Rice Plastidial *N*-Glycosylated Nucleotide Pyrophosphatase/Phosphodiesterase Is Transported from the ER-Golgi to the Chloroplast through the Secretory Pathway [W]

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A nucleotide pyrophosphatase/phosphodiesterase (NPP) activity that catalyzes the hydrolytic breakdown of ADP-glucose (ADPG) has been shown to occur in the plastidial compartment of both mono- and dicotyledonous plants. To learn more about this enzyme, we purified two NPPs from rice (*Oryza sativa*) and barley (*Hordeum vulgare*) seedlings. Both enzymes are glycosylated, since they bind to concanavalin A, stain with periodic acid–Schiff reagent, and are digested by Endo-H. A complete rice NPP cDNA, designated as *NPP1*, was isolated, characterized, and overexpressed in transgenic plants displaying high ADPG hydrolytic activity. Databank searches revealed that NPP1 belongs to a functionally divergent group of plant nucleotide hydrolases. NPP1 contains numerous *N*-glycosylation sites and a cleavable hydrophobic signal sequence that does not match with the N-terminal part of the mature protein. Both immunocytochemical analyses and confocal fluorescence microscopy of rice cells expressing NPP1 fused with green fluorescent protein (GFP) revealed that NPP1-GFP occurs in the plastidial compartment. Brefeldin A treatment of NPP1-GFP–expressing cells prevented NPP1-GFP accumulation in the chloroplasts. Endo-H digestibility studies revealed that both NPP1 and NPP1-GFP in the chloroplast are glycosylated. Collectively, these data demonstrate the trafficking of glycosylated proteins from the endoplasmic reticulum–Golgi system to the chloroplast in higher plants.

INTRODUCTION

Nucleotide pyrophosphate/phosphodiesterase (NPP) is a widely distributed enzymatic activity that catalyzes the hydrolytic breakdown of pyrophosphate and phosphodiester bonds of numerous nucleotide sugars (Rodríguez-López et al., 2000; Rodríguez-López, 2002). Experiments performed to characterize NPP-catalyzing enzymes have shown the occurrence of both intra- and extraplastidial isoforms (Baroja-Fernández et al., 2000) and intra- and extracellular isoforms (Rodríguez-López et al., 2001). These observations have evoked the idea that, essentially identical to the suggested mechanisms controlling the levels of nucleotide sugars linked to polyglucan biosynthetic processes in bacteria and animals (Hickman et al., 1985; Moreno-Bruna et al., 2001; Yagi et al., 2003), plant NPPs may play a crucial role in diverting carbon flux from starch and cell wall polysaccharide biosynthesis to other metabolic pathways in response to the physiological

needs of the cell (Baroja-Fernández et al., 2001). Furthermore, and essentially similar to the case of nucleotide sugar hydrolases from both bacteria and animals, extracellular plant NPPs may also act as a first step in the scavenging of extracellular nucleotide sugars (Baroja-Fernández et al., 2000).

Among different protein entities responsible for the NPP activity, a plastidial isoform has been partially characterized that differs from other nucleotide phosphodiesterases and pyrophosphatases described to date (Bessman et al., 1996; Bollen et al., 2000) in aspects such as molecular weight, acidic pH optima, inertness to reducing agents, resistance to high temperature, and no requirement of metal ions (Baroja-Fernández et al., 2000; Rodríguez-López et al., 2000). This enzyme was shown to preferentially cleave the starch precursor molecule ADP-glucose (ADPG). Toward the end of clarifying the nature of this enzyme, we have purified and characterized NPPs from rice (*Oryza sativa*) and barley (*Hordeum vulgare*) seedling extracts. In addition, and to further investigate its topographic distribution, we have performed confocal fluorescence microscopy and immunocyto-localization studies of transgenic rice cells expressing an NPP isoform fused with green fluorescent protein (GFP). Results presented in this work show that both rice and barley NPPs belong to a large group of structurally related and functionally divergent nucleotide hydrolases that are predicted to be glycosylated, to occur in membrane systems, to accumulate in vacuoles, or to be secreted from the cell. In addition, we show the

Table 1. Purification of 70- and 300-kD ADPG Hydrolytic NPPs from Young Barley Leaves

Purification Step	Total Volume (mL)	Total Protein (mg)	Total Activity (Units)	Specific Activity (Units/mg Protein)	Purification (Fold)
Supernatant (100,000*g*) (1)	2,900	24,186	1,195	0.05	
pH 4.2/62°C (2)	2,880	777	1,067	1.30	28
Gel filtration:					
300 kD (3)	117	97	379	3.90	79
70 kD (4)	86	164	299	1.80	37
Isoelectrofocusing:					
300 kD (5)	3	0.02	1.1	55.00	1,120
70 kD (6)	14	0.60	22.0	36.00	738

Numbers 1 to 6 correspond to Figure 1 (left panel).

plastidial localization of an *N*-glycosylated NPP and the occurrence of a traffic route of proteins from the endoplasmic reticulum (ER)–Golgi system to the plastidial compartment.

RESULTS AND DISCUSSION

Purification and Characterization of Rice and Barley ADPG Hydrolytic NPPs

Preliminary gel filtration analyses on crude extracts of young shoots of barley revealed the occurrence of ~70- and 300-kD ADPG hydrolytic NPP isoforms, whereas crude extracts of rice shoots possess a single ~300-kD NPP ADPG hydrolytic enzyme. We then attempted to purify the proteins responsible for this activity in both rice and barley. As shown in Table 1, specific activities of the purified barley enzyme preparations with respect to ADPG were 36 and 55 units/mg protein for the 70- and 300-kD isoforms, respectively, whereas the specific activity of the purified rice enzyme preparation was 180 units/mg protein (Table 2).

As illustrated in Figure 1, SDS-PAGE and subsequent staining of the two barley ADPG hydrolytic NPP isoforms revealed a single band of ~70 kD, indicating that, in our crude extracts of barley leaves, ADPG hydrolytic NPPs occur as a monomer and homopolymer made up of the same 70-kD polypeptide. SDS-PAGE and subsequent staining of the purified rice ADPG hydrolytic NPP revealed a single 70-kD band, indicating that this enzyme exclusively occurs as a homopolymer of the same 70-kD polypeptide.

Some characteristics of both barley and rice ADPG hydrolytic NPPs, such as resistance to high-temperature treatments (Table 1) and binding to concanavalin A (Con A) (Tables 2 and 3), are typical attributes of *N*-glycosylated proteins (Lamport, 1980). In

addition, *N*-glycosylated proteins are known to have a relatively high resistance to SDS denaturation and slowly migrate in SDS-PAGE (Lamport, 1980). To confirm this aspect of NPPs, we have investigated their periodic acid–Schiff (PAS) staining and Endo-H digestibility. As illustrated in Figures 2A and 2B, both rice and barley 300-kD NPPs were shown to migrate slowly in SDS-PAGE without prior denaturation (lane 1), whereas they migrate as ~70-kD bands after heat denaturation (lane 2). When SDS gels were stained with PAS reagent, the protein bands became visible (lane 3). In addition, and as illustrated in Figure 2C, 300-kD NPPs can be digested by Endo-H, yielding a protein that could not bind to Con A.

The overall results thus clearly indicate that both rice and barley ADPG hydrolytic NPPs bear *N*-linked oligosaccharide chains.

Rice and Barley ADPG Hydrolytic NPPs Are Members of a Ubiquitously Distributed Family of Plant Nucleotide Hydrolases

The 70-kD polypeptide bands obtained after SDS-PAGE of the purified rice and barley NPPs were subjected to amino acid sequencing (Table 4). These analyses revealed that the N-terminal sequences of the 70- and 300-kD barley NPP isoforms are identical, thus further confirming that in our crude extracts of barley leaves, ADPG hydrolytic NPPs occur as a monomer and homopolymer of the same 70-kD polypeptide.

Using specific primers (see Methods), a complete cDNA corresponding to a rice NPP-encoding gene was obtained, and it was designated as *NPP1*. Computer searches of databanks showed that the deduced amino acid sequence of *NPP1* shares high sequence similarity with several hypothetical or unknown proteins of both mono- and dicotyledoneous plants (see Supplemental Figure 1 online). Moreover, NPP1 shares sequence

Table 2. Purification of ADPG Hydrolytic NPP from Rice Shoots

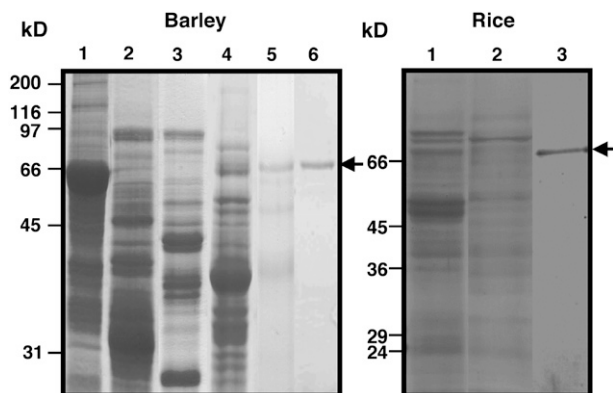| Purification Step	Total Volume (mL)	Total Protein (mg)	Total Activity (Units)	Specific Activity (Units/mg Protein)	Purification (Fold)
Crude extract	1,000	837.00	189.35	0.23	
Butyl-Toyopearl 650M	100	132.00	69.30	0.53	2.3
pH 5.0	110	46.60	67.33	1.44	6.4
Con A–Sepharose 4B	90	2.30	56.80	24.80	110.0
Microcon YM-100	0.5	0.06	10.95	182.60	807.0

Figure 1. SDS-PAGE and Coomassie Blue Staining of Protein Samples after Various Purification Steps of Both Rice and Barley ADPG Hydrolytic NPPs.

Lane numbering of barley NPP purification corresponds to Table 1. Lane numbering of rice NPP purification corresponds to (1) crude extract, (2) acid pH treatment, and (3) purified enzyme (see Table 2).

similarity with PPD1, a metallophosphatase from yellow lupin that specifically cleaves the pyrophosphate bond of diphospho-nucleotides such as ADP and GDP (Olczak et al., 2000; Olczak and Olczak, 2002). Unlike barley and rice NPPs, however, PPD1 is presented as a monomer, has strong affinity toward nucleoside diphosphates, and does not split the pyrophosphate linkage of nucleotide sugars. The overall information thus indicates that both rice and barley ADPG hydrolytic NPPs belong to a large family of structurally related nucleotide hydrolases occurring in both mono- and dicotyledonous plants.

NPP1 was expressed in *Escherichia coli* (see Methods). We found that the overexpressed NPP1 was not enzymatically active (data not shown). Assuming that NPPs need to be glycosylated for correct folding and full activity (Hammond et al., 1994; Parodi, 2000), we expressed *NPP1* in transgenic rice plants. Protein gel blot analyses using polyclonal NPP1 antisera revealed that these plants accumulate a protein that migrates as a 70-kD band in denaturing SDS gels (see Supplemental Figure 2 online). In addition, gel filtration analyses revealed that, essentially similar to the case of NPP1 from untransformed plants, this protein appeared as a symmetrical peak in a region expected for an ~300-kD protein (see Supplemental Figure 2 online). Once purified, this protein showed properties identical to those of both barley and rice NPPs (i.e., ADPG hydrolytic activity, optimal activity at acid pH, resistance to high temperature, inertness to reducing agents, and no requirement for divalent cations) (Rodríguez-López et al., 2000) (data not shown). Furthermore, young shoots of the transformed plants exhibited a much higher ADPG hydrolytic NPP activity than those of control plants (Table 5), with the overall results thus confirming the proper identification of a rice NPP encoding gene.

NPP1 Has a Plastidial Localization

NPP1 cDNA is 2201 bp in length and includes a single open reading frame of 1872 bp that encodes 623 amino acid residues, forming a 69.9-kD precursor protein (Figure 3A). Its deduced amino acid sequence contains four putative *N*-glycosylation sites, which is in agreement with results presented in Figure 2 and Table 3. Computer-assisted analyses using PSORT (http://psort.ims.u-tokyo.ac.jp/) and SignalP (http://www.cbs.dtu.dk/services/SignalP/) algorithms predicted that the immature NPP1 has a cleavable hydrophobic N-terminal signal sequence potentially acting as signal peptide to the ER. Intriguingly, however, this derived amino acid sequence does not match the N-terminal sequence determined for the mature protein (Figure 3B).

Previous studies have shown the occurrence of ADPG hydrolytic NPP activity in plastids (Baroja-Fernández et al., 2000; Rodríguez-López et al., 2000). To investigate the possible localization of NPP1 in the chloroplast, we produced plants transformed with the NPP1-GFP construct and compared the fluorescence distribution pattern of their cells with that of chlorophyll autofluorescence. These plants constitutively express a translationally fused NPP1-GFP gene under the control of the cauliflower mosaic virus 35S promoter. As control, and to detect possible artifactual mislocalization of the overexpressed protein, we also used cells expressing GFP (cytosolic marker) and mt-GFP (mitochondrial marker). As illustrated in Figure 4, GFP fluorescence in GFP-expressing cells was distributed uniformly throughout the cytosol and nucleus. By contrast, fluorescence in both NPP1-GFP– and mt-GFP–expressing cells was scattered, suggesting that GFP is localized in intracellular organelle compartments. mt-GFP fluorescence distribution pattern was different to that of chlorophyll autofluorescence. Importantly, the NPP1-GFP fluorescence distribution pattern in NPP1-GFP cells was identical to that of chlorophyll autofluoresence, with the overall results strongly indicating that NPP1-GFP occurs in the chloroplast.

To further investigate the subcellular localization of NPP1, we performed immunocytochemical analyses on ultrathin sections of both GFP- and NPP1-GFP–expressing rice cells using polyclonal GFP antiserum. Figure 5 and Supplemental Figure 3 online represent immunolocalization patterns of GFP in both GFP and NPP1-GFP cells. The positive signal of GFP, visualized as black dots, was localized specifically in the cytosol in GFP cells (Figure 5A), whereas marked labeling was specifically observed in the chloroplasts of NPP1-GFP cells (Figure 5B). Both untransformed rice cells treated with anti-GFP serum (Figure 5C) and NPP1-GFP cells treated with a preimmune serum (Figure 5D) showed no signal.

We also performed immunocytochemical analyses on untransformed leaves using polyclonal anti-NPP1 serum (see Supplemental Figure 4 online). As expected, the antiserum recognized

Table 3. Biochemical Characterization of Rice and Barley ADPG Hydrolytic NPPs

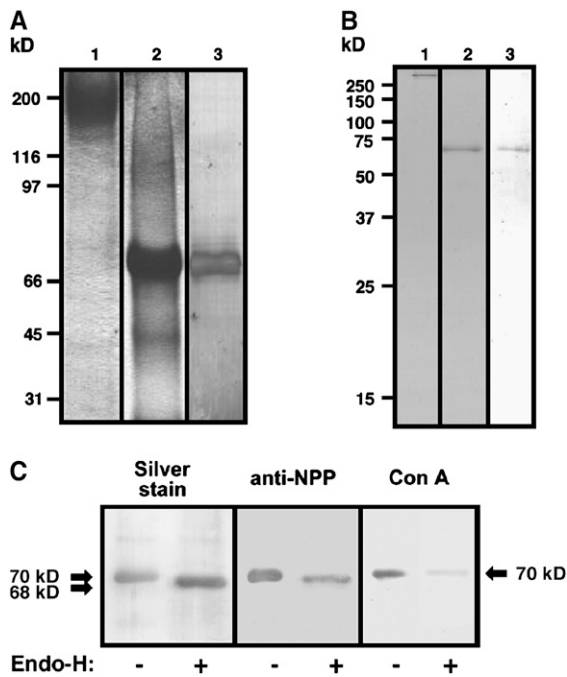| Properties	Rice NPP	Barley NPP	
		300 kD	70 kD
Optimum temperature for reaction	60°C	58°C	58°C
Optimum pH for reaction	6.0	5.7	5.6
PAS staining	Positive	Positive	Positive
Con A binding	Positive	Positive	Positive
Endo-H susceptibility	Susceptible	Susceptible	ND

ND, not determined.

Figure 2. Detection of Oligosaccharide Side Chains Conjugated to Barley and Rice ADPG Hydrolytic NPPs.

(A) and **(B)** Migrating behavior and carbohydrate staining on SDS gels of purified 300-kD ADPG hydrolytic NPPs. Lane 1, purified NPPs were applied to SDS-PAGE without a previous heat denaturation step. Lanes 2 and 3, NPPs were heat denatured prior to electrophoretic separation. In lanes 1 and 2, NPPs were stained with Coomassie blue, whereas in lane 3, NPPs were stained employing the periodic acid–Schiff reagent. Precision protein standard kits from Bio-Rad were used as reference.
(C) Endo-H susceptibility. Purified rice ADPG hydrolytic NPP was digested with Endo-H, and the heat denatured protein was subjected to SDS-PAGE, followed by silver staining (left panel), immunobloting with anti-NPP1 (middle panel), and lectinoblotting with Con A–peroxidase (right panel).

members of the NPP family occurring in both the cell wall and vacuoles. In addition, and consistent with the results presented in Figures 4 and 5 and Supplemental Figure 3 online, a marked labeling was also observed in the chloroplasts. Sections treated with either the preimmune sera or secondary antibody showed no signal (data not shown).

In attempting to further explore the possible occurrence of ADPG hydrolytic NPP in plastids, we performed cell fractionation studies of shoots as indicated in Methods. As shown in Table 6, specific activities of the plastidial marker alkaline pyrophosphatase in the chloroplast preparations were 3.5-fold higher than those of crude extracts. In addition, chloroplast preparations were virtually free from cytosolic, ER, and Golgi contaminant markers (UDP-glucose pyrophosphorylase, NADPH-cytochrome *c* reductase, and IDPase, respectively). By contrast, and in agreement with previous studies using barley endosperms (Rodríguez-López et al., 2000), specific ADPG hydrolytic activities in the chloroplast preparations were high and nearly half of those of crude extracts, thus confirming that a sizable pool of ADPG hydrolytic enzyme(s) (presumably NPP1) has a plastidial

localization in rice shoots. Highly purified chloroplasts were then loaded onto a Percoll gradient and centrifuged, and samples thus obtained were subjected to immunoblot analyses using antibodies raised against the plastidial marker ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) and NPP1. As shown in Supplemental Figure 5 online, the NPP1 distribution pattern in the Percoll gradient was nearly identical to that of Rubisco, which further strengthens the view that ADPG hydrolytic NPP has a chloroplastic localization.

The overall data thus strongly indicate that NPP1 has a plastidial localization in rice shoots.

Plastidial NPP1 Is Glycosylated

Whether chloroplastic NPP1 is glycosylated was investigated by analyzing Endo-H digestibility of NPP1 in chloroplast preparations obtained from both untransformed and NPP1-overexpressing rice leaves. In both cases, NPP1 was shown to bind to Con-A and to elute as an ~300-kD protein in gel filtration chromatography (data not shown). As illustrated in Supplemental Figure 6 online, chloroplastic NPP1 migrates slowly in SDS-PAGE when loaded without a prior denaturation step. Protein gel blot analyses using polyclonal NPP1 antisera revealed that chloroplastic NPP1 migrates as an ~70-kD band in denaturing gels with a prior heat treatment (Figure 6A). Most importantly, NPP1 can be digested by Endo-H, yielding an ~68-kD protein that could not bind to ConA (data not shown). The overall results show that NPP1 occurring in the chloroplast is glycosylated and occurs as a homopolymer of the same ~70-kD polypeptide.

We also investigated Endo-H digestibility of NPP1-GFP in chloroplast preparations obtained from NPP1-GFP–expressing leaves. GFP immunoblots of the chloroplast preparations revealed that NPP1-GFP migrates as a 97-kD band in denaturing

Table 4. N-Terminal and Internal Sequences of the Rice and Barley ADPG Hydrolytic NPPs

N-Terminal	Internal Sequences (Rice)
Rice (300 kD) GSAFVSATPALLGDQ	FQLINQRYDFSFALFTGGLENPK
Barley (300 kD) AAVRASPDLLGSRGEDTA	LVAVSEAISFK LAQGKSYDEM
Barley (70 kD) AAVRASPDLLGSRGEDTA	TAAGTLTFNR DPGFIHTAFLR APPFPGQNSLQR IIVFGDMGK DWPNTGGFFDVK FIEQCLSTVDR VYDSFTVER

Table 5. ADPG Hydrolytic NPP Activities in Young Leaves of Both Wild Type– and NPP1-Expressing Transgenic Rice Plants

Activity (Units/mg of Protein)	
Wild Type	Transgenic
0.423 ± 0.010	17.6 ± 2.7

Data are represented as the mean ± SD obtained from four independent experiments.

A

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   1  -GAAGCAGGAGAGGTAGTTAGATTGCATTAGCTAGCTGTAGAAAGCTAGCCATGGTTAGTAGGAAGAGAGGAGGAGGAGGA
                                                         M  V  S  R  K  R  G  G  G  G
  81  GGCGTGGCAATGGCGGTGGCGATGCTGCTGGCGGCGGCGAGCGCGTCGCGGCCGTCGTCGTCGCTGGAAGGGTTCCAGCCG
       G  V  A  M  A  V  A  M  L  L  A  A  A  S  A  S  R  P  S  S  S  L  E  G  F  Q  P
 162  CTGTCGAAGATCGCCGTCCACAAGGCCACCGTCGACCTCCACGGCTCCGCGTTCGTCAGCGCCACGCCGGCGTTGCTCGGC
       L  S  K  I  A  V  H  K  A  T  V  D  L  H  G  S  A  F  V  S  A  T  P  A  L  L  G
 243  GACCAGGGAGAAGACACAGAGTGGGTCACGGTGAAATACGGCTGGGCAAACCCTTCCGCTGACGACTGGATTGCTGTCTTC
       D  Q  G  E  D  T  E  W  V  T  V  K  Y  G  W  A  N  P  S  A  D  D  W  I  A  V  F
 324  TCTCCGGCCGATTTCATCTCGGGTTCTTGCCCTAATCCTTCCAGATACCCGGATGAGCCGCTGCTCTGCACTGCACCAATA
       S  P  A  D  F  I  S  G  S  C  P  N  P  S  R  Y  P  D  E  P  L  L  C  T  A  P  I
 405  AAGTATCAATTCGCAAACTACTCGGCGGAACTACGTGTACTGGGGCAAGGGCAGCATCCGGTTCCAGCTCATCAACCAGCGC
       K  Y  Q  F  A  N  Y  S  A  N  Y  V  V  Y  W  G  K  G  S  I  R  F  Q  L  I  N  Q  R
 486  TACGACTTCTCCTTCGCCCTGTTCACCGGCGGCCTGGAAAACCCTAAGCTGGTGGCGGTGTCGGAGGCGGATATCGTTCAAG
       Y  D  F  S  F  A  L  F  T  G  G  L  E  N  P  K  L  V  A  V  S  E  A  I  S  F  K
 567  AACCCCAAGGCGCCGGTGTACCCTCGGCTGGCGCAGGGCAAGTCGTACGACGAGATGACCGTCACATGGACCAGCGGCTAC
       N  P  K  A  P  V  Y  P  R  L  A  Q  G  K  S  Y  D  E  M  T  V  T  W  T  S  G  Y
 648  GACATCAGCGAGGCGTACCCGTTCGTCGAGTGGGGCATGGTCGTCGCCGGCGCCGCCGCTCCAACCCGCACCGCCGCCGGC
       D  I  S  E  A  Y  P  F  V  E  W  G  M  V  V  A  G  A  A  A  P  T  R  T  A  A  G
 729  ACGCTCACCTTCAACCGCGGCAGCATGTGCGGTGAGCCGGCACGCACTGTTGGGTGGAGACCCCGGGTTCATCCACACA
       T  L  T  F  N  R  G  S  M  C  G  E  P  A  R  T  V  G  W  R  D  P  G  F  I  H  T
 810  GCTTTCCTGAGAGACCTGTGGCCCAACAAAGAGTACTACTACAAGATTGGGCATGAACTTTCTGACGGATCAATTGTCTGG
       A  F  L  R  D  L  W  P  N  K  E  Y  Y  Y  K  I  G  H  E  L  S  D  G  S  I  V  W
 891  GGCAAGCAATACACTTTCCGGGCGCCACCCTTCCCTGGCCAGAACTCGCTGCAACGCATCATCGTCTTCGGCGACATGGGC
       G  K  Q  Y  T  F  R  A  P  P  F  P  G  Q  N  S  L  Q  R  I  I  V  F  G  D  M  G
 972  AAGGCGGAGAAGACGGATCAAACGGATTCTGGAACTACCAGCCCAGGATCTCTGAACACGACGGACAGGCTGGTCGAGGAT
       K  A  E  R  D  G  S  N  E  F  A  N  Y  Q  P  G  S  L  N  T  T  D  R  L  V  E  D
1053  CTGGACAACTACGACATTGTCTTCCACATCGGTGATCTTCCGTACGCCAATGGCTACATCTCCCAGTGGGACCAGTTCACC
       L  D  N  Y  D  I  V  F  H  I  G  D  L  P  Y  A  N  G  Y  I  S  Q  W  D  Q  F  T
1134  GCCCAGGTCGCCCCCATCACCGCCAAGAAGCCCTACATGATTGCAAGCGGTAACCATGAGAGGGACTGGCCCAACACCGGA
       A  Q  V  A  P  I  T  A  K  K  P  Y  M  I  A  S  G  N  H  E  R  D  W  P  N  T  G
1215  GGGTTCTTCGACGTCAAGGACTCCGGCGGCGAGTGCGGCGTTCCGGCAGAGACCATGTACTACTACCCGGCCGAGAATCGA
       G  F  F  D  V  K  D  S  G  G  E  C  G  V  P  A  E  T  M  Y  Y  Y  P  A  E  N  R
1296  GCCAACTTCTGGTACAAGGTGGACTACGGGATGTTCCGGTTCTGCATCGACAGCTCGGAGCACGACTGGAGGGAGGGTACC
       A  N  F  W  Y  K  V  D  Y  G  M  F  R  F  C  I  A  D  S  E  H  D  W  R  E  G  T
1377  GACCAGTACAAGTTCATCGAGCAGTGCCTGTCGACGGTGGACCGGAAGCACCAGCCGTGGCTCATCTTCGCGGCGCACCGC
       D  Q  Y  K  F  I  E  Q  C  L  S  T  V  D  R  K  H  Q  P  W  L  I  F  A  A  H  R
1458  GTGCTGGGCTACTCCTCCAACTGGTGGTACGCCGACCAGGGCTCCTTCGAGGAGCCCGAAGGGAGGGAGAGCCTGCAGCGG
       V  L  G  Y  S  S  N  W  W  Y  A  D  Q  G  S  F  E  E  P  E  G  R  E  S  L  Q  R
1539  CTGTGGCAGCGCCACCGCGTCGACGTCGCCTTCTTCGGCCACGTCCACAACTACGAGCGGACGTGCCCGATGTACCAGAGC
       L  W  Q  R  H  R  V  D  V  A  F  F  G  H  V  H  N  Y  E  R  T  C  P  M  Y  Q  S
1620  CAGTGCGTCTCCGGCGAGAGGCGCCGCTACTCCGGCACCATGAACGGCACCATCTTCGTCGTCGCCGGCGGCGGCGGGAGC
       Q  C  V  S  G  E  R  R  R  Y  S  G  T  M  N  G  T  I  F  V  V  A  G  G  G  G  S
1701  CACCTCTCGGACTACACCTCGGCGATCCCCAAGTGGAGCGTTTTCAGGGACCGGGACTTCGGGTTCGTCAAGCTCACCGCG
       H  L  S  D  Y  T  S  A  I  P  K  W  S  V  F  R  D  R  D  F  G  F  V  K  L  T  A
1782  TTCAACCACTCGTCGCTGCTGTTCGAGTACAAGAAGAGCAGCGATGGGAAGGTGTATGACTCCTTCACCGTGGAGAGGGAT
       F  N  H  S  L  L  F  E  Y  K  K  S  S  D  G  K  V  Y  D  S  F  T  V  E  R  D
1863  TACCGCGACGTGCTCAGCTGCGTGCACGACAGCTGCCTCCCCACCACGCTCGCCTCCTGATGAATGAAACAAGGGAAAGGA
       Y  R  D  V  L  S  C  V  H  D  S  C  L  P  T  T  L  A  S  *
1944  TCATTATTAGGATGCATGAGTTGATGCCTCATCGTCAAAATGCTGGCAGCTGAGAAAGAGTGATCGGTCGGTCGATCGAGT

2025  TGGGTTTTATTTTTTTTCTTCTTCTTCAACCATTTCGATCAGGTGTGGTAGTGGTCGATCGCTTGGCTCGATCGTGTTTCT

2106  CTTCCTCATGGATGGTGATGTTGTGCAATAAAATTGCTTAGCTGCTCGGGCACAAATGTCTAATAAGATTGGCAATCTGAA

2187  AAAAAAAAAAAAAAA   2201
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B

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1:  MVSRKR GGGGGVAMAVAMLLAAASA SRPSSSLEGFQPLSKIAVHKATVDLHGSAFVSATPALLGDQ  64
              TMR
```
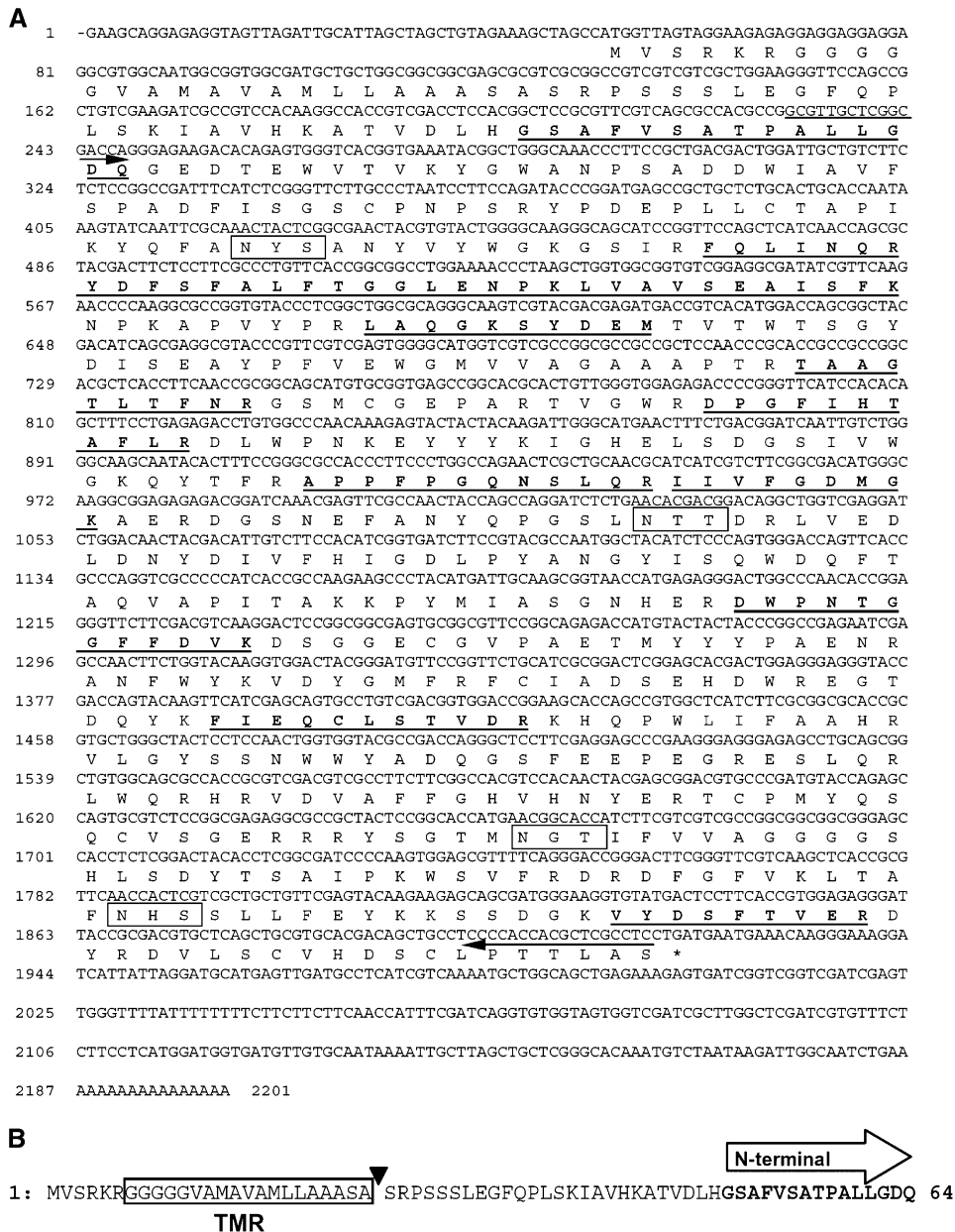
N-terminal

Figure 3. Cloning of *NPP1*.

(A) Nucleotide and predicted amino acid sequences of the deduced protein. The N-terminal and internal peptide sequences shown in Table 4 are underlined and in boldface. *N*-glycosylation sites are boxed. Arrows indicate the forward and reverse direction primers for PCR amplification.
(B) PSORT analysis of the N-terminal end of the deduced protein. The arrowhead indicates a predicted cleavage site. TMR, transmembrane region.

gels (Figure 6B). In addition, NPP1 can be digested by Endo-H, yielding an ∼95-kD protein. The overall results show that chloroplastic NPP1-GFP is glycosylated.

Targeting of *N*-Glycosylated NPP1 to the Chloroplast Involves a Vesicular Transport Pathway

The currently prevailing ideas on posttranslational protein modification assume that *N*-glycosylation occurs exclusively in the ER-Golgi system (Parodi, 2000). Having shown that *N*-glycosylated NPP1 accumulates in the chloroplast, we proceeded to investigate whether NPP1 is indeed transported through the ER-Golgi system. Toward this end, we employed cells from NPP1-GFP–expressing cells and analyzed the effect of brefeldin A (BFA) on the intracellular distribution of NPP1-GFP. This fungal antibiotic causes a block in the secretory system of eukaryotic cells by inhibiting Golgi-to-ER vesicle trafficking, leading to both the formation of large ER-Golgi hybrid stacks and fusion of
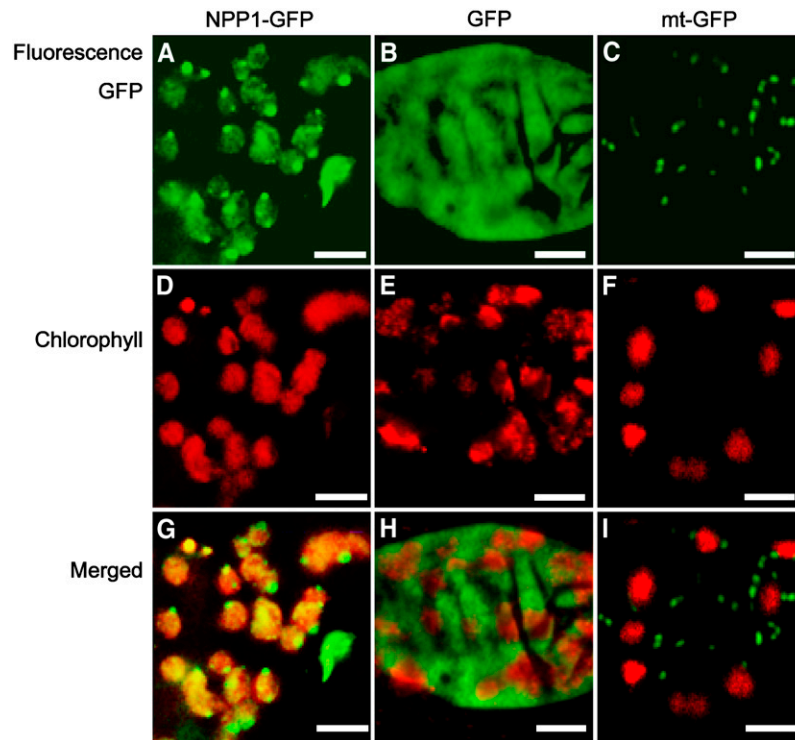
Figure 4. Fluorescence Images in Green Cells of Transgenic Rice Plants Stably Expressing Either NPP1-GFP, GFP (Cytosolic Marker), or mt-GFP (Mitochondrial Marker).

(A), **(D)**, and **(G)** represent NPP1-GFP–expressing, **(B)**, **(E)**, and **(H)** represent GFP-expressing, and **(C)**, **(F)**, and **(I)** represent mt-GFP–expressing rice green cells. Bars = 10 μm.
(A) to **(C)** GFP fluorescence.
(D) to **(F)** Chlorophyll autofluorescence.
(G) to **(I)** Merged image.

the individual Golgi cisternae with the ER (Ritzenthaler et al., 2002). As illustrated in Figure 7A, GFP fluorescence distributed in the ER network structures of NPP1-GFP–expressing cells treated with BFA. In addition, BFA prevented NPP1-GFP accumulation in the chloroplast (Figure 7B), with the overall data strongly indicating that NPP1 targeting to the chloroplast involves a BFA-sensitive vesicular transport pathway from the ER to the Golgi system. We then analyzed the fluorescence distribution pattern in cells expressing either the 66 or 108 N-terminal amino acid residues of NPP1 (which contains the predicted signal peptide for the ER) fused with GFP. As illustrated in Supplemental Figure 7 online, GFP fluorescence was distributed in ER network-like structures, indicating that the N-terminal part of NPP1 is functional and sufficient for targeting the protein to the ER.

Additional Remarks

Collectively, and essentially in agreement with previous studies showing the plastidial localization of nucleotide sugar cleaving enzymes in both cultured cells of sycamore (*Acer pseudoplatanus*) and developing barley seeds (Baroja-Fernández et al., 2000; Rodríguez-López et al., 2000), the data presented in this work

show that NPP1, a glycosylated enzyme (Figures 2 and 6), occurs in plastids.

The currently prevailing ideas on protein targeting assume that *N*-glycosylation of proteins takes place in the ER (Corsi and Schekman, 1996). In this respect, and as confirmed by the immunocytochemical analyses of Supplemental Figure 4 online, NPPs are predicted to occur in membrane systems (ER, Golgi complex, plasma membrane, and tonoplast), to accumulate in vacuoles, or to be secreted from the cells. In fact, some members of the NPP family have been predicted to be secreted or bound to the plasma or ER membranes facing the lumenal side (Olczak and Olczak, 2002). However, both fluorescence microscopy (Figure 4) and immunocytochemical analyses (Figure 7; see Supplemental Figure 3 online) of NPP1-GFP–expressing cells, as well as fractionation studies of leaf cells (Table 6; see Supplemental Figure 5 online), have shown that NPP1 occurs in the plastidial compartment.

Although consistent with recent reports showing the occurrence of *N*-glycosylated proteins in plastids (Asatsuma et al., 2005; Villarejo et al., 2005), the finding of NPP1 in the interior of a subcellular compartment unrelated to the ER-Golgi system was quite surprising and totally unpredictable in the context of the chloroplast genomics and proteomics (Miras et al., 2002; Leister,
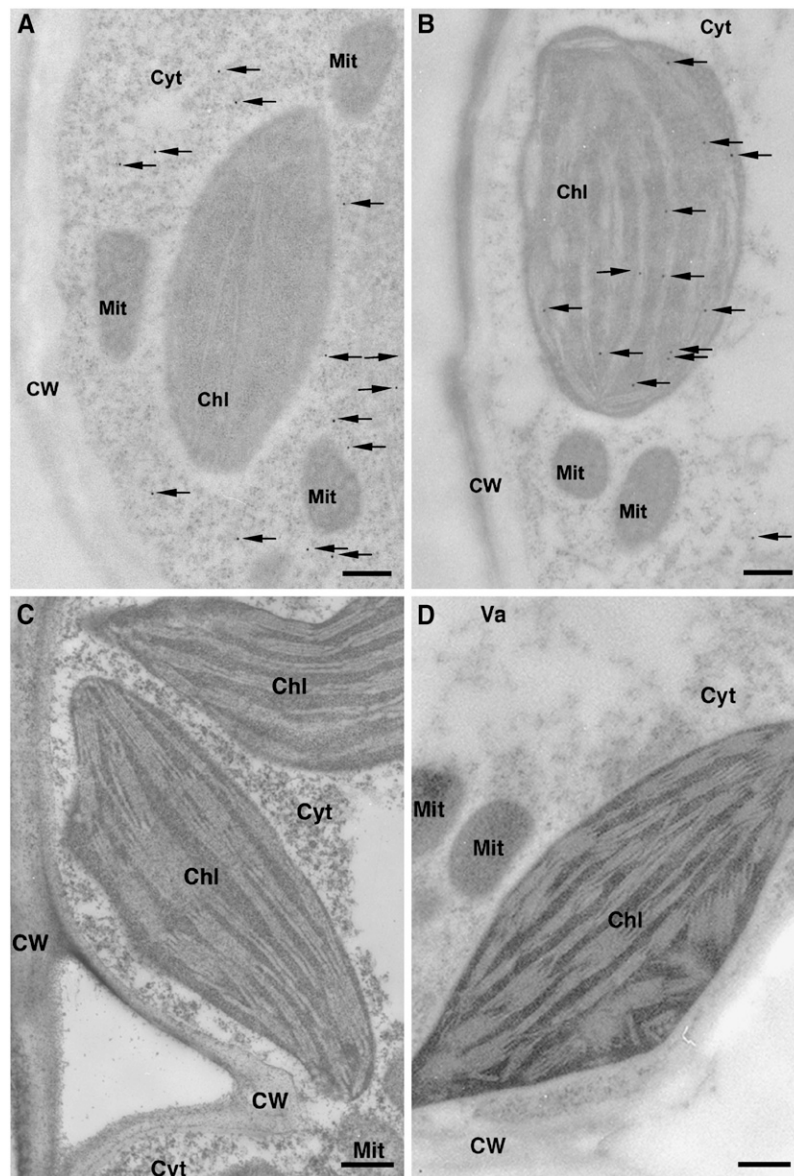
Figure 5. Intracellular Immunolocalization of GFP in Either GFP- or NPP1-GFP–Expressing Transgenic Rice Cells and Untransformed Rice Cells.

(A) GFP-expressing transgenic rice cells.
(B) and **(C)** NPP1-GFP–expressing transgenic rice cells.
(D) Untransformed rice cells.
Anti-GFP antiserum (**[A]**, **[B]**, and **[D]**) and preimmune serum **(C)** were used as the primary antibody. The arrows indicate gold particles. Wider views of **(A)** and **(B)** are shown in Supplemental Figure 3 online. CW, cell wall; Cyt, cytosol; Chl, chloroplast; Mit, mitochondrion; Nu, nucleus; Va, vacuole. Bar = 1 μm.

2003). A mechanism of protein traffic from the ER-Golgi to the chloroplast must be invoked that is distinct from the well-established pathway of protein traffic between the cytosol and plastids (Heins et al., 1998). In this respect, results presented in Figure 7 showing the accumulation of NPP1-GFP in the ER network structures of BFA-treated cells and inhibition of NPP1-GFP accumulation in the chloroplast strongly indicate that, essentially similar to the suggested mechanisms of transfer of some *N*-glycosylated proteins in *Arabidopsis thaliana* leaves (Villarejo et al., 2005), NPP1 can be transported within vesicles from the ER-Golgi system to the chloroplast. This idea is further supported by the results presented in Supplemental Figure 7 online showing the occurrence of GFP in ER network structures of cells expressing the 66 and 108 N-terminal amino acid residues of NPP1 (which contains the predicted signal peptide for the ER) fused with GFP.

Table 6. Isolation of Chloroplasts from Rice Shoots

	ADPG Hydrolytic NPP (mU/mg Protein)[a]	Alkaline Pyrophosphatase (mU/mg Protein)	IDPase (mU/mg Protein)	NADPH-cytochrome *c* Reductase (mU/mg Protein)	UDP-Glucose Pyrophosphorylase (mU/mg Protein)
Crude extract	142 ± 11	605 ± 58	6.76 ± 0.86	84.2 ± 7.4	765 ± 54
4000*g* Pellet	34.1 ± 4.8	907 ± 91	1.25 ± 0.09	20.8 ± 5.2	58.2 ± 8.8
Isolated chloroplast	89.2 ± 5.4	2138 ± 203	BDL[b]	BDL	BDL

[a] mU, milliunits.
[b] BDL, below detection limit.

Our data show that the chloroplast proteome contains *N*-glycosylated proteins and provide firm support for the existence of a targeting pathway of *N*-glycosylated proteins from the ER-Golgi system to the chloroplast (Radhamony and Theg, 2006).

METHODS

Plant Material

Barley plantlets (*Hordeum vulgare* cv Scarlett) were grown in the fields of the Public University of Navarra and harvested 5 d after germination. The rice variety used in this study was *Oryza sativa* cv Nipponbare. The seeds were supplied from the Niigata Agricultural Research Institute (Niigata, Japan). Rice seeds were sterilized in 1% sodium hypochlorite, rinsed in sterile water, and cultured for 5 d at 30°C in darkness until germination.

Extraction and Purification of NPP

Purification of Rice NPP

Shoots (200 g) of germinating rice seeds were homogenized in five volumes of 10 mM Tris-HCl, pH 8.8, and filtered through four layers of gauze. The homogenate was centrifuged at 10,000*g* for 20 min, and the resulting supernatant was adjusted to 20% (w/v) (NH_4)$_2$$SO_4$ and centrifuged at 10,000*g*. The supernatant thus obtained was applied to a Butyl-Toyopearl 650M column (2.5 × 21-cm diameter; Tosoh) equilibrated with 20% (NH_4)$_2$$SO_4$ and 10 mM Tris-HCl, pH 8.8, and eluted with 75 mL of a linear gradient of 20 to 0% (NH_4)$_2$$SO_4$ in 10 mM Tris-HCl, pH 8.8. The active fractions were adjusted to pH 5.0 with acetate buffer and centrifuged at 10,000*g*. The supernatant was readjusted to pH 7.0 with 1.5 M Tris-HCl, pH 8.8, and centrifuged at 10,000*g*. The resulting supernatant was applied to a Con A–Sepharose 4B column (1.0 × 8 cm-diameter; Amersham) equilibrated with 40 mM Tris-HCl, pH 7.0, 0.5 M NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$ and eluted with 25 mL of 0.5 M α-methyl-D-mannopyranoside in 10 mM Tris-HCl, pH 8.8. Finally, the enzyme preparation was desalted by ultrafiltration on Microcon YM-100 (Amicon).

Purification of Barley NPP

One kilogram of leaflets were homogenized using a Waring Blender with 3 liters of extraction buffer consisting of 50 mM MES, pH 6.0, 0.5 M NaCl, 1 mM EDTA, and 2 mM (±)-DTT and filtered through four layers of Miracloth. The homogenate was centrifuged at 100,000*g* for 30 min, and the supernatant was adjusted to 50% (NH_4)$_2$$SO_4$. The precipitate obtained after 30 min of centrifugation at 30,000*g* (at 20°C) was resuspended in 2.8 liters of the extraction buffer, heated in a water bath at 62°C for 20 min, cooled on ice, and centrifuged at 30,000*g* for 20 min. Proteins from the supernatant were precipitated with 50% (NH_4)$_2$$SO_4$ and resuspended in 33 mL of extraction buffer. The sample was then subjected to gel filtration on a Superdex 200 column (Amersham) preequilibrated with

50 mM MES, pH 6.0, containing NaCl 0.5 M. The partially purified enzyme preparation obtained was examined by isoelectric focusing using Amersham broad-range (pH 3.5 to 9.5) PAGplates in a Multiphor II system. After electrophoresis, the gel was cut and NPP eluted at 4°C in 50 mM MES, pH 6.0, containing 0.5 M NaCl.

The native molecular mass of NPP was determined from a plot of Kav (partition coefficient) versus log molecular mass of protein standards from a Bio-Rad kit. Protein content was measured by the Bradford method using the Bio-Rad prepared reagent.

Enzyme Assays

Enzyme activities were measured according to the methods described in the accompanying references: ADPG hydrolytic NPP (Rodríguez-López et al., 2000), alkaline pyrophosphatase (Baroja-Fernández et al., 2000), UDP-glucose pyrophosphorylase (Kimura et al., 1992), and IDPase and NADPH-cytochrome *c* reductase (Mikami et al., 2001). One unit of enzyme activity is defined as the amount of enzyme that catalyzes the production of 1 μmol of product per minute.

Amino Acid Sequencing

Purified ADPG hydrolytic NPP was digested with *Staphylococcus aureus* V8 protease in gel and separated by SDS-PAGE according to the
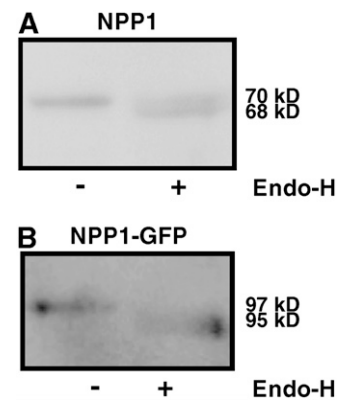

Figure 6. Endo-H Susceptibility of NPP1 and NPP1-GFP in Chloroplasts Isolated from NPP1- and NPP1-GFP–Expressing Rice Cells.

(A) NPP1-expressing rice cells.
(B) NPP1-GFP–expressing rice cells.
In **(A)**, chloroplastic proteins were digested with Endo-H, subjected to SDS-PAGE separation, and immunoblotted using NPP1 antiserum. In **(B)**, chloroplastic proteins were digested with Endo-H, subjected to SDS-PAGE separation, and immunoblotted using GFP antibodies.
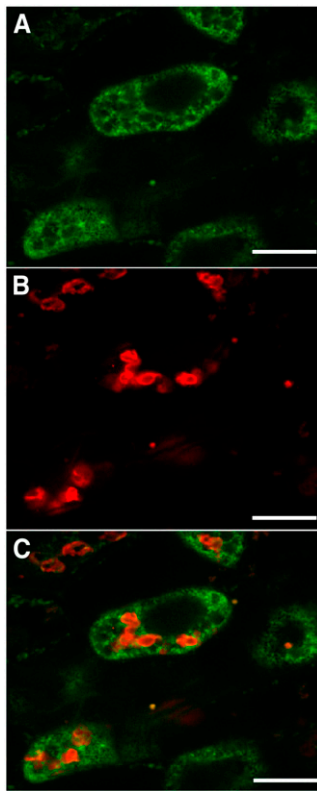
Figure 7. BFA Effect on GFP Fluorescence Distribution in NPP1-GFP–Expressing Rice Cells.

Stably transformed cells were sectioned with a vibratome (VIB3000 plus-SKR; Meiwa), incubated for 90 min with Murashige and Skoog medium in the presence of 90 μM BFA, and immediately observed by confocal laser scanning microscopy (FV1000; Olympus). GFP fluorescence **(A)**; chlorophyll autofluorescence **(B)**; merged image **(C)**. BFA treatment causes NPP1-GFP redistribution to the ER and Golgi-like structures. In addition, BFA prevents NPP1-GFP traffic to the chloroplast. Bars = 10 μm.

procedure reported by Mitsui et al. (1996). Peptide fragments were then blotted onto polyvinylidene difluoride membrane (Hybond-P; Amersham), excised, and subjected to N-terminal sequencing analysis with a Shimadzu PPSQ-21 amino acid sequencer.

Cloning and Expression of a Rice NPP Encoding cDNA

Based on the peptide sequences and analyses of the genome databases of the National Institute of Agrobiological Sciences DNA Bank (http://www.dna.affrc.go.jp), a partial NPP encoding cDNA was obtained by RT-PCR using 0.5 μg of poly(A)$^+$ RNA from 5-d-old rice shoots and the following two primers: 5′-GGCGTTGCTCGGCGACCA-3′ (forward, encoding the ALLGDQ sequence at the N-terminal part of the rice NPP) and 5′-GAGGCGAGCGTGGTGGGGA-3′ (reverse, encoding the PTTLAS sequence at the C-terminal part of NPP).

The cDNA amplified was then cloned into the pGEM-T vector (Promega) and used as a probe to screen rice and barley shoot cDNA libraries made using the ZAP-cDNA synthesis kit in the Uni-ZAPxR vector (Stratagene). The complete cDNAs thus obtained, designated as *NPP1*, was cloned into pBluescript SK− (Stratagene) to create pOsNPP1.

For *NPP1* expression in *Escherichia coli*, the *Eco*RI-*Xho*I fragment from pOsNPP1 was cloned into pET-28b(+) expression vector (Novagen) to

create pET-OsNPP1 (see Supplemental Figure 8 online). *E. coli* BL21(DE3) transformed with this plasmid were grown to an absorbance at 600 nm of ∼0.6, and then 1 mM isoprophyl-β-D-thiogalactopyranoside was added to the culture medium.

For *NPP1* overexpression in both rice plants and cultured cells of rice, the *Bam*HI-*Sac*I PCR-amplified fragment from pOsNPP1 was cloned into the same sites of the p2K-1+ plant expression vector that carried the maize ubiquitin (*Ubq*) promoter and an intron (Christensen et al., 1992; Miki and Shimamoto, 2004) to produce p2K-OsNPP1 (see Supplemental Figure 9 online). *Agrobacterium tumefaciens*–mediated transformation and regeneration of rice plants were performed according to the methods described by Hiei et al. (1994). Cultured rice cells were grown in hygromycin-selective medium for 2 weeks and then transferred to redifferentiation medium for 1 month.

Production and Expression of GFP Fusion Constructs

The NPP1 cDNA sequence was ligated to the 5′ end of the GFP gene of the modified pTN1-GFP (5′-35SΩ -sGFP-nos-3′) plant expression vector (Chiu et al., 1996; Fukuoka et al., 2000) to create pTN1-OsNPP1-GFP (5′-35SΩ -NPP1-sGFP-nos-3′) as follows: NPP1 was PCR-amplified using the BamHI-N-ter (5′-TTGGATCCATGGTTAGTAGGAAGAGAGGA-3′) and BamHI-C-ter (5′-GAAGGATCCGGAGGCGAGCGTGGTGGGGA-3′) flanking primers. The PCR product was then digested by BamHI and inserted into the BamHI site of pTN1-GFP (see Supplemental Figure 10 online).

pUC-mt-GFP (5′-35SΩ -mt-sGFP-nos-3′) (Niwa et al., 1999) was used to study the topographical distribution of mitochondria within the cell. mt-GFP is a presequence of the γ-subunit of the *Arabidopsis thaliana* F1-ATPase fused with GFP.

For stable expression of GFP and NPP1-GFP fusion proteins in rice green cells, gold particles (1-μm diameter) were coated with 2 μg of plasmid DNA. The DNA-coated gold particles were spotted on macro-carriers and used to transform rice culture cells at 1100 p.s.i. using a PDS-1000HE biolistic device (Bio-Rad). The bombarded cells were cultured with selective medium containing G418 for 2 weeks and then cultured in redifferentiation medium for 1 month. Redifferentiated cells with chlorophyll were sectioned with a vibratome (VIB3000 plus-SKR; Meiwa) to 25-μm thickness, treated for 90 min with Murashige and Skoog medium in the presence or absence of 90 μM BFA, and immediately observed by confocal laser scanning microscopy (FV1000; Olympus).

Production of Antibodies

Extracts of *E. coli* (DE3) transformed with pET-OsNPP1 were subjected to His-bind chromatography (Novagen). The purified His-tagged NPP1 thus obtained was electrophoretically separated by SDS-PAGE and stained with Coomassie Brilliant Blue R 250. An ∼70-kD protein band was then eluted and used to produce polyclonal antisera against NPP1 by immunizing rabbits. Anti-Rubisco was prepared as described previously (Nishimura and Akazawa, 1974). Anti-GFP was supplied by Invitrogen (Japan).

Endo-H Digestion, Immunoblots, and Lectinoblots

To test the possible occurrence of an *N*-linked oligosaccharide chain conjugated to NPP, the enzymatic liberation of the oligosaccharide portion from the enzyme by Endo-H digestion was performed. After the addition of 0.1 M citrate-phosphate buffer, pH 5.0, and 20 milliunits/mL Endo-H to pure proteins (0.1 μg) or chloroplast lysate proteins (20 μg), the whole mixture was incubated at 37°C for 24 h. Procedures of immunoblotting with specific antibodies and lectin blotting with Con A–peroxidase were performed essentially as described by Mikami et al. (2001). An aliquot of sample was subjected to SDS-PAGE, followed by electric

transfer of proteins separated in SDS gels to a nitrocellulose membrane (Hybond-C extra; Amersham). In immunostaining and lectinostaining, anti-NPP1 and anti-Rubisco antisera were diluted to 1:5000, anti-GFP to 1:100, [^{35}S]-labeled anti-rabbit Ig (Amersham) to 18.5 kBq/mL, peroxidase-conjugated anti-rabbit IgG to 1 μg/mL, and peroxidase-conjugated Con A to 0.4 μg/mL. The blotted nitrocellulose sheet was soaked with PBST (PBS with 0.05% Tween 20) for 15 min three times and incubated with the primary antibody in PBST containing 1% (w/v) skim milk or Con A–peroxidase in PBST for 12 h at 4°C. The sheet was then incubated with the second antibody for 3 h at room temperature as for the primary antibody. The autoradiograms were scanned using a radioisotope imaging analyzer (BAS-5000; Fuji Film), and photo-stimulated fluorescence was determined.

Immunocytochemistry

For immunocytolocalization studies, small pieces of rice tissue were fixed at room temperature in freshly prepared 4% (w/v) paraformaldehyde and 0.6% (v/v) gluteraldehyde in 60 mM Na-cacodylate buffer, pH 7.4, and placed under low vacuum for 1 h. Fixative was rinsed twice with 60 mM Na-cacodylate buffer for 30 min. The samples were then placed in a mixture of 3% potassium ferricyanide plus 2% osmium tetroxide (1:1) and fixed in the microwave for 4 min. Following fixation, tissue sections were washed twice in buffer and once in double-distilled water, dehydrated through a cold graded ethanol series up to 95% ethanol, and embedded in LR White resin following the manufacturer's protocol. Polymerization took place at 55°C for 24 h.

Ultrathin sections were collected on uncoated nickel grids and processed for immunogold labeling. Prior to incubation with either the preimmune or immune primary antibody, the sections were placed in PBS, pH 7.2, for 20 min and blocked at room temperature with PBS, 5% BSA, and 5% goat serum. Primary antibody was diluted in PBS, 0.2% BSA, and 15 mM NaN$_3$ (incubation buffer). Incubation was performed at room temperature for 1 h followed by washing with incubation buffer. Sections were then incubated for 1 h in goat anti-rabbit IgG antibody conjugated to 10-nm colloidal gold, washed in incubation buffer PBS, fixed with 2% gluteraldehyde, and rinsed with water. After staining for 10 min in 3% (w/v) uranyl acetate, sections were washed in double-distilled water. Ultrathin sections were then examined with a Hitachi H-600 transmission electron microscope at an accelerating voltage of 75 kV.

Isolation of Chloroplasts

The isolation procedure of chloroplasts was essentially as described by Tanaka et al. (2004). Ten grams of rice green cells were chopped with a razor in an isolation solution containing 50 mM HEPES-KOH, pH 7.5, 0.3 M sorbitol, 1 mM MgCl$_2$, and 2 mM EDTA. The chloroplast suspension was passed through four layers of gauze and centrifuged at 4000g for 20 s at 4°C. The pellet was gently suspended in the above isolation solution and then layered onto a discontinuous density gradient consisting of 10, 40, and 80% (v/v) Percoll in the isolation solution. The gradient was centrifuged at 8000g for 10 min at 4°C. Intact chloroplasts distributed around the 40/80% Percoll interface were isolated and reapplied to the Percoll gradient centrifugation.

Accession Numbers

Sequence data for the nucleotide sequence of *NPP1* can be found in the DDBJ and EMBL libraries under the accession number AB100451. The N-terminal end of the barley NPP has been deposited in the SWISS-PROT protein database under the accession number P83656. Sequence data of PPD1 were also found in the GenBank database under accession number AJ421009.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Amino Acid Sequence Alignments of NPP1 with Other Possible Homologs from Yellow Lupin, *Arabidopsis*, Barley and Tomato.

Supplemental Figure 2. Production of NPP1-Overexpressing Rice Plants.

Supplemental Figure 3. Wider Views of Figures 5A and 5B.

Supplemental Figure 4. Intracellular Immunolocalization of NPPs in Leaves Using Anti-NPP1 Polyclonal Antiserum.

Supplemental Figure 5. Distributions of NPP1 and Rubisco on a Density Percoll Centrifugation Gradient.

Supplemental Figure 6. Migrating Behavior on SDS Gels of Purified ADPG Hydrolytic NPP without a Previous Heat Denaturing Step.

Supplemental Figure 7. Fluorescence Images of Onion Epidermal Cells Transiently Expressing GFP-SYP31 (Golgi Marker), GFP-SYP81 (ER Marker), GFP (Cytosolic Marker), and the First 66 and 108 Amino Acid Residues of NPP1, Containing the Signal Peptide for the ER Fused with GFP.

Supplemental Figure 8. Stages to Construct pET-OsNPP1 to Express Rice NPP1 in *E. coli*

Supplemental Figure 9. Stages to Construct p2K-OsNPP1 Plasmid Necessary to Produce NPP1-Expressing Transgenic Rice Plants

Supplemental Figure 10. Stages to Construct pTN1-OsNPP1-GFP Plasmid Necessary to Produce NPP1-GFP–Expressing Green Cells of Rice.

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