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Original Paper

Differential localizations and functions of rice nucleotide pyrophosphatase/phosphodiesterase isozymes 1 and 3

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Abstract Our previous investigation demonstrated that ADP-glucose hydrolytic nucleotide pyrophosphatase/phosphodiesterase (NPP) 1 is transported from the endoplasmic reticulum (ER)-Golgi system to the plastids via a secretory pathway in rice cells [Nanjo et al. (2006) *Plant Cell* 18: 2582–2592]. In this study, we analyzed the enzymatic characteristics and subcellular localization of its isozyme, NPP3. Unlike NPP1, NPP3 exhibited no hydrolytic activity toward ADP-glucose and no plastid-targeting ability. Furthermore, there was a clear difference between their N-terminal proteolytic processing schemes to form mature enzyme proteins. NPP1 is matured to a 70-kDa protein by two-step proteolytic processing. We detected the 72 kDa form of NPP1 in the microsomes of rice cells in addition to the 70 kDa mature protein, strongly suggesting that proprotein processing occurs post-translationally in the ER-Golgi system. To clarify the existence of the plastid-targeting signal of NPP1, the plastid localization of a series of carboxy-terminal truncated NPP1 proteins fused with green fluorescence protein was tested in rice cells. The results showed that NPP1 cannot be delivered to the plastid by the N-terminal region, including the ER signal sequence and the proprotein processing site, and that the peptide region, from 308 to 478 amino acid residues, is probably important for the transport of NPP1 into plastids in rice cells.

Key words: Glycoprotein, nucleotide pyrophosphatase/phosphodiesterase, *Oryza sativa*, plastid, starch.

Nucleotide pyrophosphatase/phosphodiesterase (NPP), which exhibits hydrolytic activity toward pyrophosphate and phosphodiester bonds of numerous nucleotides and nucleotide sugars, has been identified in higher plants (Nanjo et al. 2006; Rodríguez-López et al. 2000). Although the physiological functions of NPP family enzymes are still obscure, certain NPPs may play a crucial role in diverting carbon flux from gluconeogenesis to other metabolic pathways by controlling the levels of nucleotide sugars (Baroja-Fernández et al. 2001). An NPP gene, *NPP1*, has been identified and cloned from rice (*Oryza sativa*) (Nanjo et al. 2006). The amino acid sequence deduced from the nucleotide sequence of NPP1 cDNA has revealed that the precursor polypeptide possesses a putative endoplasmic reticulum (ER) signal sequence and several *N*-glycosylation sites. Indeed, NPPs isolated from different plant tissues, including rice seedlings, contain Concanavalin A lectin-recognized and partial endo-β-*N*-acetylglucosaminidase H-susceptible *N*-linked oligosaccharide chains (Nanjo et al. 2006; Rodríguez-López et al. 2000). The majority of chloroplast proteins are synthesized in the cytosol as precursors with an N-terminal transit peptide, and are then imported post-translationally through the Toc/Tic complex into the organelle (Inoue and Akita 2008; Kessler and Schnell 2009; Lee et al. 2008). Intriguingly, however, the trafficking of glycoproteins to the plastid via the ER-Golgi system in higher plants has been mentioned recently (Asatsuma et al. 2005; Ferro et al. 2010; Inaba 2010; Jarvis 2008; Li and Chiu 2010; Villarejo et al. 2005).

Our previous studies revealed that *Oryza sativa* NPP1 bearing *N*-linked oligosaccharide chains occurs in the chloroplasts. It is transported to the organelle via a Brefeldin A-sensitive vesicular transport pathway from the ER to the Golgi system (Nanjo et al. 2006). In the present study, an isozyme NPP3 was observed to localize in extra-plastidial compartments. Here, we discuss differential functions of NPP1 and 3, and also a possible targeting signal for NPP1 that is delivered into the plastids in rice cells.

Abbreviations: ACN, acetonitrile; CaMV, *Cauliflower mosaic virus*; CHCA, α-cyano-4-hyroxycinnamic acid; DTT, dithiothreitol; ER, endoplasmic reticulum; GFP, green fluorescent protein; MALDI-TOF-MS, matrix-assisted laser desorption ionization-time of flight-mass spectrometry; NPP, nucleotide pyrophosphatase/phosphodiesterase; TFA, trifluoroacetic acid

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Materials and methods

Plant materials

Rice seeds (*Oryza sativa* L. cv. Nipponbare) were supplied by the Niigata Agricultural Research Institute (Niigata, Japan). Rice calli derived from the embryo portion of the seed were cultured as follows. About 2 g of rice callus cells were grown in a 500 ml Sakaguchi flask holding 120 ml of MS medium containing 3% (w/v) sucrose, 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid, and 5 mg l^{-1} thiamine-HCl, placed on a reciprocal shaker operated at 110 strokes min^{-1} at 70 mm amplitude, at 28°C in darkness. Established suspension-cultured cells were subcultured at 7 day intervals. All the procedures were performed under aseptic conditions (Mitsui et al. 1996).

Cloning of NPP3 cDNA

Using *NPP1* cDNA (Nanjo et al. 2006; AB100451) as a probe, we screened the rice shoot cDNA libraries to isolate other NPPs encoding cDNAs. A complete cDNA obtained, designated as *NPP3* (Oryzabase http://www.shigen.nig.ac.jp/rice/oryzabase_submission/gene_nomenclature/; McCouch 2008; AK101976) was cloned into pBluescript SK(−) (Stratagene) to create pOsNPP3.

Plasmid constructions and transformation

The constructions of pGFP, pZH2B-35S-GFP (Asatsuma et al. 2005) and p2K-Ubq-OsNPP1 (Nanjo et al. 2006) have been described previously. For overexpression of *Oryza sativa NPP3* in rice plants and cultured cells, the BamHI-KpnI PCR amplified fragments from pOsNPP3 as the DNA templates with two flanking primers, KpnI-N-ter (TTGGTACCGTTAATCATGTTCCTCCTCGCT) and KpnI-C-ter (AAGGTACCAGCAGCAGTAAGCAACCTCAG) were cloned into the same sites on the p2K-1+ plant expression vector (Christensen et al. 1992; Miki et al. 2004) to produce p2K-Ubq-OsNPP3. We also PCR-amplified *NPP3* from pOsNPP3 with specific primer sets, KpnI-N-ter (TTGGTACCATGGCGGCGGCGG) and KpnI-C-ter (AAGGTACCCGAGGCCAGGGTGGTGGT), and then digested the PCR product with BamHI. The BamHI-digested fragments were then inserted into the same site on pZH2B-35S-GFP (Asatsuma et al. 2005; Hajdukiewicz et al. 1994; Kuroda et al. 2010) to create pZH2B-35S-OsNPP3-GFP.

Full-length NPP1 cDNA and various carboxyl-terminal truncated fragments were amplified by PCR with pOsNPP1 as the template DNA and flanking primer sets: TTGGATCCATGGTTAGTAGGAAGAGAGGA and GAAGGATCCGGAGGCGAGCGTGGTGGGGA (full-length NPP1), AAGGATCCATGGTTAGTAGGAAGAGAGGA and GTTGGATCCCGTGGCGCTGACGAACGC (NPP1(Δ67–623)), AAGGATCCATGGTTAGTAGGAAGAGAGGA and TTGGATCCCGGGTATCTGGAAGGATT (NPP1(Δ110–623)), AAGGATCCATGGTTAGTAGGAAGAGAGGA and AAGGATCCCTTGCCCATGTCGCCGAAGAG (NPP1(Δ309–623)), AAGGATCCATGGTTAGTAGGAAGAGAGGA and AAGGATCCCCACCAGTTGGAGGAGTAGCC (NPP1(Δ479–623)), AAGGATCCATGGTTAGTAGGAAGAGAGGA and AAGGATCCCTTCGGGATCGCCGAGGTGTA (NPP1(Δ562–623)). The BamHI-digested fragments were then inserted into the same site on pZH2B-35S-GFP to create pZH2B-35S-OsNPP1-GFP, -OsNPP1(Δ67–623)-GFP, -OsNPP1(Δ110–623)-GFP, -OsNPP1(Δ309–623)-GFP, -OsNPP1(Δ479–623)-GFP and -OsNPP1(Δ562–623)-GFP.

The binary vectors were transformed into competent cells of *Agrobacterium tumefaciens* strain EHA101 (Hood et al. 1986) and treated with 20 mM $CaCl_2$. *Agrobacterium*-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 two weeks and then transferred to redifferentiative medium for one month. The transgenic rice lines UNP1 transformed with p2K-Ubq-OsNPP1 and UNP3 transformed with p2K-Ubq-OsNPP3 were established.

Purification of NPP3

The purification procedure of NPP3 expressed in the UNP3 transgenic rice cells transformed with p2K-Ubq-OsNPP3 was essentially identical to that of the earlier study (Nanjo et al. 2006). Rice callus cells (200 g) were homogenized in 5 volumes of 10 mM Tris-HCl (pH 8.8) and filtered through 4 layers of gauze. The homogenates were centrifuged at 20,000 *g* for 10 min, and the supernatants were adjusted to an acidic pH 5.4 with acetate buffer and then centrifuged at 20,000 *g*. After re-adjustment to pH 7.4 with 1.5 M Tris-HCl (pH 8.8) and centrifugation at 20,000 *g*, the resulting supernatants were applied to a Concanavalin A-Sepharose 4B column (ϕ 1.0×8 cm, Pharmacia) equilibrated with 40 mM Tris-HCl (pH 7.4), 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 7.4). The eluents were desalted by ultrafiltration on a Microcon YM-100 (Amicon).

Assays

The enzyme activities of NPP were measured using the methods described in an earlier study (Rodríguez-López et al. 2000). Protein content was measured by the Bradford method using a Bio-Rad prepared reagent.

Microscopic analysis

A confocal laser-scanning microscope (FV300-BX-61; Olympus) was used to image green fluorescence protein (GFP) and chlorophyll autofluorescence in rice cells (Kitajima et al. 2009). For quantitative analysis, the fluorescence intensity in plastids and in whole cells was determined using Lumina Vision imaging software. The background was always set at the maximum value of fluorescence intensity for areas in which no structural images were present. The area visualized with either chlorophyll autofluorescence was defined as the plastidial area. In analysis of the whole rice cell, each individual image from the top to the bottom of the cell, 10 to 15 frames every 0.7 to 1.4 μm, was evaluated. To evaluate the plastid-targeting abilities of GFP-labeled proteins, we determined the ratio of the fluorescence intensity of GFP in the plastidial area to that of GFP in the whole cell ($GFP_{plastid}/GFP_{total}$).

Immunoblotting analysis of microsomal NPP1

Microsomal membranes of rice (UNP1) were prepared according to the procedure described in earlier studies (Asakura et al. 2006; Mikami et al. 2001). An aliquot of the membrane sample was subjected to SDS-PAGE, followed by immunoblotting. In immunoblotting, anti-NPP1 antisera were

diluted to 1 : 5000 and peroxidase-conjugated anti-rabbit Ig IgG to 1 μg ml^{-1}, respectively. The reacted protein bands were visualized with 0.03% (w/v) 3,3′-diaminobenzidine and 0.003% (v/v) H_2O_2 in 15 mM phosphate buffer (pH 6.8) (Nanjo et al. 2006).

N-terminal amino acid sequencing analysis

The procedure of N-terminal amino acid sequencing was same as described by Nanjo et al. (2006). Purified proteins were separated by SDS-PAGE, and then blotted onto polyvinylidene difluoride membrane (Hybond-P; GE Healthcare). The excised protein bands were subjected to N-terminal sequencing analysis with a protein sequencer (PPSQ-21; Shimadzu).

MALDI-TOF-MS analysis

Tryptic digestion was performed using standard procedures (Azwan et al. 2010). Gel bands were excised, cut into smaller pieces and washed with ultrapure water. Coomassie-stained gel bands were destained by several washing steps using 25 mM ammonium bicarbonate and ACN (1 : 1). Gel pieces were dehydrated by adding ACN. After 10 min of incubation, the ACN was removed and the gel fragments were dried in a Speed-Vac. The dry gel pieces were incubated for 45 min at 56°C in 200 μl of 10 mM dithiothreitol (DTT) to reduce the disulfide bonds. The DTT was washed off and the cysteins were alkylated by incubation with 100 μl of a mixed solution of 55 mM iodoacetamide and 25 mM ammonium bicarbonate for 30 min at room temperature in the dark. After washing steps with 25 mM ammonium bicarbonate, the gel was dried and incubated overnight at 37°C with 20 ng μl^{-1} trypsin in 25 mM ammonium bicarbonate buffer. The reaction was stopped by adding 50 μl of 50% ACN/5% TFA solution. After 10 min of sonication, the supernatant was recovered and the peptides were further extracted by adding 50 μl of 50% ACN/5% TFA solution followed by 10 min of sonication. The digestion solution and extract were combined and concentrated to approximately 10 μl.

Matrix solution was prepared by mixing 300 μl of α-cyano-4-hyroxycinnamic acid (CHCA) in acetone (1 mg ml^{-1}) solution with 600 μl ethanol. One microliter of the sample was loaded onto the MTP 384 target plate ground steel T F (Bruker Daltonics) and allowed to dry in air, followed by application of 1 μl of matrix solution. After drying, the sample-matrix crystal was washed with 1 μl of 0.1%TFA solution.

MS spectra were obtained using positive reflection mode on a Bruker Daltonics Autoflex III TOF/TOF-NN without post-ionization delayed extraction. MS spectra were acquired with the following settings: ion source 1, 19 kV; ion source 2, 16.6 kV; lens, 8.75 kV; reflector, 21 kV; reflector 2, 9.7 kV; matrix suppression, 500 Da. External mass calibration was carried out using Bruker Daltonics peptide calibration standards. All spectra were obtained without application of collision gas in the collision cell. Approximately 1000 laser shots at a 100-Hz repetition rate (nitrogen laser, 337 nm) were collected over different areas of the matrix spot to generate MS spectra. The spectra were processed using FlexAnalysis 3.0 and BioTools 3.1 software tools (Bruker Daltonics).

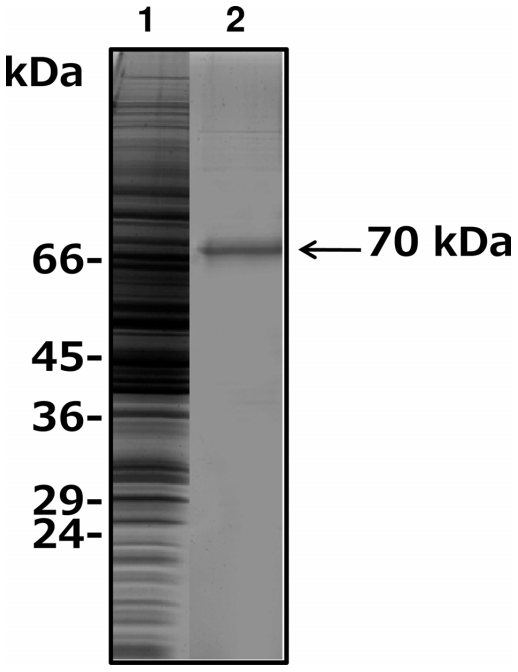

Figure 1. Purification of NPP3. NPP3 proteins were purified from rice transgenic cells with a constitutively high expression of NPP3, by employing a chromatographic process, SDS-PAGE, and Coomassie Blue staining. 1, crude extract; 2, purified enzyme preparation. Molecular size markers are shown on the left.

Results and discussion

NPP3 exhibits no ADP-glucose hydrolytic activity or plastid-targeting ability

A gene of the NPP family, *NPP3*, was identified and characterized in rice. The cDNA of *NPP3* was 2,093 bp in length and includes an open reading frame of 1,848 bp that encodes 615 amino acid residues with a calculated molecular mass of 68,133 and a pI of 5.86. The degree of similarity between NPP3 and NPP1 was 61.6%. The *NPP3* gene was located on chromosome 3.

We generated rice cells constitutively expressing *NPP3* (UNP3 line). The recombinant proteins of NPP3 were then purified to apparent homogeneity from the transgenic cells (Figure 1). SDS-PAGE and subsequent staining of the NPP3 protein revealed a single 70 kDa band. The molecular size of NPP3 as estimated by gel filtration was 524 kDa, indicating that NPP3 occurs as homopolymer made up of the same 70 kDa polypeptide. We determined the substrate specificity and kinetic parameters of the NPP3 enzyme (Table 1). NPP1 was shown to preferably cleave the starch precursor molecule, ADP-glucose (Nanjo et al. 2006). In contrast, NPP3 was highly specific to bis(*p*-nitrophenyl)phosphate and exhibited no quantifiable hydrolyzing activity toward ADP-glucose (Table 1). The physiological functions of NPP1 and NPP3 were thus clearly different. The enzymatic properties of NPP3 appeared to be similar to those of yellow lupin diphosphonucleotide phosphatase/

phosphodiesterase PPD (Olczak et al. 2000).

Our previous study revealed that NPP1 is transported to the chloroplasts via the Golgi apparatus through the secretory pathway (Nanjo et al. 2006). To determine the subcellular localization of NPP3, we produced rice cells stably transformed with *NPP3-GFP* and subjected them to confocal microscopy analyses. As shown in Figure 2, the fluorescence distribution pattern of NPP3-GFP was clearly distinguishable from that of GFP alone. However, most of the NPP3-GFP was distributed in extra-chloroplastic compartments, probably in the endomembrane system of the cells (Figure 2G). The fact that NPP3, unlike NPP1, exhibits no ADP-glucose hydrolytic activity or plastid-targeting ability strongly suggests that NPPs play differential roles in the metabolism of nucleotide pyrophosphates and phosphoric diesters in rice cells.

Proteolytic processing of NPP1

Analyses with PSORT (http://psort.hgc.jp/; Nakai et al. 1988) and SignalP (http://www.cbs.dtu.dk/services/SignalP/; Nielsen et al. 1997) algorithms predicted the precursor forms of NPP1 and NPP3 to have cleavable hydrophobic N-terminal signal sequences potentially acting as signal peptides to the ER. Determination of the N-terminal ends of NPP1 and NPP3 mature proteins indicated that, although the N-terminal amino acid residue of NPP3 mature protein was exactly matched to the predicted cleavage site of the signal sequence, the peptide removed from the NPP1 precursor protein was 24 residues longer than the signal sequence (Figure 3A). These results strongly suggest that the mature NPP1 polypeptide is formed by two-step proteolytic processing. Following the co-translational cleavage of the signal sequence in the ER, the processing of NPP1 proprotein is likely to occur post-translationally. To examine the presence of NPP1 proprotein, the microsomal membranes prepared from rice cells with a constitutively high expression of NPP1 (UNP1 line) were subjected to SDS-PAGE, followed by immunoblotting with anti-NPP1 antibodies. A 72 kDa protein recognized by anti-NPP1 antibodies was detected in rice microsomes (Figure 3B), and this protein was also confirmed to be NPP1 by means of MS analysis (Figure 3C). By contrast, the 72 kDa protein was only faintly detectable in the crude extract of whole cells (Figure 3B). These results suggest that the proteolytic processing of the NPP proprotein to the mature protein takes place in the ER-Golgi system. The carboxy side of His^{51} was determined to be the processing site of the proprotein (Figure 3A). It has been reported that chymotrypsin slowly cuts after His (Keil 1992), and a streptococcal C5a endopeptidase exhibits substrate specificity toward the carboxy side of His and Gln (Cleary et al. 1992). It is thus likely that specific endopeptidase cleavage after His operates in the secretory pathway of rice cells.

Table 1. Substrate specificity and kinetic analysis of NPP3

Substrates	K_m mM	V_{max} μmol min^{-1} (mg protein)$^{-1}$	K_{cat} s^{-1}	K_{cat}/K_m M^{-1} s^{-1}
ADP	2.19	274.34	320	1.46×10^5
UDP	1.86	270.90	316	1.70×10^5
GDP	1.80	251.95	293	1.63×10^5
IDP	1.78	252.77	295	1.65×10^5
CDP	1.57	245.23	286	1.82×10^5
TDP	3.24	272.93	318	0.98×10^5
PPi	4.66	514.56	600	1.28×10^5
APS	1.76	94.60	110	0.62×10^5
bis-*p*-NPP	0.89	554.47	647	7.52×10^5
ADP-glucose	n.q.[a]	n.q.	n.q.	n.q.

[a] n.q., not quantifiable.

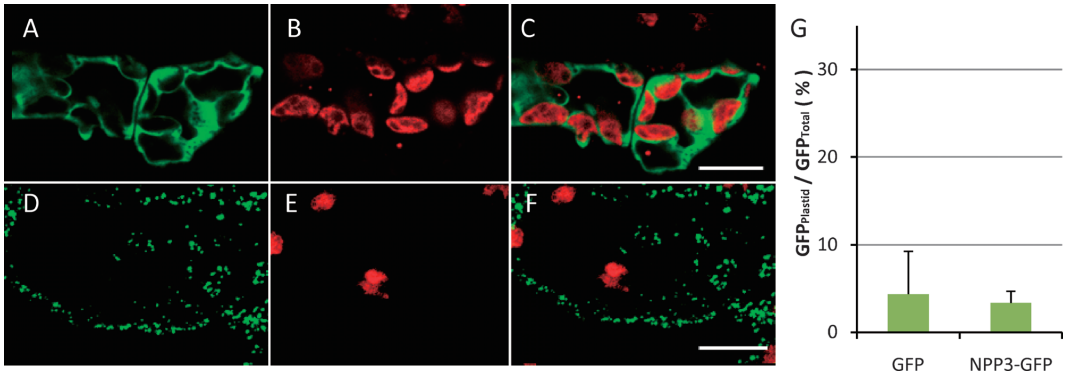

Figure 2. Fluorescent images in transgenic rice cells expressing either GFP or NPP3-GFP. The stable transformant cells were sectioned with a vibratome to 25 μm thickness and immediately observed by means of confocal laser scanning microscopy. (A to C) GFP; (D to F) NPP3-GFP. [A] and [D] GFP fluorescence, [B] and [E] chlorophyll autofluorescence, [C] and [O] GFP and chlorophyll autofluorescence merged. Bars represent 10 μm. (G) Statistical evaluation of the plastidial localization of NPP3-GFPs. Ratios of the fluorescence intensity of GFP in the plastidial area to GFP in the whole cell ($GFP_{Plastid}/GFP_{Total}$) (%) were determined. Values show means±s.d. ($n=3$).

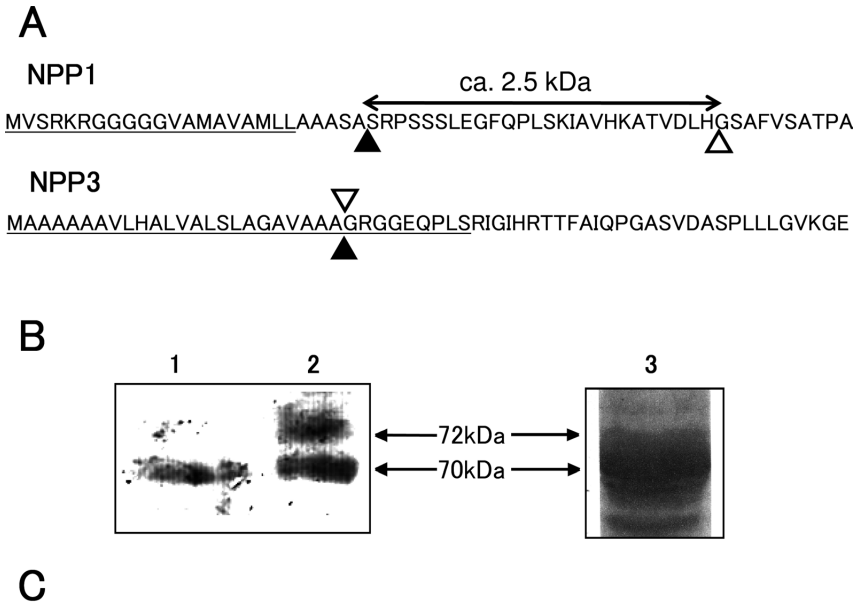

Figure 3. Identification of NPP1 proprotein in rice microsomes. (A) The N-terminal amino acid sequences of precursor proteins deduced from the nucleotide sequence of NPP1 and NPP3 cDNAs. The filled triangles represent predicted cleavage sites of the ER signal sequence, and the open triangles represent the N-terminal ends of mature proteins. (B) Immunoblotting analysis of microsomal NPP1. Crude extract and microsomes prepared from rice calli of UNP1 line were subjected to SDS-PAGE, followed by immunoblotting with anti-NPP1 antibodies. Lane 1 and 2, immunoblots of crude extract and microsomes, respectively. Lane 3, microsomal protein bands on SDS-gel visualized with Coomassie Blue staining. (C) Identification of 72 kDa NPP1 in microsomes. 72 kDa protein bands excised from SDS-gels were subjected to MALDI-TOF-MS analysis. The peptide sequences of NPP1 detected in the MS analysis are bold-faced and underlined.

Characterization of the plastid-targeting signal of NPP1

Both immunocytochemical and bio-imaging analyses of rice cells expressing NPP1-GFP revealed that NPP1-GFP occurs in the plastidial compartment (Nanjo et al. 2006; Figure 4A–C). As described above, the predicted precursor sequence of NPP1 contains the signal peptide and the additional extra-peptide that is cut off post-translationally in the secretory pathway. It has been reported that thylakoid lumen proteins are synthesized as a precursor that possesses two distinct targeting signals in tandem. The first transit peptide is removed in the stroma, after which the exposed second targeting peptide is recognized and cleaved by the thylakoid processing peptidase (Hageman et al. 1986; Ko and Cashmore 1989). We considered the possibility that the extra-peptide of NPP1 might serve as a signal for delivering NPP1 to the plastid. To test this hypothesis, we examined the subcellular localization of the N-terminal 66 amino acid residues of NPP1, NPP1(Δ67–623) fused with GFP in rice cells. As shown in Figures 4D–F and 5, NPP1(Δ67–623)-GFP frequently appeared on the surface of cells, but was only faintly detectable in the plastids. Similarly, in cells expressing NPP1(Δ110–623)-GFP, GFP fluorescence was only slightly observable in the plastids, whereas the ER network structure, in addition to the cell face, was visualized well by the fluorescent proteins (Figures 4G–I and 5). Based on these results, we concluded that the NPP1 signal peptide is needed to enter the secretory pathway, but the N-terminal extra-peptide of NPP1 is not sufficient for the plastid-targeting of this enzyme protein.

To clarify the plastid-targeting signal of NPP1, we further examined the plastid localization of a series of carboxy-terminal truncated NPP1s fused with GFP in rice cells. Although NPP1(Δ479–623)-GFP and NPP1(Δ562–623)-GFP fusion proteins were normally targeted into plastids like the full-length NPP1-GFP

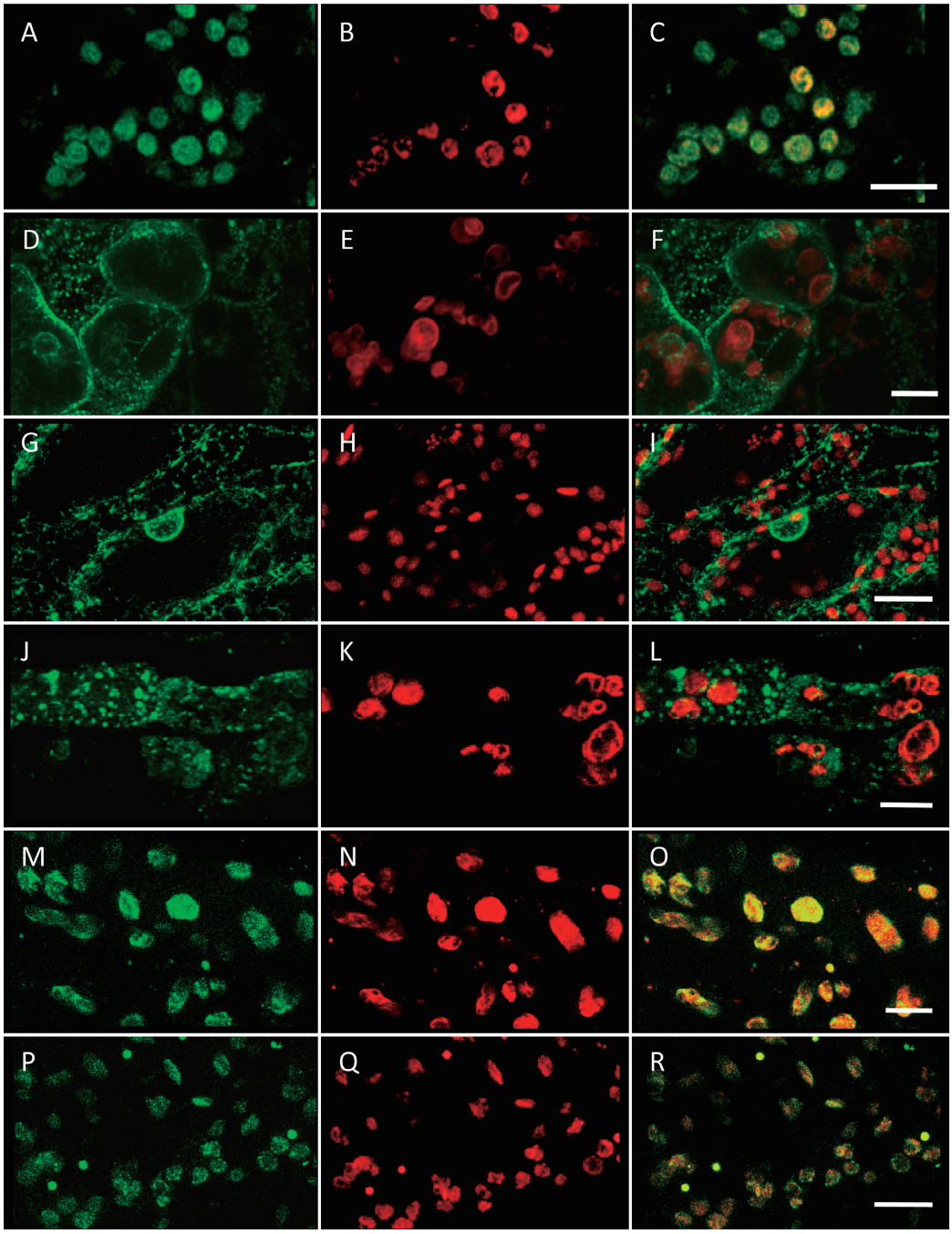

Figure 4. Plastid-targeting abilities of various C-terminal truncated NPP1-GFPs in transgenic rice cells. The stable transformant cells were sectioned with a vibratome to 25-μm thickness and immediately observed by means of confocal leaser scanning microscopy. (A to C) NPP1-GFP; (D to F) NPP1(Δ67–623)-GFP; (G to I) NPP1(Δ110–623)-GFP; (J to L) NPP1(Δ309–623)-GFP; (M to O) NPP1(Δ479–623)-GFP; (P to R) NPP1(Δ562–623)-GFP. Left panels, GFP fluorescence; middle panels, chlorophyll autofluorescence; right panels, merged image. Bars represent 10 μm.

(Figure 4A–C, M–O and P–R), more carboxyl-terminal deletion of NPP1, NPP1(Δ309–623), resulted in loss of plastid-targeting ability (Figures 4J–L, 5). Hence, we believe the peptide region from 308 to 478 amino acid residues to be important for the transport of NPP1-GFP into plastids in rice cells. There are no reports establishing that the stroma proteins with internal targeting signals are imported from the cytosol into the stroma compartment. However, the localization of ceQORH (chloroplast envelope quinone oxidoreductase; Miras et al. 2007) and IEP32 (inner envelope protein, 32 kDa; Nada and Soll 2004) to the inner envelope membrane of the chloroplast has been shown to be controlled by internal sequences. Recent investigations have demonstrated that the best characterized secretory glycoprotein AmyI-1 was transported from the Golgi

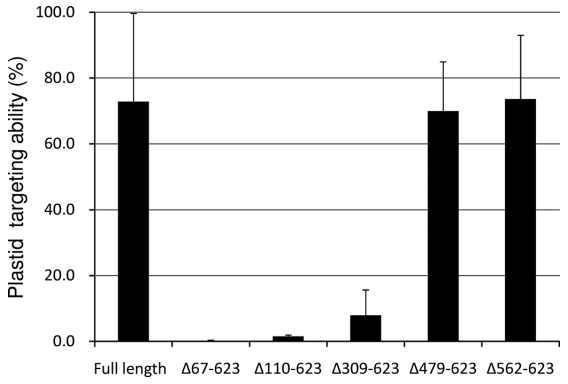

Figure 5. Statistical evaluation of the plastidial localization of various C-terminal truncated NPP1-GFPs in transgenic rice cells. Experimental details are described in Figure 4. Ratios of the fluorescence intensity of GFP in the plastidial area to GFP in the whole cell ($GFP_{Plastid}/GFP_{Total}$) (%) were determined. Values show means±s.d. ($n=3$).

complex to the plastids of rice cells (Asatsuma et al. 2005; Kitajima et al. 2009). The plastid-targeting signal of AmyI-1 was determined to be in the peptide region from 301 to 369 of the protein, consisting of 428 amino acid residues (Kitajima et al. 2009). Putative surface starch-binding sites and substrate binding subsites were located in this region (Søgaard et al. 1993). Thus, both plastid-targeting signals of NPP1 and AmyI-1 appear to be three-dimensional physical structures embedded in the mature proteins of the enzymes.

Amino acid sequence alignment between the peptides from 308 to 478 of NPP1 and the corresponding region of NPP3 revealed that identical amino acid residues occupy approximately 70% of the peptide; however, the negatively-charged residue Asp^{336} in NPP1 is substituted for the positively-charged residue Lys^{319} in NPP3. We infer that this marked difference may influence the physical structure signal for the plastid-targeting of NPP. In AmyI-1, the results obtained by site-directed mutations of Trp^{302} and Gly^{354}, located on surfaces on opposite sides of the protein, suggest that multiple surface regions are necessary for the plastid targeting of protein (Kitajima et al. 2009). It is possible that several amino acid residues in the peptide region from 308 to 478 in NPP1 also affect the delivery of NPP1 to the plastids.

Conclusion

We ascertained the presence in rice cells of intra- and extra-plastidial NPP isozymes, NPP1 and NPP3, that exhibit distinguishable substrate specificities. Although both NPP isozymes possess the N-terminal cleavable signal sequences in their predicted precursor forms, NPP1 was further converted from the proprotein to the mature protein with additional proteolytic processing in the microsomes. Similar two-step proteolytic processing has been reported in the mechanism of thylakoid luminal protein import into the chloroplast. We therefore examined whether the N-terminal extra-peptide of NPP1 can serve as a plastid-targeting signal. The localization of the N-terminal amino acid residues from 1-66 and 1-109 of NPP1 fused with GFP, NPP1(1–66)-GFP and NPP1(1–109)-GFP proved to occur in extra-plastidial compartments: the cell surface, ER network and membrane vesicles. Thus, the signal peptide of NPP1 was necessary to give it entry to the secretory pathway, but presumably the proteolytic processed extra-peptide of the proprotein is not involved in the plastid-targeting of NPP1. To determine the plastid-targeting signal, we analyzed the plastid localization of a series of carboxy-terminal truncated NPP1s fused with GFP in rice cells. Our results indicate that the peptide region from 308 to 478 amino acid residues plays a key role in delivering NPP1 protein into plastids in rice cells. The three-dimensional structure of the plastid-targeting signal of plastidial glycoprotein is as yet unknown.

Acknowledgements

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