg^{-1} seedlings); the *Nia1* activity (G5) changed from 270 to 1179 nM NO_2^- mg^{-1} seedlings (up by 909 nM NO_2^- mg^{-1} seedlings); therefore, the derived "*Nia2* activity" changed from 340 to 519 nM NO_2^- mg^{-1} seedlings (up by 179 nM NO_2^- mg^{-1} seedlings). The above calculation suggested that the increased NR activity derives predominantly from the *Nia1* gene and the contribution of the *Nia1* gene to the total NR activity is much higher after cytokinin induction. However, this calculation may not accurately reflect the *Nia2* activity in Columbia wild type because the G5 mutant may have different *Nia1* expression, but these data prompted us to study *Nia1* and *Nia2* expression at the RNA level.

Increased *Nia1* Steady-State RNA Level in Cytokinin-Treated Plants

Total RNA was extracted from 12-d-old seedlings germinated directly on hormone-containing medium. *Nia1* transcript levels were as much as 10-fold higher in both G5 and G′4–3 mutant seedlings treated with BA than in nontreated ones (Fig. 1A). *Nia1* transcript levels also increased significantly in all three wild types (Fig. 1B) grown on BA medium. However, *Nia2* RNA did not show any noticeable increase (Fig. 1C). Therefore, the increased NR activity in these plants may be due to an increased mRNA level of the *Nia1* gene, not the *Nia2* gene. However, the possible contribution of increased *Nia1* and *Nia2* mRNA stability to the high NR activity and high mRNA levels was not investigated in these experiments.

Increased *Nia1* Transcription in Cytokinin-Treated Plants

To examine the transcriptional regulation of *Nia1* in cytokinin-induced plants, nuclei were isolated from seed-

lings germinated directly on medium with or without hormone, and nuclear run-on assays were performed. *Nia1* transcripts were not detectable in mutant G5 seedlings without cytokinin treatment but were present at high levels in G5 seedlings treated with cytokinin (Fig. 2A). The positive internal control was cyclophilin, the expression of which is constant under various growing conditions and developmental stages (Lippuner et al., 1994). The meiosis-specific *AtDMC1* gene is expressed only during gametogenesis in floral development and was used as a negative internal control (Klimyuk and Jones, 1997). Similarly, *Nia1* transcription is also induced by cytokinin in Columbia wild type (Fig. 2B), whereas *Nia2* transcription did not show any significant difference in the treated versus untreated seedlings (Fig. 2B). Previous work by others with corn and barley seedlings had suggested that the induction of NR activity by cytokinin was regulated at the level of transcription (Rao et al., 1984; Lu et al., 1992). Our results here show that this is true in Arabidopsis as well. However, this transcriptional regulation is due to a specific increase only in *Nia1* transcription.

In conclusion, total NR activity dramatically increased in Arabidopsis cultures containing cytokinin or a high-cytokinin/low-auxin hormone combination. The relative contribution of the *Nia1* gene to the total NR activity increased significantly, indicating a strong cytokinin response of *Nia1* but not *Nia2*. At the same time, the steady-state levels of the *Nia1* mRNA increased significantly and specifically. Specific induction of NR activity by cytokinin is accompanied by increased transcription of the *Nia1* gene in both mutant and wild-type plants, as judged from nuclear run-on assays. The level of *Nia2* transcripts did not change as a result of cytokinin treatment. Since increased NR activity was observed during more than 2 weeks in culture, the role of cell division cannot be excluded. Epigenetic changes and/or higher *Nia1* expression preferentially in the dividing adventitious meristem cell population could also explain the observed dramatic changes in total NR activity and in transcription of the *Nia1* gene. Further investigations using a series of truncated promoter-reporter gene fusions and tissue printing to investigate the tissue-specific expression of *Nia1*, as well as epigenetic studies to search for the mechanism of the long-term effect of cytokinin on *Nia1* expression, are under way.

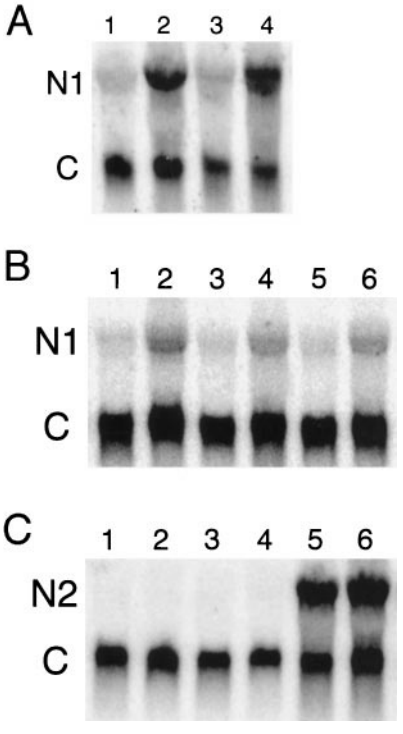

Figure 1. Autoradiograms of an RNA gel blot from wild-type and mutant plants hybridized with *Nia1*- and *Nia2*-coding region-specific probes separately. N1, *Nia1* transcript; N2, *Nia2* transcript; and C, constitutive cyclophilin control. In A, lane 1 is the total RNA extracted from G'4–3 mutant seedlings without BA treatment; lane 2 is the RNA of G'4–3 seedlings treated with BA; lane 3 is the G5 mutant without BA treatment; and lane 4 is the G5 mutant with BA. In B, lanes 1, 3, and 5 are Columbia, RLD, and Landsberg erecta wild type, respectively, without BA treatment. Lanes 2, 4, and 6 were treated with BA. In C, lanes 1, 3, and 5 are G'4–3, G5, and Columbia wild-type seedlings without BA and lanes 2, 4, and 6 are seedlings with BA.

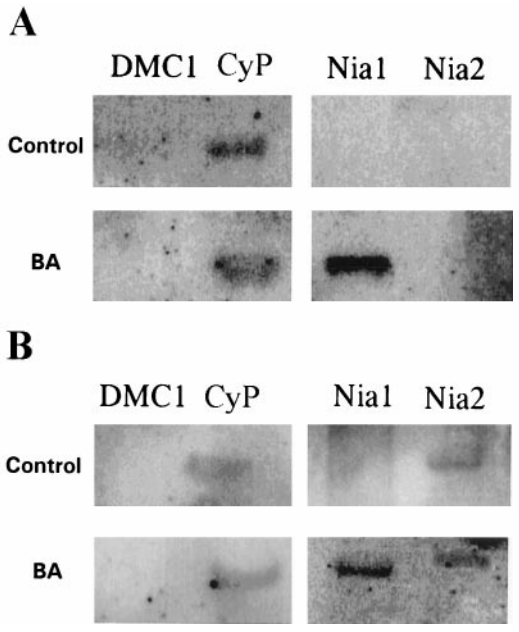

Figure 2. Nuclear run-on assay of the G5 mutant (A) and Columbia wild type (B). DMC1, Meiosis-specific gene used as a negative control; and CyP, cyclophilin constitutive control.

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