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NnSR1, a class III non-S-RNase specifically induced in *Nicotiana alata* under phosphate deficiency, is localized in endoplasmic reticulum compartments

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ABSTRACT

A combined strategy of phosphate (Pi) remobilization from internal and external RNA sources seems to be conserved in plants exposed to Pi starvation. Thus far, the only ribonucleases (RNases) reported to be induced in *Nicotiana alata* undergoing Pi deprivation are extracellular S-like RNase NE and NnSR1. NnSR1 is a class III non S-RNase of unknown subcellular location. Here, we examine the hypothesis that NnSR1 is an intracellular RNase derived from the self-incompatibility system with specific expression in self-incompatible *Nicotiana alata*. NnSR1 was not induced in self-compatible *Nicotiana* species exposed to Pi deprivation. NnSR1 conjugated with a fluorescent protein and transiently expressed in *Arabidopsis* protoplasts and *Nicotiana* leaves showed that the fusion protein co-localized with an endoplasmic reticulum (ER) marker. Subcellular fractionation by ultracentrifugation of roots exposed to Pi deprivation revealed that the native NnSR1 migrated in parallel with the BiP protein, a typical ER marker. To our knowledge, NnSR1 is the first class III RNase reported to be localized in ER compartments. The induction of NnSR1 was detected earlier than the extracellular RNase NE, suggesting that intracellular RNA may be the first source of Pi used by the cell under Pi stress.

1. Introduction

Ribonucleases (RNases) T2 are endonucleolytic enzymes, widely distributed in all kingdoms, which catalyze the cleavage of single-strand RNA [1,2]. In plants, members of the RNase T2 family have been classified into three classes, class I to class III, according to gene structure and phylogenetic relationships [3]. There are also important functional distinctions among these three groups. Class I RNases are generally induced in several species in a variety of stress scenarios, such as phosphate (Pi) starvation (described below), pathogen and pest attack [4,5], insect feeding [6], and mechanical damage [7–9]. Some physiological processes, such as the development of xylem cells [10] and senescence [11], also display an increase in class I RNase expression. The ubiquitous and usually constitutively expressed class II RNases are involved in the homeostasis of RNA metabolism [12–14]. They are also overexpressed in certain stress responses or during senescence, although to a lesser extent than class I RNases [15,16]. Class III RNases are primarily S-RNases, which are components of the self-incompatibility system in Solanaceae, Rosaceae, and Plantaginaceae and play a key role in the recognition and rejection of self and related pollen. S-RNases are constitutively expressed specifically in the pistil [17,18]. The class III RNase group also includes non-S-RNases, structurally similar to S-RNases but without participation in the pollen recognition process [18,19]. Presumably non-S-RNases, often called relic S-RNases, derived from S-RNases by duplication followed by translocation of the *S*-locus [20]. Non-S-RNases have been associated with defense [3,21] and Pi starvation responses [22]. Class III RNases, like class I and class II, have therefore also been recruited to cope with environmental stress scenarios.

The induction of RNases under Pi deficient conditions has been studied in several species, primarily *Arabidopsis*, *Nicotiana* and tomato. At least two out of the five RNase T2 family members of

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NnSR1      -----MFGSQLMFVLFILFLSLSPVYG--TFDQLQLVLTWPPSFCHGKPCTRIPKN------FTIHGLWPDEQHGMLN 65
RNase NE   MKMASNSATSLFLTLFLITQCLSVLTAAQDFDFFYFVQQWPGSYCDTKQSCCYPKTGKPASDFGIHGLWPNNNDGSYP 78

NnSR1      DCGETFTKLREPREKKELDDRWPDLKRSRSDAQEVQSFWEYEYNKHGTCCTELYDQAAYFDVAKNLKDKFDLLRNLKN 143
RNase NE   SNCDSNSPYDQSQVSDLISRMQQNWPTLACPSGTGSAFWSHEWEKHGTCSESIFDQHGYFKKALDLKNQINLLEILQG 156

NnSR1      EGIIP-GSTYTVDEIAEAIRAVTQAYPNLNCVGDPQKILELSEIGICFDRGATKVITCRRRTTCNPINKKEISFPLN 219
RNase NE   AGINPDGGFYSLNSIKNAIRSAIGYTPGIECNVDDSGNSQLYQVYICVDGSGSNLIECPVFPRGK--CGSSIEFPTF 232
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Fig. 1. *Protein sequence alignment of NnSR1 and RNase NE.* Predicted signal peptides of NnSR1 and RNase NE are underlined. The site of N-glycosylation of NnSR1 is indicated by an asterisk. Conserved active site (CAS) regions, typical of RNase T2 family members [1], are in boldface. Solid dots indicate active site amino acids. Amino acid patterns that distinguish class III RNases and S-like RNases [33] were shaded in NnSR1 (light grey) and RNase NE (dark gray) sequences, respectively.

Arabidopsis, RNS1 and RNS2, were induced in seedlings under conditions of Pi deprivation [13,15,23]. Similarly, tomato RNases LE and LX and *Nicotiana* RNase NE were induced in cultivated cells and roots growing in Pi-deficient media [24–26]. RNA or DNA supplied to the culture medium rescued the growth inhibition caused by the absence of Pi in *Arabidopsis* seedlings [27]. Concordantly, ribonucleosides were produced in a culture medium of tomato cells supplied with RNA [28]. Thus, the induction of RNases under Pi deprivation contributed to Pi mobilization from nucleic acid sources. This induction appears to be crucial: RNS1 was the most abundant protein accumulating in the secretome of *Arabidopsis* cells growing under Pi starvation [29].

Interestingly, RNases induced during Pi starvation have different subcellular locations. While RNS1, RNase NE and RNase LE are extracellular enzymes [4,30,31], RNS2 and RNase LX reside mainly in the endoplasmic reticulum (ER) and vacuoles [10,12,32]. These results indicate that in plant cells, Pi may be scavenged from both external and internal sources of RNA. It is unknown whether these different pools of RNA are mobilized simultaneously under any Pi stress scenario or whether the magnitude of the Pi stress regulates the selective mobilization of one or the other RNA source.

Recently, a non-S-RNase of *Nicotiana alata*, NnSR1 (*Nicotiana non S-RNaseI*), was found to be induced in roots and stems of hydroponic plants cultivated under Pi deprivation [22]. NnSR1 appeared to be induced to recycle Pi because it was expressed in a catalytically active form and its expression was repressed when exogenous Pi was added to the culture medium. NnSR1 has sequences characteristic of S-RNases [33] and, presumably, the *NnSR1* gene was originated from the S_{70}*-RNase*, a functional allele of the self-incompatibility (SI) system. Both RNases share about 80% amino acid identity and are placed together in a phylogenetic tree, with a robust bootstrap value [19]. Like S-RNases, NnSR1 expression is specific and constitutive in styles under normal conditions of growth [22]. *Non-S-RNase* genes derived from the *S*-locus have been found in several self-incompatible species [19,34–36] although no function had been described previously for these genes until recently [22]. The induction of NnSR1 and its capacity to recycle Pi in roots exposed to Pi deficit seems to be similar to that of the extracellular RNase NE [26]. However, previous evidence suggests that NnSR1 may have an intracellular localization [22]; thus, rather than being redundant, NnSR1 and RNase NE activities may be complementary, scavenging Pi from intracellular and extracellular sources of RNA, respectively. The distinctive features of NnSR1 and RNase NE amino acid sequences are shown in Fig. 1.

Here, we compared the expression of several RNases of *Nicotiana alata* under conditions of Pi deprivation. We confirmed that NnSR1 and RNase NE are the only RNases examined so far that are induced by Pi stress, and that they are differentially expressed in self-incompatible and self-compatible *Nicotiana* species. This apparent redundancy in the response to Pi-starvation, i.e. induction of different enzymes with the same activity, could be explained if these enzymes carry out their activity in different cellular locations or at different times during the stress response. Given that RNase NE is secreted to the apoplast, we tested the subcellular localization of NnSR1. Both confocal fluorescence microscopy and immunochemical approaches consistently showed that NnSR1 was predominantly in ER compartments. Thus, in *Nicotiana alata*, like in *Arabidopsis* and tomato [12,30–32], a combined strategy of Pi remobilization from internal and external RNA sources is used, even if the evolutionary origin of the enzymes involved in this process differs between species. Results also show that the NnSR1 protein accumulation is detected earlier than RNase NE, suggesting that intracellular sources of RNA are mobilized before extracellular sources under Pi starvation. The adaptation of class III NnSR1 to a new function and subcellular localization is discussed.

2. Materials and methods

2.1. Plant material and growth conditions

Nicotiana alata, *Nicotiana longiflora* [19], *Nicotiana tabacum* and *Nicotiana benthamiana* were germinated and hydroponically cultivated in conditions as previously described [22]. *Nicotiana benthamiana* used for transient expression experiments was soil-grown in a greenhouse at 28 °C and long day photoperiod, without humidity control.

Arabidopsis thaliana ecotype Columbia-0 plants were grown in chambers at 21 °C with 16 h light and 60% humidity. Mesophyll protoplasts were prepared from rosette leaves according to [37].

2.2. RNA extraction and RT-PCR conditions

Total RNA from roots and leaves was prepared using TRI Reagent Solution (Ambion) following the manufacturer's instructions. RNA was then treated with TurboDNase (Life Technologies) to remove genomic DNA contamination. For RT-PCR amplification, 1 mg of total RNA and 5.5 mM oligo (dT)15 were incubated for 5 min at 70 °C, chilled in ice water for 5 min, and mixed with 6 mM $MgCl_2$, 0.5 mM dNTPs, 1 μL of ImProm-II Reverse Transcriptase and ImProm-II buffer (Promega) in a total volume of 20 μL. Single-stranded cDNA synthesis was performed for 1 h at 42 °C, according to the manufacturer's instructions. A 2-μL aliquot of cDNA synthesis reaction was used as template for the amplification step, performed in a 20-μL reaction containing 0.4 units of GoTaq Flexi DNA polymerase (Promega), Green GoTaq Flexi buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 0.4 μM of each primer, and 12.5–33 pg DNA. The reaction was incubated at 95 °C for 5 min and then at 32 cycles of 1 min at 94 °C, 45 s at the annealing temperature indicated in Supplementary Table 1 and 1 min at 72 °C, followed by a final extension

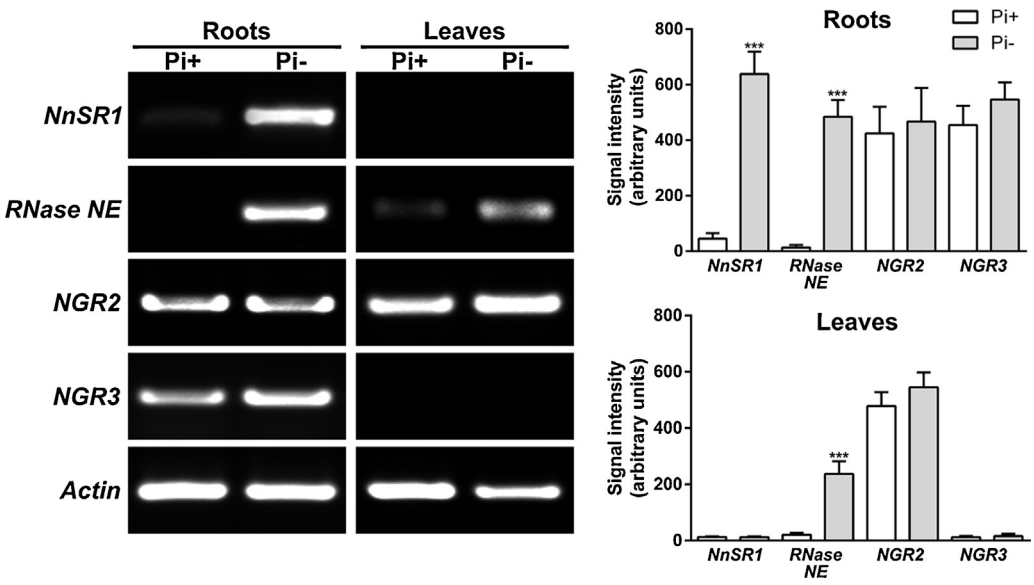

Fig. 2. *Transcript differential expression of four RNases of Nicotiana alata under Pi sufficient and Pi deprived conditions.* Specific primers for RNases *NnSR1, RNase NE, NGR2*, and *NGR3* were used for RT-PCR amplification of roots and leaves exposed to 14 days to Pi deprivation. A representative gel and the statistical analysis are shown. Signal intensity values represent the mean ± S.E.M. of three independent experiments. Data were statistically analyzed using a *t*-test. $^{***}P < 0.001$. Pi+, Pi-sufficient medium; Pi−, Pi-deficient medium.

step of 5 min at 72 °C. Details of primer sequences, and PCR product sizes are indicated in Supplementary Table 1. Amplified fragments were analyzed on 1% agarose gels and assessed by densitometry with Gel-ProTM Analyzer 3.0 software. Transcript abundances are indicated relative to an actin transcript.

2.3. Plasmid construction and transformation procedures

The *NnSR1* coding sequence was amplified from pistil cDNA avoiding the stop codon and introducing the Xba1 and BamH1 restriction sites. Cerulean (Cer) fluorescent protein and *nos* 3′ sequences were extracted from pAN578 [38] by digestion with BamH1 and Kpn1 enzymes. After assembly, the NnSR1-Cer fusion protein was cloned into Xba1 and Kpn1 sites of pCAMBIA1300MCS1, a binary expression vector derived from pCAMBIA1300 [39] (Supplementary Fig. A1). ER-YFP and Golgi-YFP markers [40] were obtained from the Arabidopsis Biological Resource Center (Ohio State University).

Nicotiana benthamiana leaves were agroinfiltrated following the procedure described by Ma et al. [41]. Infiltrated plants were incubated under the growth conditions described above for 2–3 days before visualization by confocal microscopy.

Transient transformation of *Arabidopsis* rosette leaf protoplasts was performed according to [37]. Ten to 20 μg of ER-YFP, Golgi-YFP, or NnSR1-Cer plasmid DNA was used for each transformation. Transformed protoplasts were incubated 2–3 days at 22 °C in darkness on an orbital shaker at 50 rpm and visualized using confocal microscopy.

2.4. Fluorescence microscopy

Confocal microscopy was performed using a Leica SP5 X MP confocal multiphoton microscope at the Iowa State University Confocal and Multiphoton Facility. *Nicotiana* leaves were imaged using an HCX PL APO CS 40.0 × 1.25 oil objective and *Arabidopsis* protoplasts were imaged using an HPX PL APO CS 63.0 × 1.40 oil objective. Confocal microscopy used an excitation/emission of 405 nm/458–490 nm for Cer and 514 nm/525–600 nm for YFP. For co-localization analysis, Fiji software was used to quantify pixel intensity and Pearson's coefficient [42].

2.5. Tissue extraction and subcellular fractionation

For each culture period, roots from three representative plants cultivated with or without Pi were mixed and extracted as described in Rojas et al. [22]. Homogenates were centrifuged at 16,000 × *g* for 15 min, the pellet was discarded and the supernatant was subjected to additional centrifugation at 110,000 × *g* for 50 min. The supernatant was saved and microsomes were washed and resuspended in centrifugation buffer (10 mM Tris-HCl, pH 7.5; 2 mM EDTA; and 1 mM DTT). Microsomes were then subjected to a discontinuous sucrose gradient prepared by layering 0.5 ml each of 45%, 40%, 35%, 28%, 20% and 10% sucrose (w/w) in centrifugation buffer. For samples in the presence of Mg^{2+}, 7 mM $MgCl_2$ was added to the centrifugation buffer. The gradient was centrifuged at 110,000 × *g* for 12 h at 4 °C and fractions of 0.25 ml were collected and assayed by SDS-PAGE and western blot as detailed below. Protein content was estimated with Bradford reagent (Bio-Rad, Richmond, CA, USA).

2.6. In gel ribonuclease activity, deglycosylation, and Western blot assays

Electrophoresis for in-gel RNase activity staining was performed according to Yen and Green [43] with modifications described by Rojas et al. [22]. Band signals were visualized with a UVP EC3 bioimaging system (UVP Inc., Upland, CA, USA) and assessed by densitometry with Gel-ProTM Analyzer 3.0 software.

Western blot analysis was performed following the procedure previously described [22]. The antibody recognizing NnSR1 (1:2000) was described earlier [22]. RNase NE antibody (1:800) was kindly gifted by Dr. Eric Galiana (Sophia Agrobiotech Institute, University of Nice Sophia Antipolis, CNRS, France). BiP antibody (ac-19; 1:500) was from Santa Cruz Biotechnology (Dallas TX, USA). Band signals were visualized with an Odyssey infrared imaging

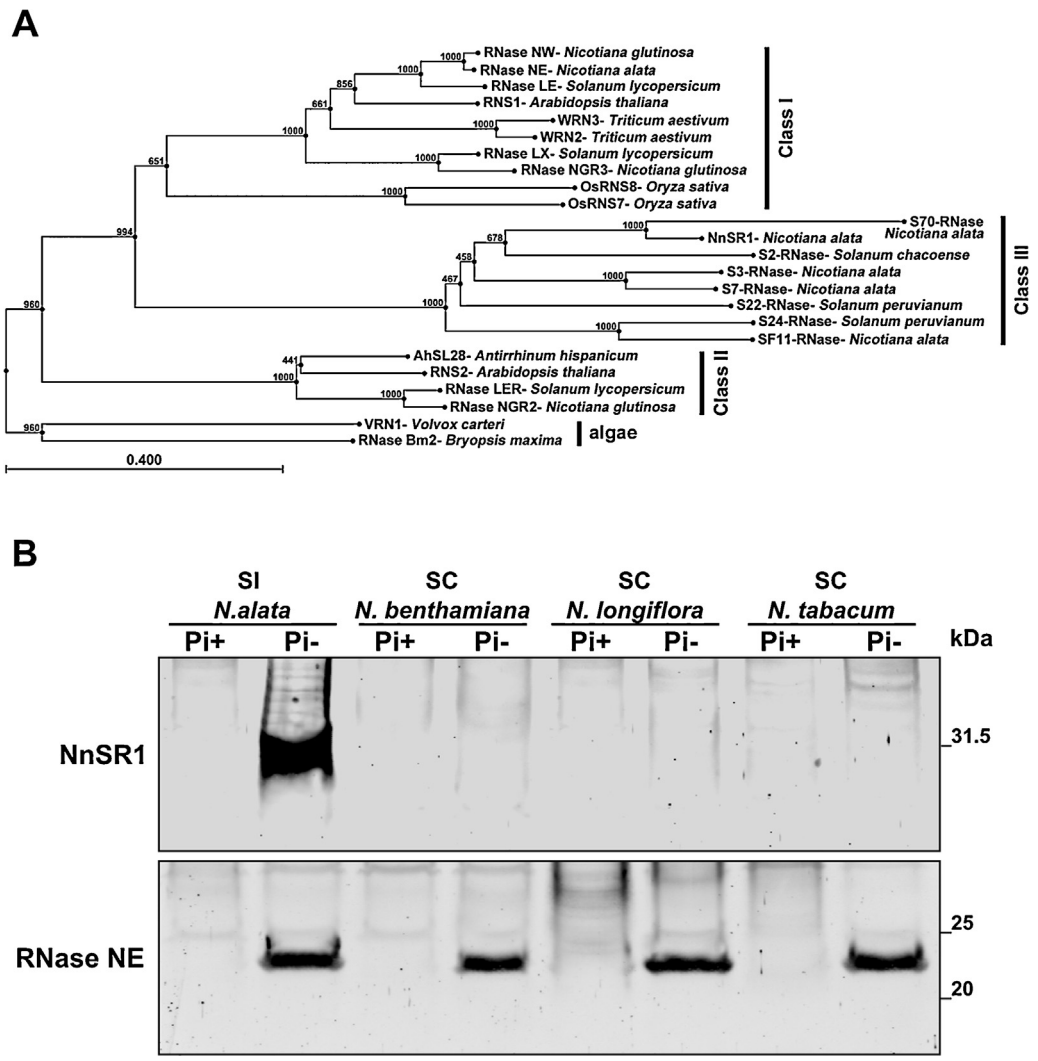

Fig. 3. *Phylogenetic relationships and differential expression of RNase NE and NnSR1 in Nicotiana.* (A) A phylogenetic tree was estimated by the Neighbor-Joining method using only conserved regions. The tree was rooted using algal sequences. Bootstrap values are indicated for each branch. Scale bar: amino acid substitutions per site. Accession number**** for sequences used, from top to bottom, are BAC77613, AAA21135, NP_001234195, P42813, AY528721, AY517470, NP_001234551, BAA84469, LOC_Os07g43640 and LOC_Os07g43600 from Rice annotation project, ADG57008, BAA24017 (NnSR1), CAA40216, AAB07492, Q40381, BAC00930, BAC00932, Q7SID5, CAC50874, NP_030524, CAL64053, BAA84468, BAA95359 and BAE06157****. (B) NnSR1 and RNase NE expression was assayed by Western blot in root extracts of four *Nicotiana* species subjected to Pi deprivation for 14 days. Thirty μg of protein were loaded in each lane. SI, self-incomptible; SC, self-compatible. Pi+, Pi-sufficient medium; Pi-, Pi-deficient medium.

system (LICOR Biosciences) and then assessed by densitometry with Gel-ProTM Analyzer 3.0 software.

Deglycosylation was performed as described by MacIntosh et al. [44].

2.7. Phylogenetic analysis

Protein sequences were obtained from GenBank. Sequence alignment was carried out using ClustalW2 [45], followed by manual adjustment. A phylogenetic analysis with the Neighbor Joining (NJ) method was performed using the CLC bio software package, with 1000 bootstrap replications.

2.8. Statistical analysis

The data shown are the average ± standard error means (SEM) of at least three independent experiments. Statistical analyses were carried out through Student's *t*-test using the GraphPad Prism V5.0 statistical software.

3. Results

3.1. Differential expression of RNase T2 genes from Nicotiana alata under Pi deprivation

Remarkable differences were observed in four RNase T2 genes of *Nicotiana alata* when their expression patterns were compared in roots and leaves grown with and without Pi (Fig. 2). *NnSR1* and *RNase NE* [22,26] were clearly induced in conditions of Pi deprivation, while *NGR2* and *NGR3* [46], were expressed at similar levels both in Pi-sufficient and Pi-deficient conditions. Two other RNase T2 genes, S_{c10}*-RNase* and *NnSR2* [19,22] were not expressed either with or without Pi (results not shown). Thus, NnSR1 and RNase NE appear to be the main members of RNase T2 family involved in Pi recycling during Pi starvation. Regardless of this common function, we focused on the distinctive features of NnSR1 and RNase NE to gain more insight into how the Pi-rescue system is regulated in *Nicotiana alata*. Phylogenetic analysis showed that RNase NE and NnSR1 grouped in different clades (Fig. 3A); RNase NE is a class I S-like RNase expressed in

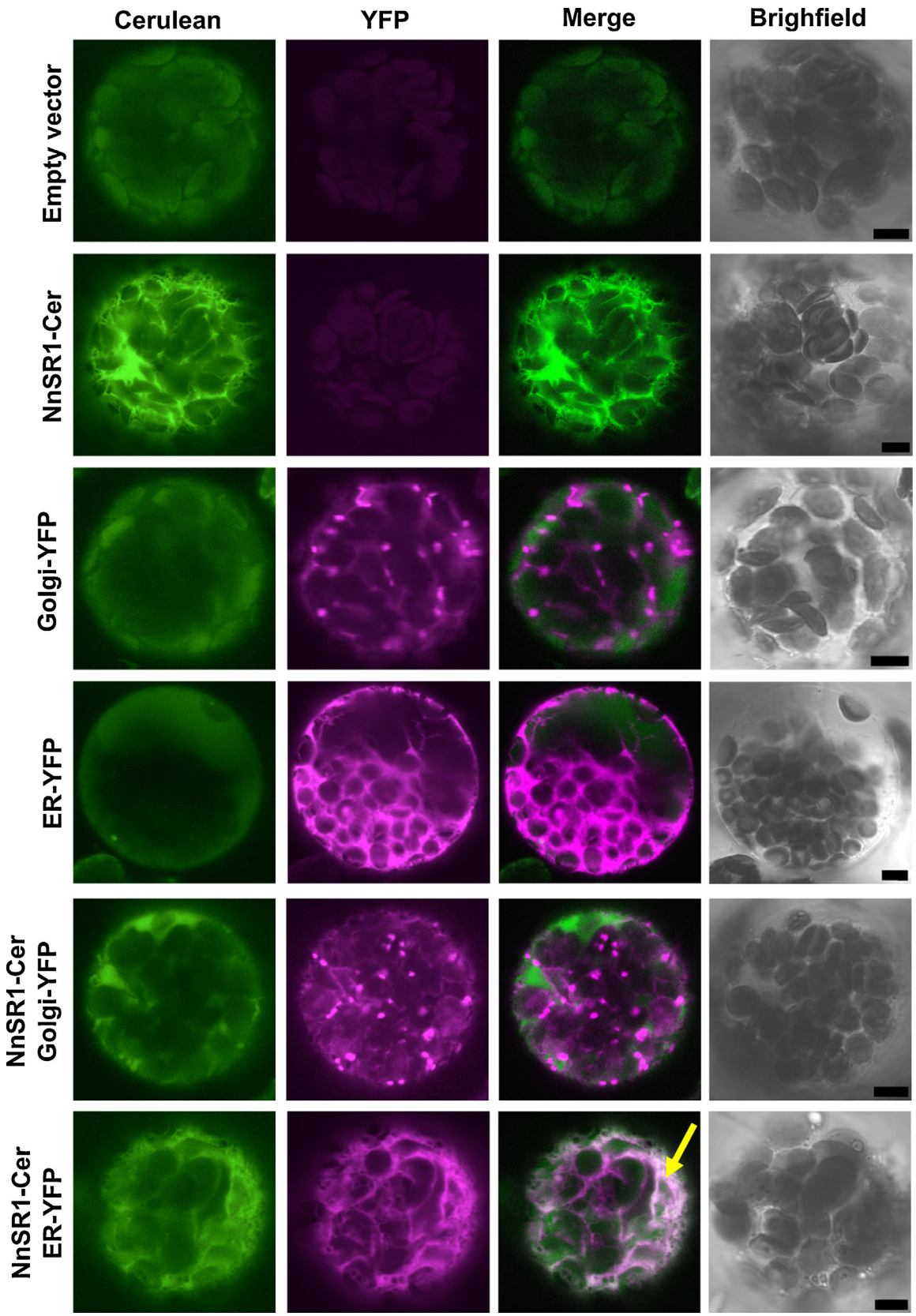

Fig. 4. *Localization of NnSR1-Cer in endoplasmic reticulum of Arabidopsis leaf protoplasts*. Protoplasts prepared from *Arabidopsis* rosette leaves were transiently transformed with the constructs indicated on the left. Cer and YFP fluorescence were visualized by confocal microscopy. Transformation with the empty vector pCAMBIA1300MCS1 was used as a fluorescence control. Arrow points to the colocalization of NnSR1-Cer and YFP-ER marker. Scalebar = 5 μm.

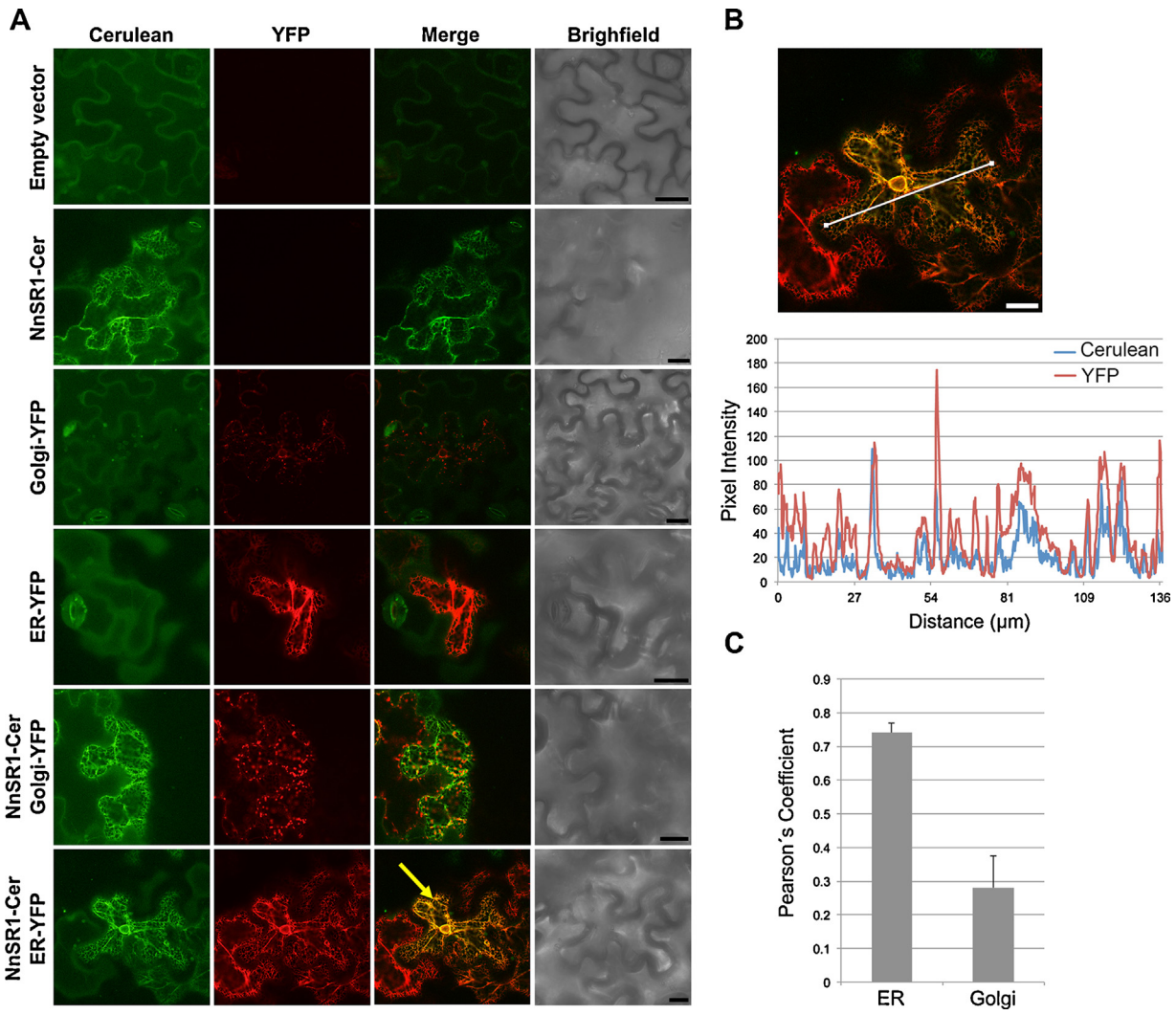

Fig. 5. *Localization of NnSR1-Cer in endoplasmic reticulum of Nicotiana benthamiana leaves.* (A) Leaves were transiently transformed by agroinfiltration with the constructs indicated on the left. Cer and YFP fluorescence were visualized by confocal microscopy. Transformation with the empty vector pCAMBIA1300MCS1 was used as a fluorescence control. Arrow indicates colocalization of NnSR1-Cer and the ER-YFP marker. Scale bar = 25 μm. (B) Colocalization analysis using Fiji software to quantify pixel intensity in each fluorescence channel following the path indicated in the figure. Scale bar = 25 μm. (C) Pearson's colocalization coefficient analysis for ER-YFP with NnSR1-Cer and YFP-Golgi-YFP with NnSR1-Cer using Fiji software. Pearson's coefficient was derived from 11 to 12 images of three independent experiments. Error bars represent standard deviation.

several self-compatible species and in self-incompatible *Nicotiana alata* (Fig. 3B). In contrast, NnSR1 was presumably derived from the self-incompatibility system by duplication from the functional S_{70}-*RNase* allele [19], and it was expressed in self-incompatible *Nicotiana alata* but it is absent or not expressed in self-compatible species.

3.2. Subcellular localization of NnSR1

Previous results showed that when *Nicotiana alata* is hydroponically grown in Pi-limiting conditions, NnSR1 is not secreted to the culture medium and is present in a microsomal fraction, suggesting that it may be an intracellular RNase [22]. To determine the subcellular localization of NnSR1, we first expressed a recombinant NnSR1 protein in *Arabidopsis* leaf protoplasts, fused at its C terminus to the Cer fluorescent protein (Supplementary Fig. A1) [38]. Confocal microscopy imaging of NnSR1-Cer fusion protein revealed a reticular labeling pattern, similar to that exhibited by the ER marker HDEL-YFP (Fig. 4). This reticular pattern was clearly different from the typical punctate pattern displayed by the Golgi-YFP marker. Coexpression of NnSR1-Cer and ER-YFP marker showed an almost complete overlap between the two signals indicating that NnSR1-Cer resides in ER compartments. No colocalization was seen when NnSR1-Cer was coexpressed with the Golgi-YFP marker, revealing that NnSR1-Cer was not localized in Golgi compartments of *Arabidopsis* protoplasts.

NnSR1-Cer fusion protein was then transiently expressed in leaves of *Nicotiana benthamiana*, a close relative of *Nicotiana alata*. Confocal laser microscopy once again showed the fusion protein in a dense fluorescent network in epidermal cells of infiltrated leaves (Fig. 5A and B). Coexpression experiments revealed a significant overlap between NnSR1-Cer and the ER-YFP marker fluorescence. Similar results were obtained by examining the fluorescence at 36 and 60 h after leaf infiltration, suggesting that ER localization of NnSR1-Cer was not due to transient protein accumulation within the secretory pathway. Some overlap between NnSR1-Cer and the Golgi-YFP signals was also detected; however, this level of colocalization was almost three fold lower than that observed between NnSR1-Cer and the ER marker, as determined by comparison of Pearson's coefficient values (Fig. 5C). Thus, the subcellular localization of NnSR1-Cer, visualized by fluorescence microscopy both in protoplast- and *in planta*-expression systems,

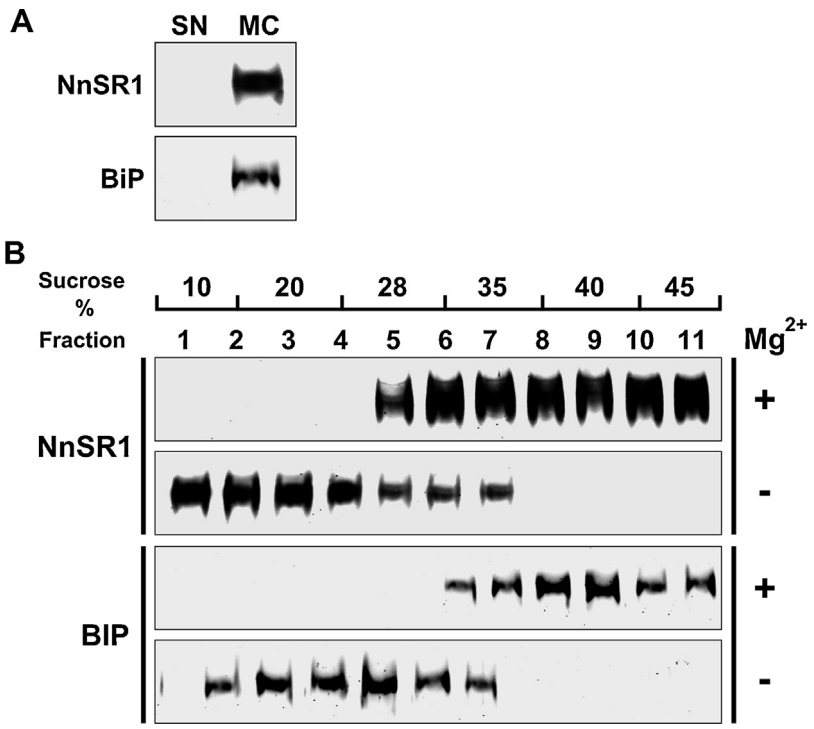

Fig. 6. *NnSR1 colocalizes with ER structures in roots exposed to Pi deprivation.* Subcellular fractionation of roots grown for 14 days without Pi. (A) Root extracts were centrifuged at 110.000 × *g* for 50 min. NnSR1 and the ER marker BiP were immunodetected by western blot in supernatant (SN) and microsomal (MC) fractions. Thirty μg of protein were loaded in each lane. (B) Microsomal fraction was subjected to high speed centrifugation in a discontinuous sucrose gradient, in the presence (+) or absence (−) of Mg^{2+}. Twenty five μL of each fraction were assayed by western blot using antibodies against NnSR1 and the ER marker BiP.

showed that this fusion protein was primarily associated with ER structures.

To confirm the subcellular localization of endogenous NnSR1, a biochemical approach was carried out using roots grown under Pi deprivation. A microsomal fraction isolated from these roots was subjected to ultracentrifugation in a sucrose density gradient with and without the addition of Mg^{2+} (Fig. 6). The density of the ER is increased in the presence of Mg^{2+} due to its stabilization of ribosome binding to ER membranes [47]. Under these conditions, NnSR1 and the ER marker BiP (Binding Protein) were detected in fractions migrating to 30–40% (w/w) sucrose. The absence of Mg^{2+} induced a shift in both NnSR1 and BiP mobilities, which migrated to lower density fractions, between 10 and 20% (w/w) sucrose. Taken together, subcellular localization by fluorescence microscopy and immunoblot of subcellular fractions obtained by high speed centrifugation indicated that NnSR1 was predominantly localized in the ER.

3.3. NnSR1 is produced in a glycosylated form

The predicted molecular weights of precursor and mature NnSR1 proteins are around 25 and 23 kDa, respectively (GenBank accession no. D63887.1****). However, using western blot analysis we estimated a molecular weight of 31 kDa for NnSR1 [22]. This difference may be caused by N-glycosylation at the Asn 49 site of the NnSR1 precursor. This site, included in the conserved active site I (CAS I), is commonly glycosylated in T2 class III RNases [2]. Thus, we tested the hypothesis that NnSR1 is glycosylated by incubating root extracts from plants exposed to Pi deprivation with N-glycosidase F (Fig. 7). While the signal of NnSR1 became fainter, at least two to three activity bands of lower molecular weight, corresponding to different glycosylation states, were evident after deglycosylation treatment, indicating that NnSR1 is produced in a glycosylated form.

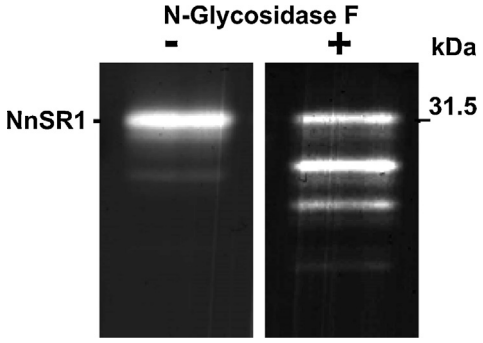

Fig. 7. *Deglycosylation assay of NnSR1.* Root extracts from plants exposed to 14 days of Pi deprivation were incubated at 37 °C for 5 min with (+) or without (−) the addition of N-glycosidase F. After the incubation, the extracts were analyzed by in gel-RNase activity assay.

3.4. Time course of NnSR1 and RNase NE expression from roots under Pi deprivation

Given that both RNase NE and NnSR1 were induced in root by Pi deprivation, and that they displayed a different subcellular localization, we were interested in comparing the temporal pattern of accumulation for each of these RNases. A time course of transcript relative abundance, assayed by semi-quantitative RT-PCR, showed that *NnSR1* and *RNase NE* were induced in parallel during Pi starvation (Fig. 8A). The signal increase was gradual between 3 and 5 days of culture. However, a more pronounced increase of transcript expression was observed earlier for *NnSR1* (day 7) than for *RNase NE* (day 9). The detection of both RNases by protein gel blot analysis showed that NnSR1 gradually increased from days 3–6 onwards while RNase NE was observed only from days 9 to 12 onwards (Fig. 8B). The induction pattern of RNase activity also showed earlier detection of NnSR1, consistent with the western blot assay (Fig. 8C).

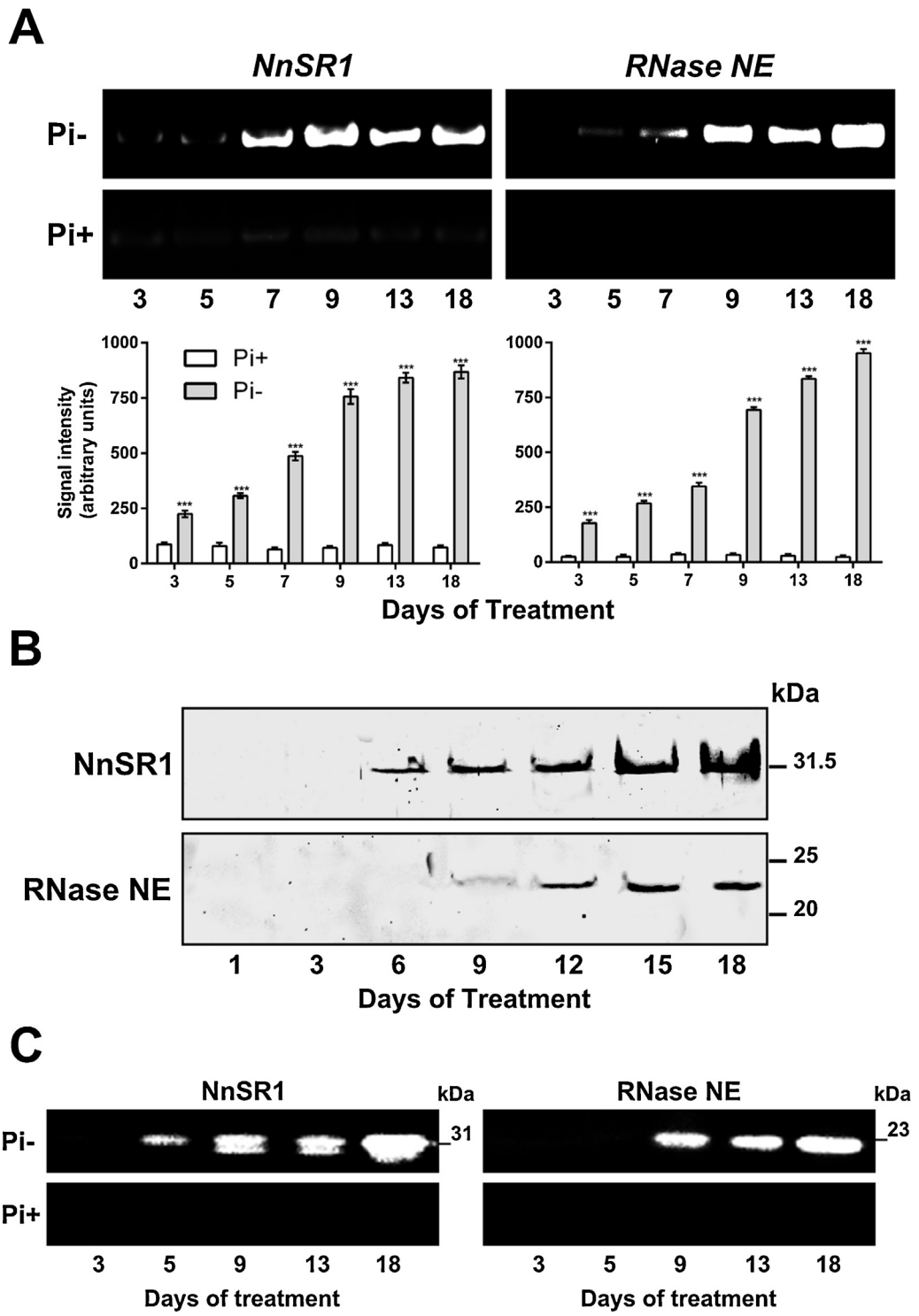

Fig. 8. *Temporal expression of RNase NE and NnSR1 in hydroponically grown roots under Pi sufficient and Pi deprived conditions.* (A) Specific primers were used for RT-PCR amplification of *RNase NE* and *NnSR1* transcripts in Pi sufficient (Pi+) and Pi deficient (Pi−) conditions. Representative gels and the statistical analysis are shown. Signal intensity values represent the mean s.e.m. of three independent experiments. Data were statistically analyzed using a *t*-test. $^{***}P < 0.001$. (B) RNase NE and NnSR1 protein accumulation was assayed by western blot on roots grown in Pi deficient medium. (C) In gel-RNase activity of NnSR1 and RNase NE assayed on roots cultivated for different days in Pi deprived (Pi−) and Pi-sufficient (Pi+) conditions. The apparent molecular weight of each RNase is indicated. Thirty μg of protein were loaded in each lane of B and C.

These results suggest that accumulation of the intracellular NnSR1 protein could occur before that of the extracellular RNase NE protein. As expected, the relative *NGR2* and *NGR3* transcript abundance was constant throughout the culture period, and no difference was observed with or without the addition of Pi to the culture medium (Supplementary Fig. A2).

4. Discussion

In this work we studied the expression pattern of several RNase T2 genes of *Nicotiana alata* under Pi deficiency, focusing on *NnSR1* and *RNase NE*. We confirmed that, thus far, *NnSR1* and *RNase NE* are the only RNase T2 genes induced by Pi starvation in this species.

Using confocal microscopy and biochemical approaches, we studied the subcellular localization of NnSR1, demonstrating that it is predominantly an ER-resident RNase. The detection of NnSR1 protein accumulation slightly preceded that of the extracellular RNase NE, which suggests that intracellular RNA may be the first source of Pi used by the cell under Pi stress.

The duplication of S-RNase genes, followed by translocation from the *S*-locus and loss of functionality in the pollen recognition, is a common event in species with S-RNase based self-incompatibility. The products of these duplication events were called relic- or non-S-RNases [18–20,34–36]. Based on the 81% identity shared by the two RNases and the phylogenetic analysis (Fig. 3A), we propose that *NnSR1* was derived from duplication of S_{70}*-RNase*, a functional allele in the self-incompatibility system [19]. The induction of NnSR1 in ER compartments in response to Pi starvation also implies that duplication was followed by the acquisition of a new subcellular localization and function. To our knowledge, NnSR1 is the first class III RNase reported to be localized in the ER. This result implies that ER compartment is important in the Pi-scavenging mechanism, a function so far thought to occur mainly in the vacuole [12]. The other members of class III group, the S-RNases involved in the self-incompatibility reaction, enter the secretory pathway and are secreted to the extracellular matrix of the transmitting tissue, where they are subsequently internalized by pollen tubes [48,49]. On the other hand, the fact that NnSR1 is only present in *Nicotiana alata* but not in its self-compatible relatives suggests that the acquisition of the new function by NnSR1 may have occurred after speciation of the *Nicotiana* genus (Fig. 3B).

In a previous report, NnSR1 was detected in a root microsomal fraction but not in the extracellular medium of a hydroponic culture, suggesting an intracellular location for this RNase [22]. Further characterization by fluorescence microscopy clearly demonstrated that the fusion protein NnSR1-Cer colocalized with ER compartments. Similar results were obtained by the coexpression of NnSR1-Cer and the ER marker YFP-HDEL either in *Arabidopsis* leaf protoplasts or *N. benthamiana* leaf epidermal cells (Figs. 4 and 5). Moreover, after subcellular fractionation of Pi-deprived roots, native NnSR1 was immunodetected in the same fractions as the ER marker BiP (Fig. 6). This is consistent with results obtained by fluorescence microscopy and argues against an artifact caused by the addition of the Cer tag to NnSR1. Overall, these results provide convincing evidence that NnSR1 is an ER-resident class III RNase. The exact localization of NnSR1 in the ER cannot be inferred by our experiments. However, given that S-RNases are cytotoxic when they are localized in the cytoplasm [49], this enzyme should be located inside the ER (either associated to the membrane or in the ER lumen) and not exposed to the cytoplasmic side of the membrane.

Several class I and II S-like RNases are ER resident proteins, although the ER retention signal is not clearly identified in all of them. In some RNases, this signal is related to a C-terminal extension of their sequences. For instance, tomato RNase LX [10] and *Nicotiana* NGR3 [46] are retained in ER compartments by virtue of the tetrapeptide HDEF, included in such a C-terminal extension. The amino acid sequence of *Arabidopsis* RNS2 also possesses this extension, although the ER retention signal is not as conserved [12,15], while tomato RNase LER lacks any known ER retention signal [14]. NnSR1 has an N-terminal signal peptide that directs the protein to the secretory pathway, but does not contain a recognizable ER-localization signal.

It has been demonstrated recently that Golgi mediated glycosylation determines the subcellular fate of Rny1, the RNase T2 in *Saccharomyces cerevisiae* [50]. A similar mechanism may operate in NnSR1 which has an N-glycosylation site (Asn 49) that seems to be glycosylated, since its migration pattern was changed upon incubation with N-glycosidase F (Fig. 7). Further studies involving the use of point mutation experiments may allow the direct testing of the role of the Asn49 linked glycan in NnSR1 localization.

Active NnSR1 accumulated in response to Pi deficiency, and its expression was suppressed in the presence of Pi [22]. Considering that the induction of RNases under Pi starvation has been related to nucleic acid degradation [27,28], we propose that NnSR1 may be degrading RNA during Pi stress for Pi recycling in the ER. This recycling mechanism could be similar to the one proposed for *Arabidopsis* RNS2, a class II RNase found in ER and vacuoles that is constitutively expressed and further induced by Pi starvation [12,31]. Hillwig et al. [12] suggested that a ribophagy-like process could occur in plant cells to scavenge Pi from rRNA. Through this process, ribosomes are targeted for degradation, as was observed in yeast undergoing nitrogen starvation [51]. Regardless of this evidence, the existence of a ribophagy-like process in plants is not yet conclusive [52], and the potential involvement of NnSR1 in RNA degradation requires further study.

Why was NnSR1 maintained in *Nicotiana alata*, despite the apparent redundancy with other RNases with similar functions? Plants appear to have a dual strategy under Pi starvation, scavenging Pi from both intracellular and extracellular sources of RNA [2]. NnSR1 may have acquired a novel function after duplication from an ancestral gene involved in the self-incompatibility system, and was maintained because it provided *Nicotiana alata* with greater plasticity in resilience to Pi stress, through mobilization of intracellular RNA. This function may be critical because, considering the time course expression of NnSR1 and RNase NE (Fig. 8), intracellular RNA appears to be the first source of Pi used by the cell under Pi stress. Similar results were obtained in tomato when comparing the expression profile of extracellular RNase LE and intracellular RNase LX under Pi starvation [25,53].

In summary, we report here that NnSR1, a class III RNase derived from the self-incompatibility system, is an ER-resident RNase induced by Pi starvation in *Nicotiana alata*. It is very likely that NnSR1 recycles Pi from endogenous rRNA, the main reserve source of cellular Pi. The localization and function of NnSR1 resemble those of S-like RNases, suggesting that after duplication of the S-locus, NnSR1 regained ancestral functions of class I or class II RNases, to adapt to environmental challenges [54,55]. Further research is required to determine whether this RNase is specifically expressed under Pi deficit or, like class I S-like RNases, is also induced under other environmental stresses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2015.04.012

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