Expression Patterns of the *NPP1* Protein Phosphatase Gene and Biochemical Activity of Its Encoded Protein

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The *NPP1* cDNA encoding the catalytic subunit of a type 1 serine/threonine protein phosphatase (PP1) was previously cloned and characterized in *Nicotiana tabacum* (Plant Mol Biol 36, 315-322, 1998). In this study, the expression patterns of *NPP1* mRNA in response to various stimuli were examined to gain insight on the cellular function of the NPP1 protein. *NPP1* mRNA accumulation was stimulated by Ca^{++}, wounding, fungal elicitiors, and chitosan in leaves. However, with abscisic acid treatment, no change in transcript levels was observed. The recombinant NPP1 protein was catalytically active and could dephosphorylate the autophosphorylated recombinant NtCDPK1, a calcium-dependent protein kinase from tobacco.

Keywords: dephosphorylation, NPP1, stress-inducible, type 1 Ser/Thr protein phosphatase

The reversible phosphorylation of proteins, catalyzed by protein kinases and phosphatases, is a major mechanism for the regulation of almost all cellular functions from metabolism, signal transduction, and cell division (Mumby and Walter, 1993; Shenolikar, 1994). While a large number of protein kinases involved in these regulatory processes has been studied for many years, the number and variety of protein phosphatases have only been appreciated more recently. Phosphatases are a complex family of proteins, which is divided into protein serine/threonine phosphatases (PPs) and protein tyrosine phosphatases (PTPs). Based on their biochemical and structural characteristics, the protein Ser/Thr phosphatases are further divided into PP1, PP2A, PP2B, and PP2C. In the past few years, our knowledge on PPs has grown significantly, and the data have revealed that Ser/Thr phosphatases are a complex family of proteins and regulate many diverse cellular functions (Mumby and Walter, 1993; Shenolikar, 1994). The functional diversity of individual PPs results from the association of various regulatory and targeting subunits to the highly conserved catalytic subunits (Shenolikar, 1994; Faux and Scott, 1996). Thus the regulatory and targeting subunits play important roles in substrate-recognition, targeting of the holoenzyme to the appropriate subcellular compartment, and modulating the holoenzyme activity (Mayer-Jaekel and Hemmings, 1994; Shenolikar, 1994; Faux and Scott, 1996). In animals, many genes encoding the catalytic and regulatory subunits of Ser/Thr protein phosphatases have been cloned, and the results suggest that both the catalytic and regulatory subunits are encoded by multiple isogenes (Arino et al., 1993; Smith and Walker, 1993; Mayer-Jaekel and Hemmings, 1994; Corum III et al., 1996).

Similarly, multiple genes encoding PP1 (Rundle and Nasrallah, 1992; Smith and Walker, 1993; Pay et al., 1994; Zimmerlin et al., 1995) and PP2A proteins (Arino et al., 1993; Pirck et al., 1993; Casamayor et al., 1994) have been cloned from various plant species. In *Arabidopsis*, a type 1 PP is encoded by at least 5 genes, designated *TOPP1* through *TOPP5* (Smith and Walker, 1993). All of the *TOPP* genes are expressed in rosettes, roots, and flowers, though the level of the transcripts varies between the clones (Smith and Walker, 1993). Type 2A PPs of *Arabidopsis* have also been encoded by at least five genes, each of which appears to be expressed in all tissues, albeit at different levels (Arino et al., 1993; Casamayor et al., 1994). *Arabidopsis* A and B regulatory subunits of PP2A have also been encoded by a small multigene family (Slabas et al., 1994; Rundle et al., 1995; Corum III et al., 1996; Garbers et al., 1996).

The functions of PPs in plants are likely as diverse as those of the PPs in animals. Plant PPs have been shown to play a role in auxin transport (Garbers et al., 1996), metabolism (Carter et al., 1990; MacKintosh, 1992), and the abscisic acid signaling pathway (Leung

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et al., 1994; Meyer et al., 1994). PPs may also participate in plants' defense system (MacKintosh et al., 1994), cell cycle control (Nitschke et al., 1992; Ferreira et al., 1993; Arundhati et al., 1995), and guard cell regulation (Thiel and Blatt, 1994).

We previously isolated five cDNAs, designated *NPP1* to *NPP5*, encoding the catalytic subunit of PPs from *Nicotiana tabacum*, as a first step to investigate the functions of PPs in plants (Suh et al., 1998). The five tobacco PP cDNA clones exhibited high sequence identity in the PP core region to the corresponding genes from animals and plants. However, they showed differential expression patterns in various plant tissues and during flower developmental stages. Among them, *NPP1*, which encodes a type 1 PP, was predominantly expressed in flowers with no significant change of the mRNA level throughout flower developmental stages (Suh et al., 1998).

In this study, the expression patterns of the *NPP1* mRNA in response to various stimuli were examined to facilitate the effort to understand the function of NPP1. In addition, biochemical activity of the NPP1 protein was investigated using a dephosphorylation assay with the phosphorylated NtCDPK1 protein kinase as a substrate.

To examine *NPP1* gene expression upon wounding, tobacco leaves were pressed against needles, and RNA was extracted after 0.5, 2, 11, 28, and 48 h (Fig. 1). The RNA gel blot was hybridized with a *NPP1*-specific probe. The *NPP1*-specific probe was a 420 bp PCR product which corresponded to the 3' untranslated region of the *NPP1* cDNA. The same blots were stripped and hybridized with an *rDNA* probe to show the relative amounts of RNA in each lane. Accumulation of *NPP1* transcripts was significantly stimulated at 11 h after wounding, and then the mRNA level was maintained up to 48 h (Fig. 1).

To examine the change of *NPP1* mRNA level upon $CaCl_2$ treatment, petioles of young leaves were put into a 10 to 200 mM solution of $CaCl_2$ or into water for 12 h. The *NPP1* mRNA accumulation in leaves was not stimulated by 10 to 50 mM $CaCl_2$, but at concentrations of 100 to 200 mM the mRNA level significantly increased (Fig. 2).

When tobacco BY2 cells were treated with fungal elicitors (30 μg/mL), the *NPP1* transcript level increased at 2 h after the treatment, but the level returned to its basal level at 24 h after the treatment. However, with chitosan treatment (100 μg/mL), the transcript level slightly increased at 2 h after treatment, and the level was maintained up to 6 h, then increased significantly at 24 h after treatment (Fig. 3).

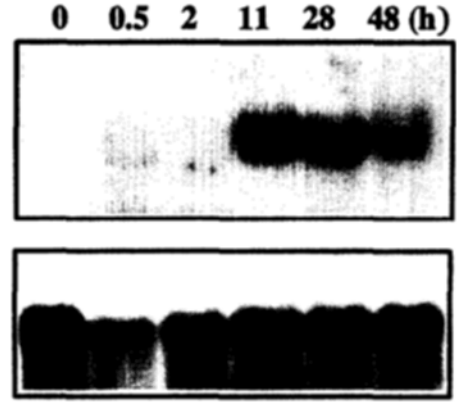

Figure 1. *NPP1* expression upon wounding. Tobacco leaves were pressed against needles, and total RNA was extracted after 0.5, 2, 11, 28, and 48 h. Approximately 50 μg of total RNA were electrophoresed in a 1% formaldehyde/agarose gel and transferred to Hybond-N nylon membrane (Amersham, USA). The membrane was hybridized at high stringency with a probe that was specific for the *NPP1* gene. Prehybridization, hybridization, and washing conditions were carried out as described by Suh et al. (1998). After removing the *NPP1* probe, the same blot was hybridized with a 25S *rDNA* probe to check the amount of RNA loaded in each lane. To generate the *NPP1*-specific probe, PCR was carried out with the upstream primer (CCAGATACTGAAGCC) located at the 3' end of the coding region and T7 primer in the pBluescript vector using the *NPP1* cDNA in the pBluescript vector (SK) as a template. The target sequences were amplified with the following temperature cycle: after denaturation of the cDNA at 94°C for 5 min, temperature was cycled at 94°C for 1 min, at 55°C for 1 min, then at 72°C for 1 min for 30 cycles, and at 72°C for 10 min for 1 cycle. The 420 bp PCR product was separated on a 1% agarose gel, eluted using ZETsorb (Genomed Inc, USA), and used as a probe. Approximately 50 μg of total RNA was loaded in each well. (Top) *NPP1* probe; (bottom) *rDNA* probe.

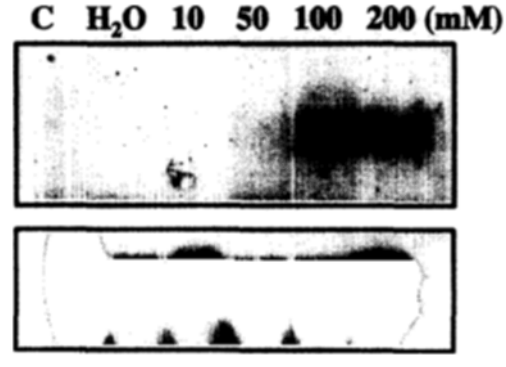

Figure 2. *NPP1* expression upon $CaCl_2$ treatment. The petioles of leaves were put into a 10 to 200 mM solution of $CaCl_2$ or into water for 12 h. Approximately 50 μg of total RNA was loaded in each lane. (Top) *NPP1* probe; (bottom) *rDNA* probe.

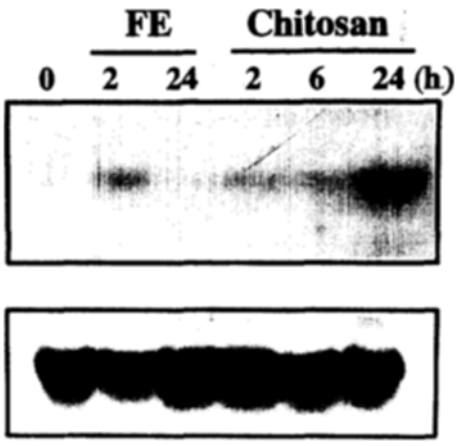

Figure 3. *NPP1* expression upon treatments with fungal elicitors (FE) and chitosan. Tobacco BY2 cells were treated with chitosan (100 µg/mL; 2, 6, and 24 h) and fungal elicitors (30 µg/mL; 2 and 24 h). (Top) *NPP1* probe; (bottom) *rDNA* probe.

Upon treatment with abscisic acid (100 µM; 0.5, 1, 1.5, 2, 4, and 6 h), there was no significant change in the *NPP1* transcript levels (results not shown).

Since *NPP1* encodes a catalytic subunit of a type 1 PP, we examined whether NPP1 possessed phosphatase activity. It has been shown that catalytic subunits of PPs do not exhibit strong substrate specificity, thus we used NtCDPK1, a calcium-dependent protein kinase isolated from tobacco (Yoon et al., 1999), as a substrate. The cDNA fragment encoding NPP1 was cloned into the pMAL™-c2 vector using the XbaI/PstI sites to generate the recombinant proteins fused to the C-terminus of maltose-binding proteins (MBP). The cDNA fragment encoding NtCDPK1 was also cloned into the pMAL™-c2 vector. The two fusion proteins, MBP-NPP1 and MBP-NtCDPK1, were expressed in *Echerichia coli* and purified using amylose resin. After purification, the two recombinant proteins were visualized by Coomassie blue staining (Fig. 4A). Autophosphorylation assays were performed with the recombinant NtCDPK1 and NPP1 under various conditions (Fig. 4B). Four µg of the recombinant NPP1 or BSA was added at the beginning of the autophosphorylation reaction of NtCDPK1, or added after the termination of the reaction followed by further incubation. As a control, 4 µg of the recombinant NPP1 alone was subjected to phosphorylation reaction. The recombinant NPP1 alone did not produce any labeled products, while the NtCDPK1 incubated with BSA resulted in a radioactive band of 87 kDa whose position matched the position of the purified NtCDPK1 protein shown in Figure 4A. Thus the radioactive band represented the phosphorylated recombinant NtCDPK1. When autophosphorylation of NtCDPK1 was carried out in the presence of NPP1, it resulted in a much reduced signal, suggesting that NtCDPK1 was dephosphorylated by NPP1. However, NPP1 added after termination of the phosphorylation reaction reduced the signal less visibly, which may be due to a relatively short incuba-

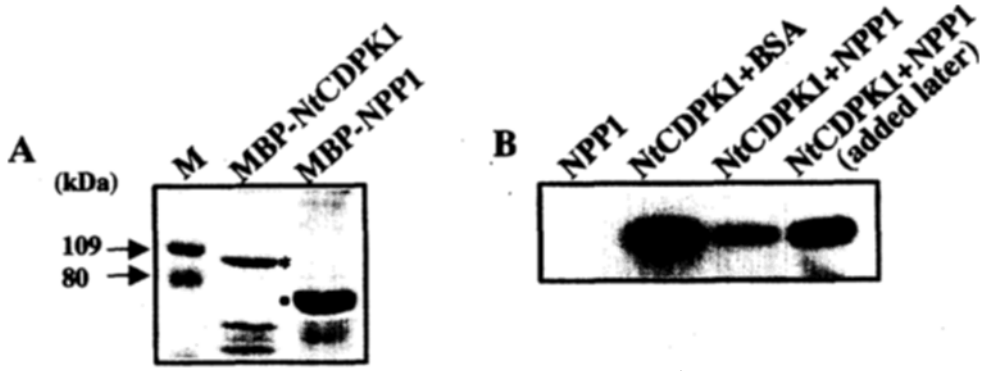

Figure 4. Dephosphorylation Assay **A**. Expression of the recombinant NPP1 and NtCDPK1 in *E. coli*. NPP1 and NtCDPK1 were expressed as fusion proteins with maltose-binding proteins (MBP). After SDS-PAGE, the purified recombinant proteins were visualized with Coomassie blue staining. Asterisks (*) indicate full length MBP-NtCDPK1 proteins. Filled circles (●) indicate MBP-NPP1 protein. **B**. Dephosphorylation of MBP-NtCDPK1 by MBP-NPP1. Two µg of purified MBP-NtCDPK1 were incubated in a 20 µL kinase buffer [25 mM HEPES, pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 200 µM $CaCl_2$, 10 µCi of γ-^{32}P-ATP (6000 Ci/mmol)] overnight at 30°C for autophosphorylation. Four µg of the recombinant NPP1 or BSA were added at the beginning of the autophosphorylation reaction, or 4 µg of NPP1 was added after the termination of the reaction followed by further incubation for 3 h at 30°C. As a control, 4 µg of NPP1 alone was incubated in the kinase buffer. The reactions were terminated by the addition of 5X Laemmli sample buffer and electrophoresed on a 10% SDS-polyacrylamide gel. The gel was blotted to nitrocellulose and exposed to X-ray film.

tion time. Based on these results, we concluded that NPP1 possessed phosphatase activity.

In plants, Ser/Thr protein phosphatases are involved in regulating cellular processes. The *Arabidopsis rcn1* mutant, which is defective in auxin transport, is caused by a mutation in the gene encoding a PP2A regulatory subunit A (Garbers et al., 1996). The *Arabidopsis ABI1* gene, which is involved in the abscisic acid signaling pathway, encodes a PP2C with a calcium-binding site in its N-terminus (Leung et al., 1994; Meyer et al., 1994). PP1-At could rescue conditional cell cycle mutants of the *dis2-11 Schizosaccharomyces pombe* strain, which contains a cold-sensitive allele of the phosphatase 1 gene (Nitschke et al., 1992). Recently, AtPP1bg, highly expressed in reproductive organs of *Arabidopsis*, has been found to complement a conditional cell cycle mutant of *Aspergillus* (Arundhati et al., 1995).

Our results showed that gene expression of *NPP1* was stimulated by various stimuli, including $CaCl_2$, wounding, fungal elicitiors, and chitosan. However, the *NPP1* mRNA levels did not change in response to abscisic acid. In many cases, the expression of genes encoding components of a given signaling cascade is upregulated by the corresponding stimulus, as examplified by *CTR1* and *Nr* in ethylene signaling (Kieber et al., 1993; Wilkinson et al., 1995), *NIM1* in pathogen signaling (Ryals et al., 1997), and *CIP1* in light signaling (Yamamoto et al., 1998). Though the biological function of the *NPP1* gene product is unknown, this enhanced gene expression in response to abiotic stresses indicates that the *NPP1* gene product may play a role in stress signal transduction. The protein may take part in the stress signaling pathway through interaction with its regulatory subunits and other signaling components. Reverse genetic approaches, such as antisense RNA techniques and using dominant negative mutants, will be direct approaches to determine functions of NPP1. In addition, probing their subcellular localization and identifying interacting regulatory or targeting subunits may also provide insights into cellular functions of this phosphatase.

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