DOI: 10.1007/s10535-018-0783-6 BIOLOGIA PLANTARUM 62 (2): 261-268, 2018

# *NaPi/$S_X$-RNase* segregates as a functional *S-RNase* and is induced under phosphate deficiency in *Nicotiana alata*

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## Abstract

In plants, class III T2 RNases involves two groups of structurally similar proteins, but with different biological functions: S-RNases and non-S-RNases. S-RNases have been involved in self-incompatibility whereas non-S-RNases have been implicated in stress responses. Here, we report a novel class III RNase termed NaPi/$S_x$-RNase, which works both in self-incompatibility and in response to phosphate deficiency. The *NaPi/$S_x$-RNase* gene was identified in roots of *Nicotiana alata* grown in the absence of inorganic phosphate. Phylogenetic analysis showed that NaPi/$S_x$-RNase was included within the class III RNase T2 group. The *NaPi/$S_x$-RNase* was expressed in styles and its temporal expression increased in parallel to stylar development, with a slight decrease after anthesis. Progeny analysis showed that *NaPi/$S_x$-RNase* and *$S_{107}$-RNase*, a functional allele of the self-incompatibility system, segregated in a 1:1 ratio. The progeny segregation of a semicompatible cross, in which *NaPi/$S_x$-RNase* was shared by the two parents, exhibited a pattern consistent with a functional *S-RNase* allele. Considering genetic segregation, primary structure, and physiological role, the NaPi/$S_x$-RNase may be either an S-RNase with diversified functions or a non-S-RNase linked to the *S*-locus. To our knowledge, this is the first evidence for a specific function of the *S*-locus other than the self-incompatibility reaction. These results support the hypothesis that the self-incompatibility and stress responses may have evolved from a common origin.

*Additional key words:* ribonuclease, self-incompatibility, S-locus, stress responses.

## Introduction

During the course of evolution, angiosperms have developed different strategies that favored cross fertilization over self-fertilization. In this manner, the genetic diversity has been preserved, avoiding the deleterious consequences of inbreeding. At the molecular level, the most well known strategies is the self-incompatibility (SI), a prezygotic reproductive barrier based on the pistil recognition and rejection of self- and closely related pollen (Rea and Nasrallah 2008, Bedinger *et al.* 2017). The genetic basis of this recognition is determined by the highly polymorphic *S*-locus, which carries the genes codifying for female and male specificity factors (Iwano and Takayama 2012, Fujii *et al.* 2016). These genes are inherited as one single unit and the different variants of the *S*-locus are known as haplotypes (Kao and Tsukamoto 2004, Takayama and Isogai 2005). A plant cross is compatible when the two haplotypes of the diploid pistil are different from the pollen haplotype. Conversely, when the pollen haplotype and any of the pistil haplotypes are the same, the cross is called incompatible.

In *Solanaceae*, the SI system specificity of the female side is given by the *S-RNase* gene (Lee *et al.* 1994, Murffet *et al.* 1994), which encodes a ribonuclease (S-RNase) included in the class III of T2 RNase superfamily (Luhtala and Parker 2010, MacIntosh 2011). On the male side, the SI specificity is defined collaboratively by a collection of genes termed *SLF*

*Submitted* 6 April 2017, *last revision* 22 August 2017, *accepted* 24 August 2017.
*Abbreviations*: Na - *Nicotiana alata*; Pi - inorganic phosphate; RNase - ribonuclease; RT-PCR - reverse transcription polymerase chain reaction; SI - self-incompatibility.
*Acknowledgements*: We thank Gabriela Diaz Cortez for editorial and language assistance. This work was supported by the Agencia Nacional de Promoción Científica y Tecnológica (PICT 12/1322), and the Secretaría de Ciencia y Técnica (SECyT) of Universidad Nacional de Córdoba (366/16). [a] -These authors contributed equally to this work.
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(S-locus of F-box) (Sijacic *et al.* 2004, Kubo *et al.* 2010). The SLF proteins contain an F-box domain and they are a part of the SCF (Skp1-Cullin1-F-box) ubiquitination complex (Hua and Kao 2006, Li *et al.* 2014). S-RNases are secreted by the transmitting tissue and enter massively the pollen tube when it penetrates the style (Luu *et al.* 2000, Goldraij *et al.* 2006). The SI recognition event takes place in the pollen cytoplasm through the molecular interaction between S-RNase and SLF (Hua and Kao 2006, Liu *et al.* 2014). In compatible crosses, cytoplasmic non-self S-RNases are ubiquitinated by the $SCF^{SLF}$ complex and subsequently destroyed by the proteasome 26S machinery (Sun and Kao 2013, Boivin *et al.* 2014, Entani *et al.* 2014, Liu *et al.* 2014). A large pool of S-RNases stored in pollen vacuoles remains compartmentalized (Goldraij *et al.* 2006, Meng *et al.* 2011, Fujii *et al.* 2016) and pollen tubes grow towards the ovary without interference. Instead, in incompatible crosses, self S-RNases are resistant to ubiquitination and exert a cytotoxic action that degrades pollen RNA, inhibits apical growth, and promotes massive cellular disorganization of self-pollen tubes (Goldraij *et al.* 2006, Roldán *et al.* 2012, 2015). In addition to S-RNase and SLF, several additional factors, unlinked to the S-locus, appear to be indispensable for the full SI manifestation (McClure *et al.* 1999, Hancock *et al.* 2005, Jimenez Durán *et al.* 2013).

Although the recognition and rejection of self-pollen is the unique role of S-RNases reported so far, it has been suggested recently that some functional S-RNase alleles in *Petunia* may also act as antimicrobial agents to prevent contamination in floral nectar (Hillwig *et al.* 2010, 2011). This suggestion, based on the S-RNase accumulation in the nectar secretion, is consistent with the hypothesis that the SI mechanisms evolved from an ancestral floral defense system to prevent microorganisms from invading the stigma style (Hiscock *et al.* 1996, Nasrallah *et al.* 2005).

Besides S-RNases, the class III group of RNases also includes the non S-RNases, which have been effectively involved in several defense responses (Igic and Kohn 2001, Hillwig *et al.* 2010, Rojas *et al.* 2013). S-RNases and non-S-RNases are both expressed in pistils and share conserved features of primary structures (Lee *et al.* 1992, Liang *et al.* 2003, Banović *et al.* 2009, Rojas *et al.* 2013). However, unlike S-RNases, the examined non-S-RNases were not functional in the SI system and their expression was not individual-specific (Roldán *et al.* 2010). It is thought that the genes encoding non-S-RNases are derived from duplication events of S-RNases followed by translocation from the *S*-locus (Golz *et al.* 1998, Igic and Kohn 2001, Kao and Tsukamoto 2004). At least in *Nicotiana*, a change of function has been associated to this translocation event from the *S*-locus, as reported for the class III RNase *NnSR1*gene (*Nicotiana non-S-RNase*1) (Rojas *et al.* 2013). The induction of the *NnSR1*gene under phosphate (Pi) deficiency indicates that class III RNases are also recruited to cope with some stresses, in a similar way to previously characterized class I S-like-RNases (reviewed in MacIntosh 2011).

Here, we report a new class III RNase gene in *Nicotiana alata*, termed *NaPi/$S_x$-RNase* (*Nicotiana alata/phosphate inorganic/$S_x$-ribonuclase*). The aims of this work were to carry out a comparative phylogenetic analysis of the NaPi/$S_x$-RNase and to examine the expression and the functional role of the *NaPi/$S_x$-RNase* gene both in the SI system and under conditions of Pi deficiency.

## Material and methods

**Plants and growth conditions:** *Nicotiana alata* Link & Otto plants used in this study come from a natural population described earlier (Roldán *et al.* 2010). Plants were grown in a growth chamber at day/night temperatures of 28/22 °C, relative humidity of 50 %, 16-h photoperiod, and irradiance of 150 - 200 μmol $m^{-2}$ $s^{-1}$. Seeds were germinated and grown for 20 d in small pots containing sterilized 3:1 soil:sand mixture purchased from a local supplier (*Jardín de las Flores*, Córdoba, Argentina). Sets of nine 3-week-old plants were removed from soil and then hydroponically cultivated for 14 d in containers with complete Hoagland's solution containing 1.5 mM $KNO_3$, 1.25 mM $Ca(NO_3)_2$, 0.75 mM $MgSO_4$, 0.5 mM $KH_2PO_4$, 50mM KCl, 10 mM $MnSO_4$, 2 mM $ZnSO_4$, 1.5 mM $CuSO_4$, 72 mM Fe-EDTA, and 75 nM $(NH_4)_6Mo_7O_{24}$, at pH 6. Nutrient solution was changed every 10 d and deionized water was added every 2 d to replenish water loss. After 15 - 20 d of acclimatization, plants at the vegetative stage with 6 - 8 leaves were grouped in two sets and cultured in complete Hoagland solution or in Hoagland solution without Pi. After 14 d, leaves and roots were separated, frozen in liquid nitrogen, and stored at -80 ºC until used.

**Nucleic acid extraction and PCR conditions**: Genomic DNA was obtained from leaves by phenolic extraction and ethanol precipitation of DNA or using a *Wizard* genomic DNA *Miniprep* kit (*Promega*, Madison, WI, USA). Roots from three representative plants were mixed and total RNA was extracted either following Roldán *et al.* (2010) or using a *SpectrumTM* plant RNA total kit (*Sigma*, St. Louis, MO, USA). RNA was treated with DNase (*RQ1* RNase-free DNase; *Promega*) to remove contaminating genomic DNA. Reverse transcription (RT)-PCR amplification was performed according to Rojas *et al.* (2013). Specific and degenerate primers were used at 0.4 and 1.2 μM, respectively. The reaction mixture was incubated at 95 ºC for 5 min and then 32 cycles at 94 ºC for 1 min were used, followed with annealing at temperature indicated in Table 1 Suppl. for 45 s, and a final extension at 72 ºC for 5 min. Amplified fragments were analyzed on agarose gels and quantified

by densitometer with *Gel-ProTM Analyzer 3.0* software. Transcript expressions of *NaPi/$S_x$-RNase* were normalized to actin transcription. For sequencing, amplified fragments were cloned into pGEM-T easy vector (*Promega*). Thirty plasmids were analyzed by restriction enzyme digestion and at least two plasmids of each digestion pattern were selected to sequence both DNA strands with standard SP6 and T7 promoter primers (*Macrogen*, Rockville, MD, USA). Partial cDNA sequence of *NaPi/$S_x$-RNase* gene has been deposited with the GenBank data libraries under accession number KY711337.

**Crossing, segregation analysis, and statistical analysis**: Flowers were emasculated 2 d before anthesis and heavily pollinated within 24 h after anthesis. The *S*-functionality was established by crossing plants that shared *NaPi/$S_x$-RNase* gene and by subsequent genotyping of the progeny by three independent assays of allele-specific PCR (35 cycles) on DNA extracts, following the procedure detailed in Roldán *et al.* (2010). The $\chi^2$ test was used to analyze the progeny distribution in the *NaPi/$S_x$-RNase* functionality test (Roldán *et al.* 2010).

The data of *NaPi/$S_x$-RNase* induction under Pi deficiency were obtained from at least three independent experiments. The Student's t-test was carried out by *GraphPad Prism V5.0* software.

**Sequence alignment and phylogenetic analysis:** Selected RNase sequences spanning the region between the conserved domains C2-C5 (Ioerger *et al*. 1991) of *Nicotiana alata, Nicotiana glutinosa, Solanum chilense, Arabidopsis thaliana*, *and Aspergillus oryzae* were aligned using *MUSCLE* (Edgar 2004). The resulting alignment was manually curated using *Jalview* (Waterhouse *et al*. 2009). Phylogenetic analyses were performed on the amino acid level, where sites with over 50 % gaps were filtered out. *Prottest* (Abascal *et al*. 2005) was used to select the best evolutionary model by the *AICc* criterion. The best model was the *WAG* model with γ-distributed site rate variation (WAG+G). Tree construction was performed with *RAxML 8.2.10* (Stamatakis 2014) using the rapid hill-climbing algorithm with the WAG+G model using four discrete γ categories. A search for the best scoring maximum likelihood tree was performed, and percentages of 1 000 rapid bootstraps were plotted onto that tree as support values for each node. Accession numbers of all sequences used are provided in Table 2 Suppl.

## Results and discussion

Degenerate primers based on the S-RNase conserved domains C2, C4, and C5 of *Nicotiana alata* (Roldán *et al.* 2010; Table 1 Suppl.) were used to amplify class III RNase genes induced in Pi-deprived plants. This approach was previously used to identify *NnSR1,* a non-S-RNase gene constitutively expressed in pistils of *Nicotiana alata* which is induced in turn, in roots and stems under Pi deficiency (Rojas *et al.* 2013, 2015). Using both C2-C4 and C2-C5 primer combinations, RT-PCR from roots revealed a single band in each amplification only in hydroponic plants grown without Pi (Fig. 1*A*). Cloning and sequencing analysis of these bands showed that the main PCR product was the *NnSR1* transcript (Rojas *et al.* 2013) present in 90 % of the analyzed colonies. The other PCR product amplified by both C2-C4 and C2-C5 primers corresponded to a new putative RNase sequence. In a subsequent RT-PCR with primers based on this new sequence (Table 1 Suppl.), a single band of the expected size was amplified, confirming the induction of this novel RNase gene expressed in roots grown under Pi starvation (Fig. 1*B*). We have tentatively named this new class III RNase gene *NaPi/$S_x$-RNase*. Like *NnSR1*, the cDNA fragment of *NaPi/$S_x$-RNase* gene was also isolated from styles of plants grown under normal conditions (see below). The sequence of this cDNA fragment was identical to that found in roots cultivated in hydroponic solution without Pi.

The identification of NaPi/$S_x$-RNase was examined first by *BLASTP* search of the non redundant protein database at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). Using the deduced amino acid sequence of NaPi/$S_x$-RNase as a query, we found that the top seven hits with a *BLAST* score over 200 corresponded to S-RNases sequences from different species of *Solanum* with identity values ranging from 64 to 74 % (results not shown). The rest of the sequences included among the 100 top *BLAST* hits were *Solanaceae* S-RNases of *Solanum*, *Lycium*, *Iochroma*, *Petunia*, and *Nicotiana* species with identity values ranging from 46 to 73 %. The values of *Nicotiana* S-RNases ranged between 47 and 51 % identity with NaPi/$S_x$-RNase. These apparently low identity values are in fact consistent with early reports establishing that an ancient S-RNase polymorphism predated speciation events in *Solanaceae*. Consequently, interspecific identities were very frequently higher than intraspecific ones (Ioerger *et al.*1990, Richman *et al.* 2000).

To provide additional support that the NaPi/Sx-RNase is a *bonafide* class III RNase, sequences of *Nicotiana* and *Solanum* protein from the three classes of T2 RNases were selected to carry out a comparative phylogenetic analysis. We also included sequences of class I and class II RNases from *Arabidopsis*. Importantly, in this test we used a collection of *Solanum chilense* S-RNase sequences that covers a wide, if no the entire, range of ancient *S*-RNase lineages (Roldán *et al.* 2010). The resulting maximum likelihood tree displayed a robust bootstrap value for all clades, strongly supporting the inclusion of NaPi/$S_x$-RNase as a class III RNase (Fig. 2).

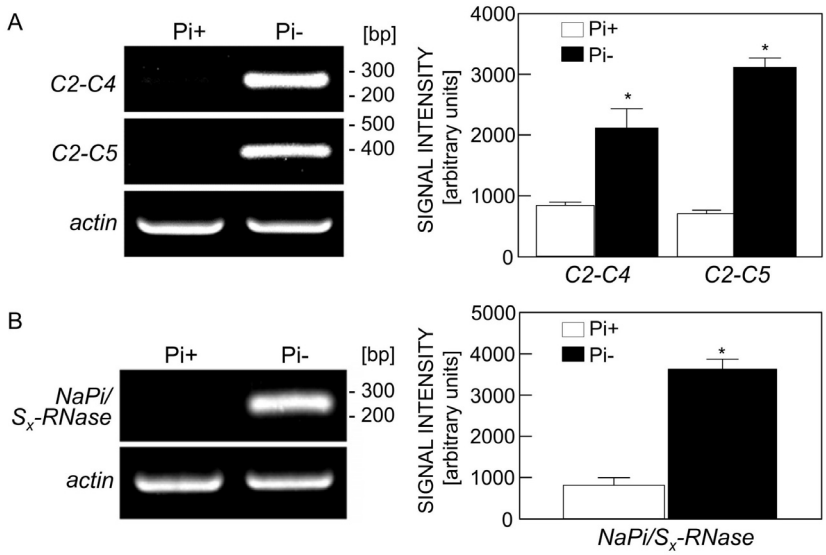


Fig. 1. Expression of *NaPi/S$_x$-RNase* in *Nicotiana alata* roots exposed to Pi deprivation (Pi-) for 14 d. *A* - semiquantitative RT-PCR amplification using degenerate primers designed from *C2*, *C4*, and *C5* conserved domains of *Nicotiana S-RNases*. *B* - similar to *A* but using *NaPi/S$_x$-RNase*-specific primers. Thirty two PCR cycles were used in each case. Signal intensity values quantified by densitometry represent the means ± SEs of three independent experiments, * - $P < 0.001$ according *t*-test.

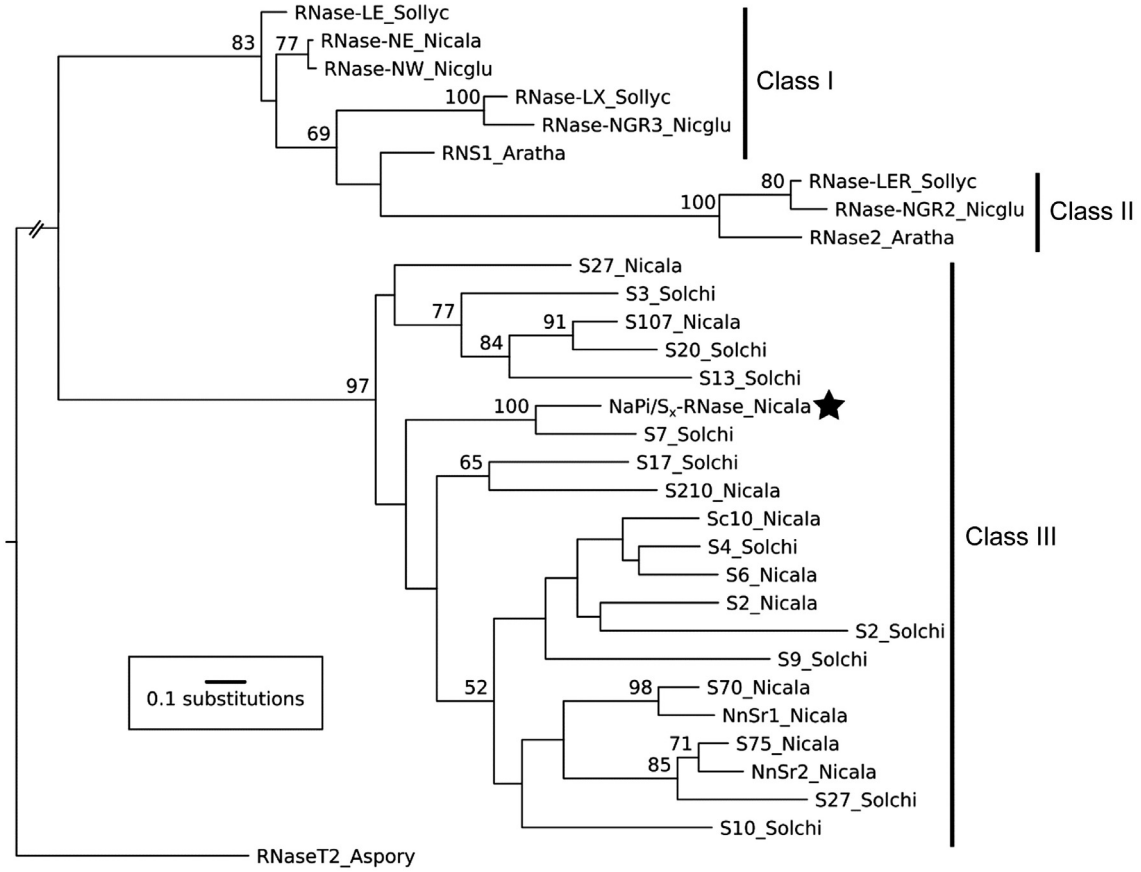


Fig. 2. Phylogenetic analysis of NaPi/S$_x$-RNase. NaPi/S$_x$-RNase was compared to T2 RNases of *Nicotiana alata* (Nicala), *Nicotiana glutinosa* (Nicglu), *Solanum lycopersicum* (Sollyc), *Solanum chilense* (Solchi), and *Arabidopsis thaliana* (Aratha). Maximum-likelihood tree topology showing phylogenetic relationships of class I, II, and III T2 RNases. The NaPi/S$_x$-RNase is highlighted with a *star*. Posterior probabilities and bootstrap values are shown for corresponding nodes when their value exceeds 50 %. The tree was rooted with a RNase T2 from *Aspergillus oryzae* (Aspory).

The deduced partial amino acid sequence of NaPi/$S_x$-RNase was compared to functional S-RNases and non-S-RNases of *Nicotiana alata*. The NaPi/$S_x$-RNase sequence exhibited the active site domains C2 and C3 (also called conserved active sites CAS I and CAS II) present in all RNases T2, as well as the other conserved domains and hypervariable regions HVa and HVb, typical of class III S-RNases (Fig 3; Ioerger *et al.* 1991, Luthala and Parker 2010). Domains C2 and C3 contain the two histidine residues that are essential for RNase activity suggesting that NaPi/$S_x$-RNase may be catalytically active, consistent with a function of Pi mobilization from RNA sources.

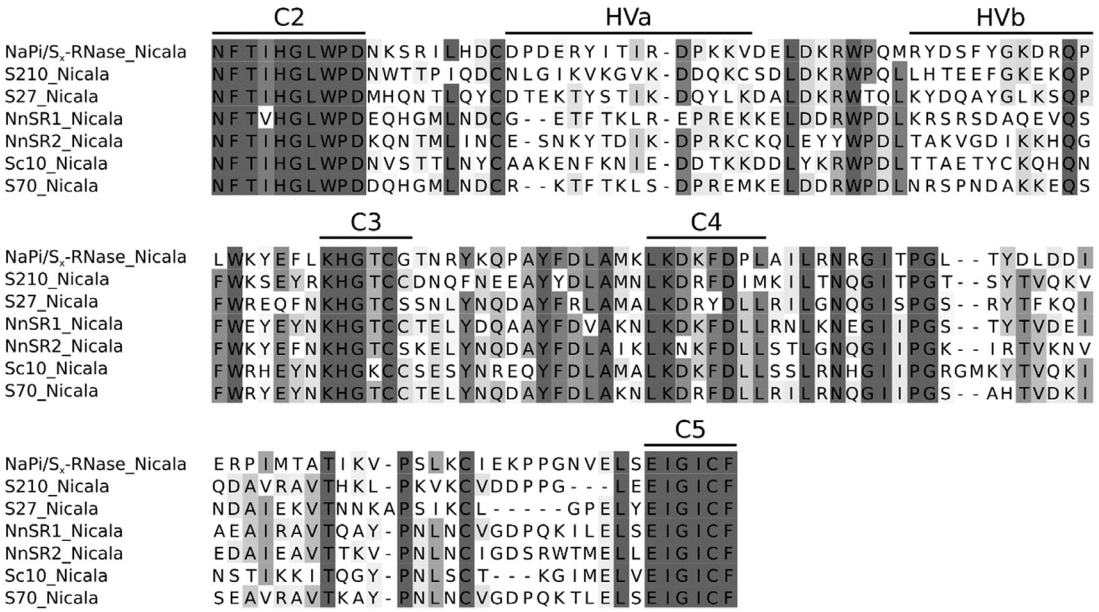


Fig. 3. Comparison of amino acid sequences of class III S-RNases and non S-RNases of *Nicotiana alata*. The alignment spans the conserved domains C2 - C5 and the two hypervariable regions (HVa and HVb), characteristic of RNases T2 grouped in class III. Amino acids are *shaded* according to percentage agreement. Accession numbers are shown in Table 2 Suppl.

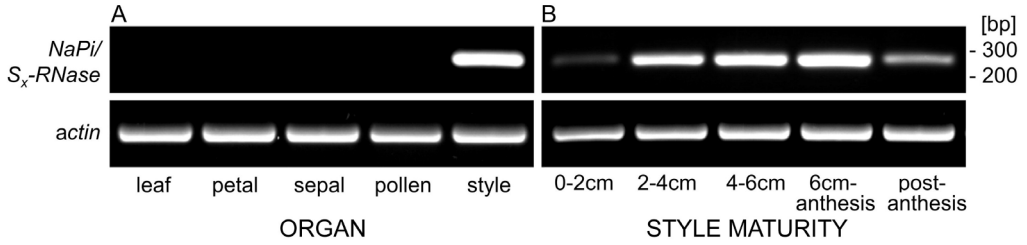


Fig. 4. Spatial and temporal expression of *NaPi/$S_x$-RNase* gene. Specific primers were used for semiquantitative RT-PCR amplification of *NaPi/$S_x$-RNase*. The gels are representative of three experiments. *A* - organ specificity. *B* - stylar developmental stages. Transcript relative abundances were compared to *actin* transcription.

Additional characterization of *NaPi/Sx-RNase* gene was tested by a semiquantitative RT-PCR assay to evaluate the spatial and temporal expression pattern in different organs and stages of style maturity. Transcription of *NaPi/$S_x$-RNase* increased in parallel with stylar development. The expression peaked before anthesis and after anthesis slightly decreased (Fig. 4*A*,*B*). Overall, the deduced amino acid sequence of the *NaPi/$S_x$-RNase* gene and its spatial and temporal expression in styles was typical of *S-RNase* genes (Roldán *et al.* 2010). However, the expression of these genes in roots under Pi starvation has not yet been reported (Rojas *et al.* 2013, 2015). To gain more insight into the relationships between the *NaPi/$S_x$-RNase* gene and the *S-RNase* alleles, we first crossed a plant carrying the *S*-genotype $S_{c10}S_{210}$ with a plant carrying the putative *S*-genotype *NaPi/$S_x$-RNase*$S_{107}$, $S_{c10}$-, $S_{210}$-, and $S_{107}$-*RNases* are three functional *S* alleles of *Nicotiana alata* tested elsewhere (Roldán *et al.* 2010). We expected that if *NaPi/$S_x$-RNase* was a part of the *S*-locus, it would be present in 50 % of the progeny and it would segregate with respect to $S_{107}$-*RNase.* The progeny segregation assayed by genomic PCR fitted the expected 1:1 ratio, which thus suggests that *NaPi/$S_x$-RNase* may effectively be the *S*-allele (Fig. 5; Table 3 Suppl.).

Next, we tested the progeny segregation of a half-compatible cross in which the *NaPi/$S_x$-RNase* gene was shared by the two parents. This type of crossing is useful

to determine the functionality of *S*-allele candidates because all the progeny of a half-compatible cross will bear the single compatible paternal allele (Roldán *et al.* 2010). Functionality test of *NaPi/$S_x$-RNase* demonstrated that this gene behaved as a fully functional *S*-allele, since the hypothesis of a full compatible cross was clearly rejected (Table 1).

Table 1. Segregation of pollen-derived *S*-alleles in semi-compatible reciprocal crosses to test SI functionality of *NaPi/$S_x$-RNase* gene; (+:-) means presence or absence of the paternal allele assayed by PCR; expected ratio for the *S*-allele assayed by PCR if the shared *NaPi/$S_x$-RNase* gene is non-functional and the cross is fully compatible. If the shared *NaPi/$S_x$-RNase* gene was a functional *S*-allele, it would not be inherited from the paternal parent.

| Parental *S*-genotype | Population size | Paternal allele assayed | Observed ratio (+: -) | Expected ratio (+:-) | $X^2$/*P*-value |
|---|---|---|---|---|---|
| ♀ *NaPi/$S_x$ $S_{27}$* × ♂ *NaPi/$S_x$ $S_{107}$* | 22 | $S_{107}$ | 21:1 | 1:1 | 18/<0.0001 |
| ♀ *NaPi/$S_x$ $S_{107}$* × ♂ *NaPi/$S_x$ $S_{27}$* | 20 | $S_{27}$ | 20:0 | 1:1 | 20/<0.0001 |

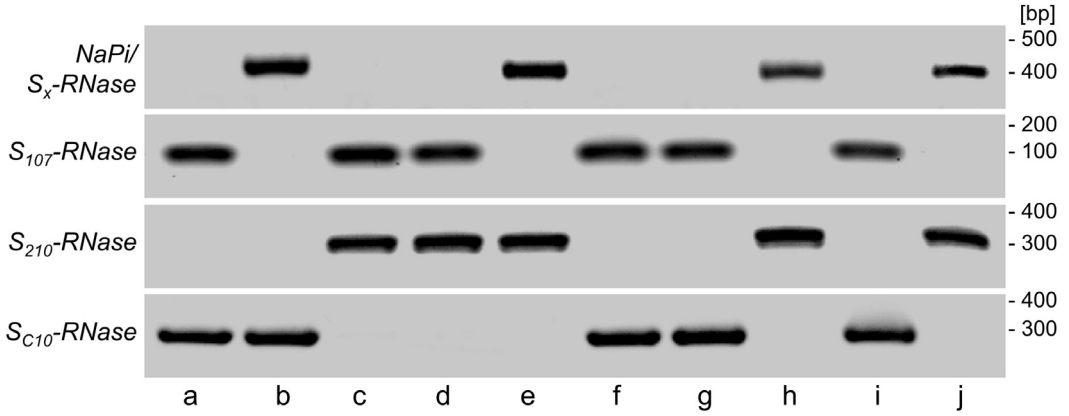


Fig. 5. Segregation of *NaPi/$S_x$-RNase* and functional *$S_{107}$-, $S_{210}$ -,* and *$S_{c10}$-RNases*. Progeny individuals of the cross *$S_{107}$ NaPi/$S_x$-RNase* × *$S_{210}S_{c10}$* were genotyped by allele specific PCR using primer pairs shown in Table 1 Suppl.

The first interpretation of these results is that the product of the *NaPi/$S_x$-RNase* gene is a functional *S*-RNase, capable of acting in both self pollen rejection as well as in Pi remobilization under Pi deficiency. In the former function, the NaPi/$S_x$-RNase exhibited a robust SI response, beyond a weak leakage in one progeny of the crosses tested in Table 1. This leakage is not rare in natural populations of *Solanacea* species, which display some variation in the strength of the SI response (Roldán *et al.* 2010). On the other hand, the Pi mobilization from nucleic acids has been reported in the three different classes of T2 RNases of several species, especially in class I S-like-RNases (Dodds *et al.* 1996, Köck *et al.* 2006, Tran and Plaxton 2008), to a lesser extent in the class II S-like-RNases (MacIntosh *et al.* 2010), and in the class III non S-RNases (Rojas *et al.* 2013). Although no conclusive evidence has been shown for some specific function of S-RNases other than the SI reaction, a possible role in defense against microorganisms has been proposed for *Petunia* S-RNases. This possibility was raised taking into account the expression of $S_x$-RNase and $S_1$-RNase in nectaries and nectar, along with other defense proteins (Hillwig *et al.* 2010, 2011). Interestingly, minor expressions of *Petunia* S-RNases were also reported early in immature pollen and other non-stylar tissues, which suggests the existence of a function not related to the SI system for these S-RNases (Clark and Sims 1994). The putative dual function of *NaPi/$S_x$-RNase* gene, both in SI and in an abiotic stress response, may be consistent with the proposition that the SI system evolved from an ancestral defense system (Hiscock *et al.* 1996, Nasrallah 2005, Hillwig *et al.* 2011). Early reports from the genera *Pisum*, *Luffa,* and *Momordica* also suggested a putative role in plant defense for class III RNases (Igic and Kohn 2001). In any case, a gain of function assay will be necessary to demonstrate convincingly that the NaPi/$S_x$-RNase is a functional S-RNase. We have previously found that *$S_{c10}$-RNase*, a functional haplotype of *Nicotiana alata* SI system, shows no induction in roots under the conditions in which *NaPi/$S_x$-RNase* was induced (Rojas *et al.* 2015). Alternatively, *NaPi/$S_x$-RNase* may be either linked to the *S*-locus or close to it, without being a functional S-RNase allele in itself. If this was the case, *NaPi/$S_x$-RNase* would co-segregate with a so far unknown functional *S-RNase*. The *NaPi/$S_x$-RNase* would have been originated by the duplication of the *S*-locus or part of it. Subsequently, it would have been translocated to a site close to *S*-locus, where *NaPi/$S_x$-RNase* could have evolved independently into a new function related to Pi mobilization under stress (Golz *et al.* 1998, Igic and Kohn. 2001, Rojas *et al.* 2013). In fact, the *S*-locus in *Solanaceae* is a huge

multigene complex, bearing genes related to SI, *i.e*. those encoding multiple SLF proteins (Wheeler and Newbigin 2007, Kubo *et al.* 2010) as well as other genes non-related to SI (Wang *et al.* 2003).

In conclusion, we reported here a cDNA sequence of a new class III RNase that we named *NaPi/$S_x$-RNase*. Identical *NaPi/$S_x$-RNase* cDNAs were isolated both from styles and from roots exposed to Pi deprivation. The protein alignment showed that the primary structure of NaPi/$S_x$-RNase was similar to class III RNases. The stylar expression pattern of *NaPi/$S_x$-RNase* and the genetic segregation analysis were similar to those expected for S-RNases. Moreover, the semi-compatible cross to test SI functionality suggested that NaPi/$S_x$-RNase was a functional S-RNase allele. This result may be the first evidence for a specific function of the locus *S* other than the SI reaction. The focus of our next research will be to confirm whether NaPi/$S_x$-RNase is a conventional non-S-RNase, or a functional S-RNase involved both in the SI reaction as well as in the response to Pi deficiency.

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