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A novel dual-specificity protein kinase targeted to the chloroplast in tobacco[1]

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Abstract The *NtDSK1* cDNA encoding a novel chloroplast-targeted protein kinase was identified in *Nicotiana tabacum*. It contains the kinase domain at the C-terminus and a putative regulatory domain at the N-terminus. The recombinant NtDSK1 underwent autophosphorylation of serine, threonine, and tyrosine residues, indicating that *NtDSK1* encodes a functional dual-specificity protein kinase. The NtDSK1–green fluorescent protein fusion protein was targeted to chloroplasts. Furthermore, the NtDSK1 protein was immunodetected in chloroplast fractions isolated from tobacco seedlings. The *NtDSK1* mRNA expression was developmentally regulated in different tissues, including anthers and germinating seeds, and strongly stimulated by gibberellin. The mRNA was rapidly light responsive during seedling growth. NtDSK1 may play a role in a light-regulated signaling process in tobacco. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Chloroplast-targeted; Dual-specificity protein kinase; Light- and gibberellin-stimulated expression; NtDSK1; Tobacco

1. Introduction

Protein kinases comprise the largest known family of proteins. Various cellular processes are controlled by phosphorylation of proteins on tyrosine and serine/threonine residues, carried out by protein tyrosine kinases and protein serine/threonine kinases. Dual-specificity protein kinases are a group of protein kinases which are able to phosphorylate both tyrosine and serine/threonine residues [1]. In plants, characterization of dual-specificity protein kinases that do not belong to the MAP kinase kinase family is restricted to ADK1 from *Arabidopsis* [2], PRK1 from petunia [3], and PK12 from tobacco [4]. PRK1 is a predominantly pollen-expressed receptor-like kinase which autophosphorylates on serine and tyrosine [3]. PRK1 has been shown to play an essential role in pollen and embryo sac development in *Petunia inflata* [5,6]. PK12 is a member of the LAMMER kinases. PK12 activity is rapidly and transiently increased when plants are treated with ethylene [4]. The PK12 kinase phosphorylates serine/arginine-rich (SR) splicing factors in vivo to alter their activity and the splicing of target mRNAs [7]. ADK1 was isolated by screening an *Arabidopsis* expression library with anti-phosphotyrosine antibodies. ADK1 autophosphorylated serine, threonine, and tyrosine residues and was also capable of phosphorylating tyrosine residues on poly(Glu/Tyr) as a substrate. However, the function of ADK1 is unknown [2].

In this study, we attempted to isolate kinase cDNAs that are preferentially expressed in the tobacco anthers. As a result, a cDNA encoding a novel dual-specificity protein kinase targeted to chloroplasts was obtained. The cDNA was designated *NtDSK1* (*Nicotiana tabacum* dual-specificity kinase 1). This is the first dual-specificity protein kinase that is localized in chloroplasts. *NtDSK1* mRNA expression was stimulated by gibberellin (GA), by light, and during various developmental processes including seed germination. The possible function of NtDSK1 is discussed based on these results.

2. Materials and methods

2.1. Plant materials

Tobacco plants (*N. tabacum* cv. Xanthi) were cultivated in a greenhouse under a regime of 16 h light and 8 h dark.

2.2. Autophosphorylation and phosphoamino acid analysis

A PCR product corresponding to the kinase domain (residues 294–610) of NtDSK1 was obtained using Pwo DNA polymerase (Boehringer-Mannheim, Germany). It was subcloned into the pET32a vector (Novagen, USA) using *Bam*HI/*Sal*I sites. The construct was introduced into *Escherichia coli* in order to express the kinase domain fused to the C-terminus of thioredoxin. After purification using nickel resin (New England Biolabs, UK), the fusion protein was digested with thrombin for 12 h at 4°C to remove the thioredoxin moiety. Autophosphorylation assays and phosphoamino acid analyses were carried out according to [8].

2.3. Subcellular localization of the NtDSK1–green fluorescent protein (GFP) protein

The *NtDSK1* cDNA corresponding to amino acid residues 1–610 was PCR-amplified using Pwo DNA polymerase to generate *Bam*HI sites at both the 5′ and 3′ ends. This PCR-amplified DNA was cloned into the CD3-326 plasmid using *Bam*HI sites to generate pNtDSK1-soluble modified (sm) GFP, which contained NtDSK1–smGFP in-frame fusion under the control of the cauliflower mosaic virus 35S promoter. The pNtDSK1–smGFP and control CD3-326 plasmids were introduced into protoplasts prepared from *Arabidopsis* seedlings by polyethylene glycol treatment as described in [9]. After 16 h incubation, the protoplasts were viewed under a fluorescence microscope.

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2.4. Chloroplast preparation

Crude chloroplasts were prepared from tobacco seedlings as described in [10]. The day before chloroplast isolation, plants were kept in the dark to deplete chloroplasts of accumulated starch grains. The seedlings were harvested with cold grinding buffer (0.33 M sorbitol, 50 mM HEPES–KOH, pH 7.3, 2 mM EDTA, and 0.1% bovine serum albumin (BSA)) on ice. The seedlings were homogenized with a Polytron (Kinematica GmbH, Switzerland) set at 70% power by three bursts of 5 s each. The homogenate was filtered through two layers of Miracloth into precooled polycarbonate centrifuge tubes. Chloroplasts were pelleted by centrifugation at 3000×*g* for 5 min. The supernatant was removed and stored as the supernatant fraction. Chloro-

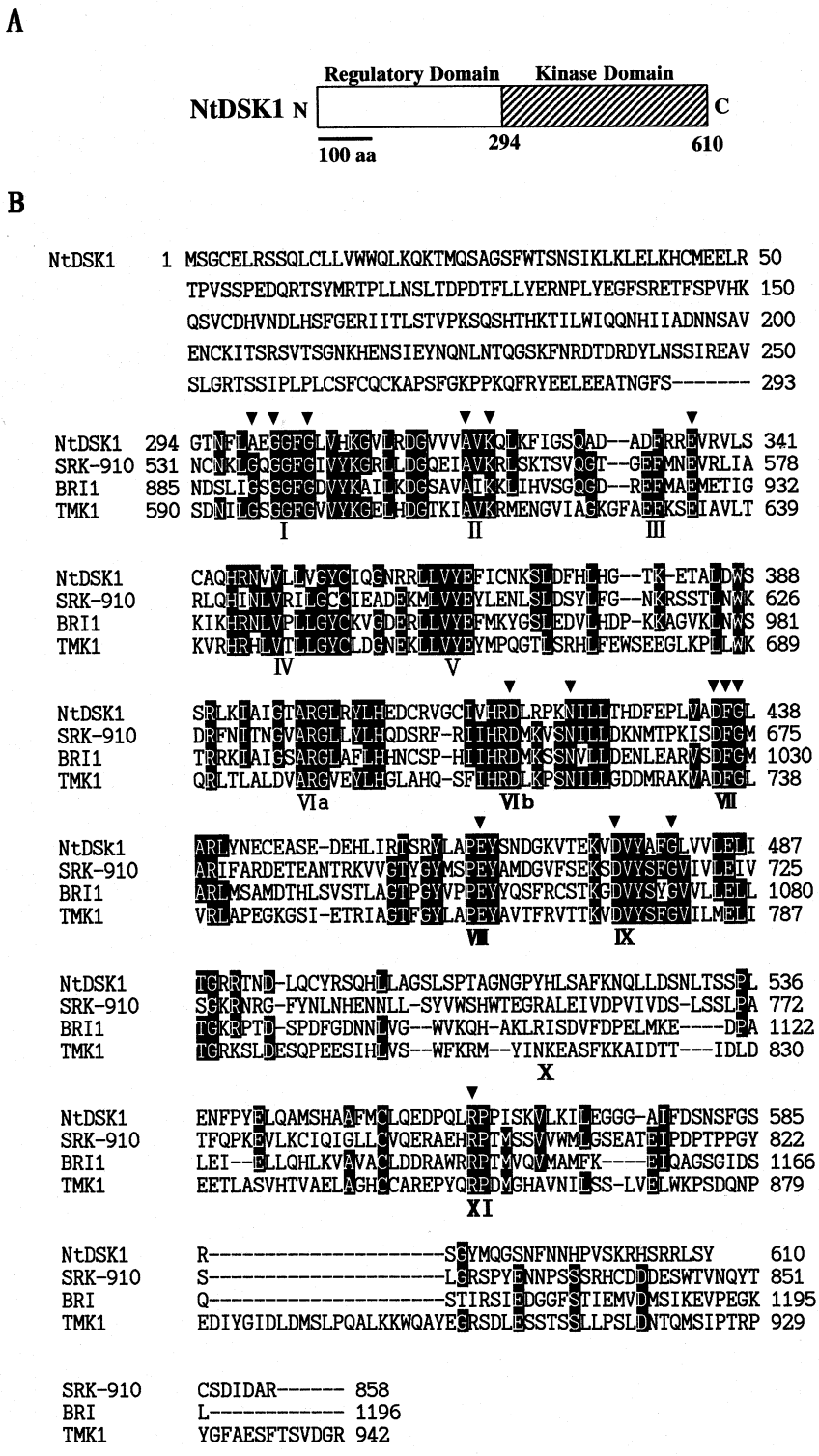

Fig. 1. Structure and amino acid sequence comparison of NtDSK1. A: Schematic representation of NtDSK1. B: Deduced amino acid sequence of NtDSK1 and alignment with related sequences. The NtDSK1 sequence is aligned with the kinase domain of SRK-910, TMK, and BRI1. The number on the right indicates the amino acid residues. Gaps are indicated by dashes (–). Residues that are conserved among at least three of the compared sequences are highlighted in reverse contrast letters. Arrowheads (▼) indicate invariant residues in eukaryotic serine/threonine-type kinases.

plasts were gently suspended in the same buffer with a fine brush. The volume was increased to 30 ml, the suspension was transferred to sterile Corex tubes, and chloroplasts were pelleted at 3000×*g* for 5 min. The chloroplast fraction was examined under a light microscope. Protein concentration was measured by the Bradford method [11] using the Bio-Rad protein assay kit and BSA as the standard.

2.5. Immunoblotting

The NtDSK1-specific polyclonal rabbit antibody was prepared using the recombinant regulatory domain of NtDSK1 in Iyhyo Biotech (Kwangju, South Korea). Immunoblotting was carried out as described in [12]. Nitrocellulose membranes containing 10 μg each of the total proteins from uninduced (UI) and induced (I) *E. coli* cells that carry pMAL plasmid (New England Biolabs, UK) containing NtDSK1 regulatory domain, or 70 μg each of proteins from chloroplast fractions and the supernatant containing non-chloroplastic materials isolated from tobacco seedlings were prepared. The membranes were blocked with 5% skim milk in Tris-buffered saline and 0.05% Tween 20 (TBST), reacted with anti-NtDSK1 antibody (1:2000 dilution) in TBST, and washed with TBST. They were then reacted with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:5000 dilution; Amersham, USA), and the signal was detected by ECL+Plus (Amersham, USA).

3. Results and discussion

3.1. Isolation of a cDNA clone encoding a protein kinase in N. tabacum

Reverse transcription-PCR with degenerate kinase primers [8] and the subsequent screening of a tobacco flower cDNA library with one of the PCR-amplified DNA fragments resulted in isolation of a cDNA clone, designated *NtDSK1*. Among four independent clones obtained, the longest cDNA was 2414 bp in length and encoded a protein of 610 amino acids with the structural features of a protein kinase (Fig. 1A,B). Its predicted molecular weight was 68 870 Da. The N-terminal region of NtDSK1 (residues 1–293) contained a novel sequence that did not show any significant sequence similarity with proteins in the plant database. This region may constitute a regulatory domain that modulates the kinase activity of NtDSK1. The C-terminal region contained 12 conserved subdomains of eukaryotic protein kinases (Fig. 1B). Fifteen invariant amino acid residues for protein kinases were conserved in the kinase domain of NtDSK1, with the exception of one glycine residue that was replaced by alanine in NtDSK1 (Ala299 in subdomain I). The amino acid sequence of this clone was analyzed by SMART comparative sequence analysis (http://coot.embl-heidelberg.de/smart-103/), and was predicted to encode a dual-specificity protein kinase.

The amino acid sequence of NtDSK1 and comparison of the sequence with other plant kinases using the CLUSTAL W multiple sequence alignment program (version 1.7: http://dot.imgen.bcm.tmc.edu:9331/multi-align/) are shown in Fig. 1B. The kinase domain of NtDSK1 is most closely related to the kinase domains of TMK1 [13] from *Arabidopsis* (44% identity), SRK-910 [14] from *Brassica* (37% identity), and BRI1 [15] from *Arabidopsis* (36% identity). BRI1 and TMK1 both belong to the group of LRR receptor-like kinases that contain leucine-rich repeats in their extracellular domain, while SRK-910 belongs to the SRK class of receptor-like kinases that contain SLG-like sequences in the extracellular domain.

3.2. NtDSK1 encodes a functional dual-specificity protein kinase

In order to determine if NtDSK1 encodes a functional dual-specificity protein kinase, an autophosphorylation assay was performed with the recombinant kinase domain (residues 294–610) of NtDSK1. After removal of the thioredoxin moiety by thrombin, the resulting kinase domain was assayed for autophosphorylation. A single 35-kDa band was detected by autoradiography (Fig. 2A), the position of which matched to that of the kinase domain, as determined by Coomassie blue staining. In order to determine if autophosphorylation requires an active kinase, the mutant form of the kinase domain, which carries a mutation in the essential ATP binding site (Lys320 to Asn), was prepared in the same way and was analyzed for kinase activity. The mutation drastically reduced radiolabeling of the 35-kDa band, indicating that autophosphorylation activity is dependent on a functional kinase (Fig. 2A). To study the specificity of kinase activity, the phosphoamino acid analysis was carried out (Fig. 2B). Major ^{32}P-labeled spots corresponded to the positions of phosphoserine, phosphothreonine, and phosphotyrosine, indicating that NtDSK1 possesses dual-specificity kinase activity.

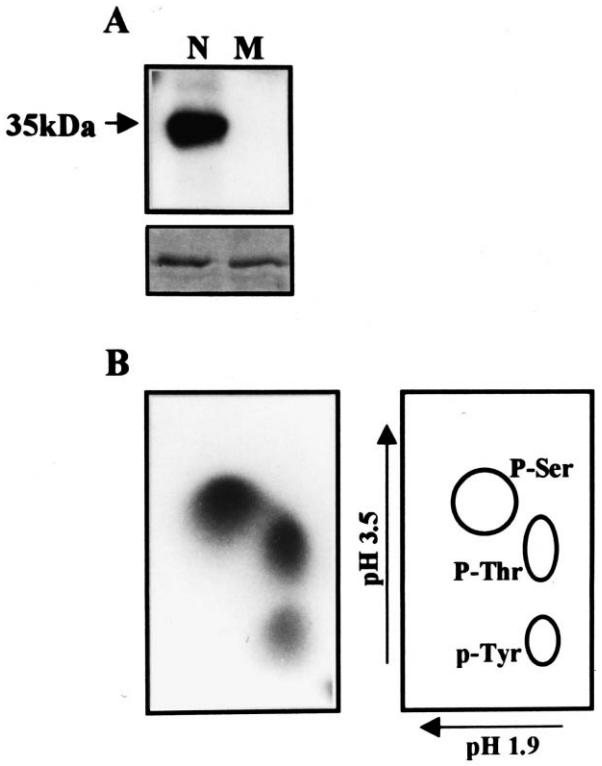

Fig. 2. Phosphorylation of the recombinant kinase domain of NtDSK1. A: Autophosphorylation. Top: autoradiography; bottom: a Coomassie blue-stained gel showing relative amounts of the recombinant proteins. Normal (N) and mutant (M) forms of the kinase domain are indicated. In the mutant kinase the essential ATP binding site Lys320 was mutated to Asn. B: Phosphoamino acid analysis of the autophosphorylated NtDSK1. The ^{32}P-labeled kinase domain was hydrolyzed with HCl and subjected to two-dimensional thin-layer electrophoresis. The positions of phosphoserine, phosphothreonine, and phosphotyrosine are indicated.

3.3. Genomic DNA gel blot of NtDSK1

DNA gel blot analysis was performed with tobacco genomic DNA digested with restriction enzymes (Fig. 3). The probe was the 0.4-kb PCR fragment corresponding to the C-terminal region of NtDSK1. With the probe, both *Eco*RI and *Hin*dIII digestion resulted in two hybridizing bands. Con-

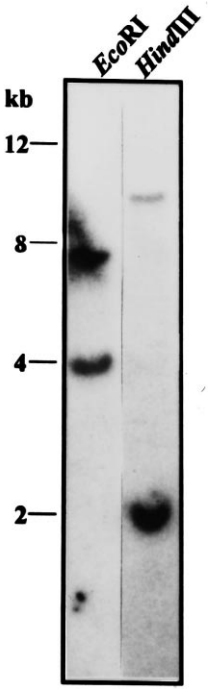

Fig. 3. Genomic DNA gel blot analysis of the *NtDSK1* gene. Each lane contains 10 μg of plant genomic DNA digested with restriction enzymes. DNA preparation, prehybridization, hybridization, and washing were carried out as described in [8]. DNA markers are indicated in kb.

sidering that *N. tabacum* is amphidiploid between *Nicotiana tomentosiformis* and *Nicotiana sylvestris*, these results indicate that the tobacco genome contains a single copy of the *NtDSK1* gene (Fig. 3).

3.4. *NtDSK1 mRNA accumulation is developmentally regulated and strongly stimulated by gibberellin*

Using RNA gel blot analysis, a 2.6-kb transcript was detected at a low level in leaves, stems and flowers, but in roots the transcripts were barely detectable (Fig. 4A). The size of the transcript was consistent with the size of the isolated cDNA. During flower development, the *NtDSK1* mRNA

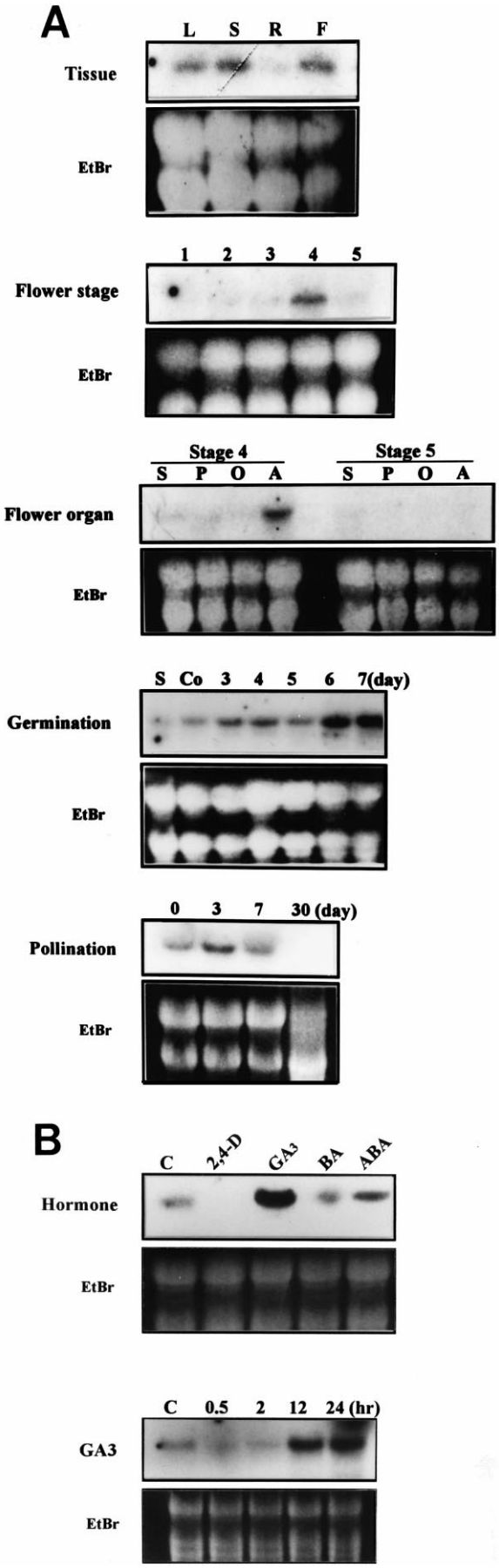

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Fig. 4. Expression of the *NtDSK1* mRNA. RNA preparation, prehybridization, hybridization, and washing were carried out as described in [8]. The 0.4-kb PCR fragment corresponding to the C-terminal end of the *NtDSK1* cDNA was used as a probe. Each lane contains 100 μg of total RNA. A: Expression in plant tissues at different developmental stages. (Tissue-specific expression) roots (R), stems (S), leaves (L), and flowers (F); (expression during flower development) stage 1, <1 cm buds; stage 2, 1–2 cm; stage 3, 2–3 cm; stage 4, 3–4 cm; stage 5, open flowers; (expression in floral organs) sepals (S), petals (P), pistils (O), and anthers (A); (expression during germination) mature seeds (S), 3-day cold-treated seeds (Co), and water-imbibed seeds for the indicated time (3–7 days) after 3-day cold treatment; (expression after pollination) ovaries before anthesis (0), and ovaries at 3, 7, and 30 days after pollination are indicated. B: Expression of the *NtDSK1* mRNA in response to GA. Top: young tobacco leaves were cut and their petioles were put into hormone solutions at 100 μM of auxin (2,4-D), abscisic acid (ABA), GA (GA_3), and cytokinin (BA) for 12 h. Control leaves were treated with water in the same manner. Bottom: petioles were put into GA_3 solution (100 μM) for indicated times.

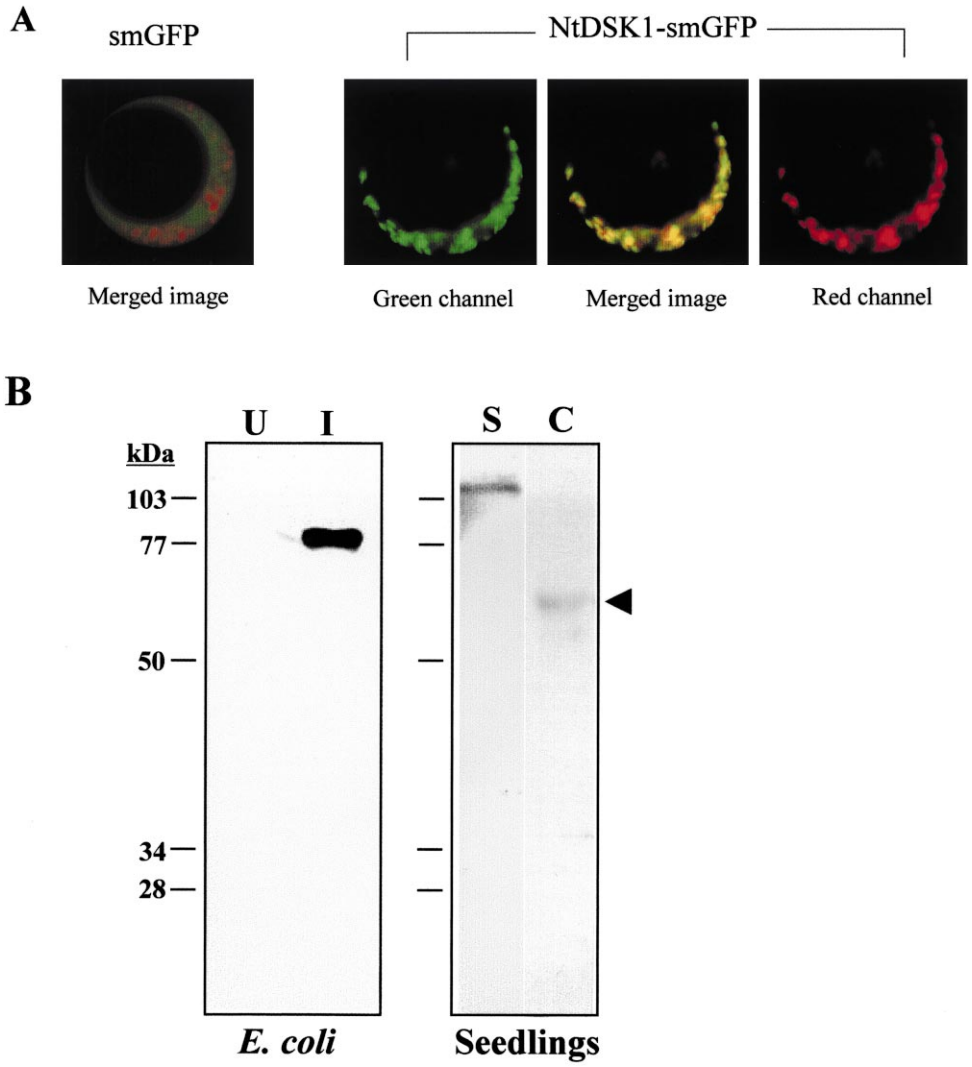

Fig. 5. Subcellular localization of the NtDSK1 protein. A: Localization of the NtDSK1–smGFP fusion protein. B: Immunodetection of the NtDSK1 protein in the chloroplast fractions. Left: protein extracts from uninduced (U) and induced (I) *E. coli* cells that carry the pMAL–NtDSK1 plasmid were subjected to immunoblotting with the NtDSK1 antibody. The fusion protein between maltose binding protein and NtDSK1 regulatory domain was detected only after induction. Right: proteins of chloroplast (C) and supernatant (S) fractions prepared from tobacco seedlings were subjected to immunoblotting with the antibody. The putative NtDSK1 protein is marked with the arrowhead.

was most highly expressed in stage 4 (just prior to anthesis). In stage 4 *NtDSK1* mRNA was predominantly expressed in the anther. Mature seeds contained a barely detectable level of the *NtDSK1* mRNA, but the mRNA levels increased after 3 days of cold treatment and kept increasing up to 7 days of imbibition. Just before anthesis the ovaries contained a low level of *NtDSK1* mRNA, but the level increased at 3 days after pollination, then decreased to a barely detectable level at 30 days (Fig. 4A).

Next we examined changes in the *NtDSK1* mRNA level in response to various stimuli (Fig. 4B). GA (GA_3) treatment resulted in strong induction of *NtDSK1* expression. Abscisic acid and cytokinin did not affect the transcript level significantly, while auxin treatment reduced the mRNA level. GA_3 at the concentration of 10–100 μM was effective in stimulating *NtDSK1* mRNA expression (results not shown). GA_3 (100 μM) treatment caused an increased transcript level at 12 h after treatment, which was maintained until 24 h after treatment (Fig. 4B).

3.5. Localization of the NtDSK1 protein in chloroplasts

Cellular localization of NtDSK1 was examined by constructing a fusion protein between NtDSK1 and a GFP (NtDSK1–smGFP). After transient expression of DNA constructs, encoding NtDSK1–smGFP or smGFP, an individual protoplast was examined under a fluorescence microscope. In contrast to the cytosol-localized staining pattern of smGFP, fluorescence of the NtDSK1–smGFP protein was associated with chloroplasts, indicating that NtDSK1 is primarily targeted to the chloroplast (Fig. 5A).

We further investigated localization of NtDSK1 in the chloroplast using immunoblotting. Polyclonal rabbit antiserum was raised using the recombinant regulatory domain of NtDSK1. After purification, the antiserum specifically recognized the fusion protein between the maltose binding protein and the NtDSK1 regulatory domain from *E. coli* total proteins, only after induction (Fig. 5B, left). Crude chloroplast fractions and the supernatant containing non-chloroplastic materials were prepared from tobacco seedlings. The antise-

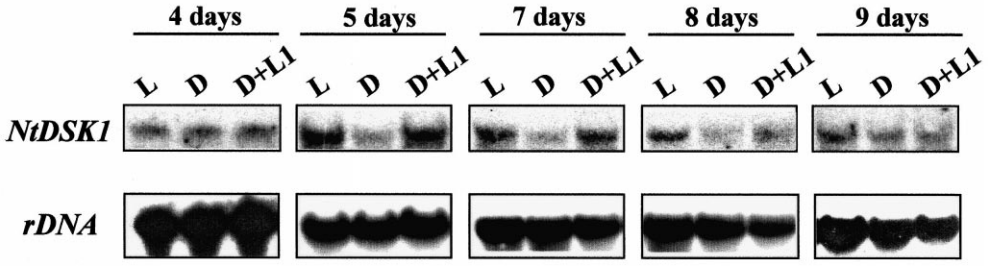

Fig. 6. Light-stimulated expression of *NtDSK1*. RNA gel blot analysis was carried out with RNA from 4–9-day-old tobacco seedlings grown for indicated times after 3-day cold treatment under continuous light (L), under dark (D), or grown under dark and then transferred to the light for 1 h (D+L1). The 0.4-kb *NtDSK1* probe and the ribosomal DNA probe were used.

rum detected a single protein of approximately 65 kDa in the chloroplast fraction (Fig. 5B, right), while a protein of approximately 120 kDa was detected in the supernatant. Preimmune serum did not detect any signal (results not shown). Based on the expected size of NtDSK1 and localization of the NtDSK1–GFP protein in the chloroplast, the 65-kDa protein in the chloroplast fraction likely represents NtDSK1. The 120-kDa protein detected in the supernatant may be a NtDSK1-unrelated protein containing similar epitopes.

3.6. NtDSK1 expression is rapidly stimulated by light

Using RNA gel blot analysis, *NtDSK1* mRNA levels were examined in 4–9-day-old tobacco seedlings grown on MS media under continuous light, under dark, or grown under dark and then transferred to the light for 1 h (Fig. 6). In 5–8-day-old seedlings, the *NtDSK1* mRNA level was lower in dark-grown seedlings than light-grown seedlings, but exposure to light for 1 h after the dark-grown period increased the *NtDSK1* mRNA level to the level of light-grown seedlings. Interestingly, there was no visible difference in the mRNA levels between light- and dark-grown seedlings at 4 days of growth when cotyledons were not fully opened, and at 9 days of growth. The results indicate that *NtDSK1* expression is rapidly stimulated by light, but to different degrees depending on the growth stage.

Using NtDSK1–GFP fusion and immunoblotting with the NtDSK1 antibody (Fig. 5A,B), it has been shown that the NtDSK1 protein is targeted to the chloroplast. The number and identity of the protein kinases and phosphatases localized in chloroplasts are still unclear. NtDSK1 is the first dual-specificity protein kinase targeted to chloroplasts. In chloroplasts, light-driven redox reactions provide signals for self-regulation of photosynthetic electron flow, regulating energy transfer via reversible phosphorylation of LHCII antenna [16]. More recent data suggest that redox-controlled protein phosphorylation in chloroplasts has a much wider physiological significance as exemplified by its involvement in the regulation of gene expression and stress-related responses [17]. *NtDSK1* mRNA accumulation is stimulated by light (Fig. 6) and H_2O_2 treatment (Cho and Pai, unpublished) in tobacco seedlings, implicating a role of NtDSK1 in light-induced stress responses.

Alternatively, NtDSK1 may be involved in GA signal transduction. GAs regulate various cellular processes, including seed germination, internode elongation, mobilization of seed reserves in cereals, seed development, and anthocyanin biosynthesis [18,19]. The expression patterns of the *NtDSK1* mRNA during plant development and upon GA treatment indicate that expression of *NtDSK1* may be regulated by changes of endogenous GA levels in tobacco. The GA content increases during seed germination and the initial stage of seed development [18,20]. GA levels also increase in stamens prior to anthesis in a number of species [21]. NtDSK1 may be an example of a signaling component whose expression is upregulated by the corresponding signals. GA response of plants is influenced by light [22]. Previous studies have suggested that light enhances both GA biosynthesis and tissue sensitivity to GA in various plant species [22,23]. NtDSK1 may mediate cross-talk between light and GA signal transduction. Interestingly, copalyl diphosphate synthase and *ent*-kaurene synthase, the enzymes catalyzing the first step of GA biosynthesis, are translocated into plastids [24]. It would be interesting to examine if NtDSK1 has the ability to regulate GA biosynthesis and/or signaling in response to light. Despite these possibilities, in vivo functions of NtDSK1 remain to be demonstrated. Molecular genetic approaches, such as antisense RNA techniques and dominant negative mutants, would be direct approaches to determine the functions of NtDSK1. In addition, identifying interacting signaling components may also provide insights into cellular functions of NtDSK1.

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