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ORIGINAL ARTICLE

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Molecular analysis of the conserved C4 region of the S_{11}-RNase of *Solanum chacoense*

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Abstract The stylar component to gametophytic self-incompatibility in Solanaceae is an S-RNase. Its primary structure has a characteristic pattern of two hypervariable regions, involved in pollen recognition, and five constant regions. Two of the latter (C2 and C3) constitute the active site, while the highly hydrophobic C1 and C5 are believed to be involved in protein stability. We analyzed the role of the C4 region by site-directed mutagenesis. A GGGG mutant, in which the four charged residues in the C4 region were replaced with glycine, did not accumulate the protein to detectable levels in styles, suggestive of a role in protein stability. A R115G mutant, in which a charged amino acid was eliminated to reduce the potential binding affinity, had no effect on the pollen rejection phenotype. This suggests the C4 does not interact with partners such as potential pollen tube receptors facilitating S-RNase uptake. Finally, a K113R mutant replaced a potential ubiquitination target with arginine. However, this RNase acted as the wild type in both incompatible and compatible crosses. The latter crosses rule out the role of the conserved C4 lysine in ubiquitination.

Keywords Self-incompatibility · Site-directed mutagenesis · *Solanum chacoense* · S-RNase

Introduction

Self-incompatibility (SI) is a prezygotic reproductive barrier used by angiosperms to promote outcrossing, assure intraspecific genetic variability and prevent inbreeding. This cell–cell recognition mechanism involves interactions between gene products expressed in the pollen, and those expressed in specialized cells of the pistil. As a result, self-incompatible plants reject self- but accept non-self pollen (de Nettancourt 1977, 2001). Among the various SI systems, the most widespread is the gametophytic, or GSI, where the genotype of the haploid pollen determines its own incompatibility phenotype.

Generally characterized by stylar rejection and typically found in species with wet stigmas and binucleate pollen, GSI is present in more than 60 families, and has been studied at the molecular level in the Solanaceae, Rosaceae, Scrophulariaceae, Campanulaceae, Poaceae and Papaveraceae. With the exception of the Poaceae and Papaveraceae, all these families share an RNase-based GSI controlled by a highly complex *S*-locus with multiple S-haplotypes containing, among other elements, both the pistillar and the pollen determinants to SI (for recent reviews see Franklin-Tong et al. 2003; Kao et al. 2004). Pollen rejection occurs when the S-haplotype of the haploid pollen matches either of the two S-haplotypes of the diploid pistil. The components of the SI reaction must be tightly linked or the system would break down, suggesting that the style and pistil components are physically close.

The pistillar determinant to SI was identified almost 15 years ago as a highly polymorphic stylar extracellular ribonuclease (S-RNase) (McClure et al. 1989). The alignment of the deduced amino acid sequences of solanaceous S-RNases reveals a distinct pattern of five short conserved (designated C1 through C5) and two longer hypervariable (HV) regions. These latter contain the S-haplotype-specific sequences that constitute the determinants for pollen recognition (Matton et al. 1997). The conserved C2 and C3 regions are hydrophilic and constitute the active site of the protein, as they contain the two conserved histidines essential for RNase activity (Green 1994). The conserved C1, C4 and C5 regions are thought to be involved in stabilizing the core of the protein (Ioerger et al. 1991). While this seems likely from the highly hydrophobic nature of the C1 and C5 regions, it is less evident for the C4 region where half of the

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residues are charged. Intriguingly, although the C1, C2, C3, C5 regions of the Solanaceae and Rosaceae S-RNases share similar positions and sequences, a region conserved among the Rosaceae, and named RC4, has no homology to its counterpart in solanaceous species (Ushijima et al. 1998). Thus, the role of the C4 region remains to be further investigated.

RNase activity is needed for self- pollen rejection (Huang et al. 1994; Royo et al. 1994). This finding suggests that SI is caused by the cytotoxic action of the S-RNases degrading pollen tube RNA in an S-haplotype-specific manner. Two models have been proposed to account for this. The receptor model proposes that entry of the S-RNases inside the pollen tube is S-haplotype-specific, i.e. does not allow entry of S-RNases of different haplotypes, and implies that the pollen determinant to SI (pollen S-gene product) is membrane- or cell wall-bound (Dodds et al. 1996). The inhibitor model permits penetration of the S-RNases inside the pollen tube in S-haplotype-independent manner, and implies that the pollen S-gene product acts as a cytoplasmic inhibitor of non-cognate S-RNases (Kao et al. 1996; Thompson et al. 1992). The finding that an S-RNase of one haplotype accumulates in the cytoplasm of pollen tubes of different S-haplotypes has provided experimental support for the inhibitor model (Luu et al. 2000). This finding draws attention to the mechanism, still unknown, by which S-RNases penetrate inside the pollen tubes. It is tempting to speculate that a conserved domain of the protein might play such a role.

The pollen determinant to S-RNase-mediated SI has remained elusive for many years, but has finally been identified as an F-box family member (Sijacic et al. 2004). The general role of F-box proteins in ubiquitin-mediated protein degradation (for review see Pickart 2004) suggests a novel biochemical basis for the SI reaction, in which inhibition of activity is replaced by protein degradation. This new model requires the F-box protein not to trigger degradation when bound specifically to the recognition domain of its cognate S-RNase (incompatible crosses) and to cause degradation when not bound in an allele-specific manner (compatible crosses). The breakthrough has occurred with the discovery, in Rosaceae and Scrophulariaceae, of several F-box genes physically linked to the S-RNase gene (Ikeda et al. 2004; Lai et al. 2002; Ushijima et al. 2003; Yamane et al. 2003). Some were expressed in the pollen, and displayed levels of allelic sequence variability high enough to be considered good candidates for being the pollen *S* gene. These were designated as *S*-haplotype-specific F-box genes (SFB) (Ikeda et al. 2004; Ushijima et al. 2003) or *S*-locus F-box genes (SLF) (Entani et al. 2003; Lai et al. 2002). The formal implication of F-box genes in SI has very recently been provided by the transgenic approach in Petunia. In a first study, the transformation of plants S_1 S_1, S_1 S_2 and S_2 S_3 haplotype with a transgene containing the pollen-expressed S_2 allele of the *P. inflata* SFL, has resulted in self-compatibility through the production of S-heteroallelic pollen (Sijacic et al. 2004). Similarly, the transfer of S_2 SLF from *Anthirrhinum hispanicum* into an SI line of *Petunia hybrida* converted the latter into SC (Qiao et al. 2004b).

The aim of the present study was to investigate the role of the C4 region in solanaceous S-RNases. We were particularly interested in testing the hypothesis that the C4 region could be involved in S-RNase entry into the pollen tube or in mediating the compatibility response by either inhibiting S-RNase activity or by targeting the protein itself for degradation. In particular, assuming that ubiquitination is responsible for degradation of S-RNase in Solanum, we wanted to see if substituting the lysine in the S_{11}-C4, one of the few lysine residues conserved among solanaceous S-RNases, would disrupt the pathway leading to S-RNase inhibition. In Anthirrhinum, degradation of S-RNases is proposed to occur through F-box protein-mediated ubiquitination (Qiao et al. 2004a). Our analyses showed no involvement of the C4 region in any of these functions, and support the idea that it plays a structural role.

Materials and methods

Plant materials

The diploid ($2n = 2x = 24$) *Solanum chacoense* self-incompatible genotypes used in the present study include two parental lines (obtained from the Potato Introduction Station at Sturgeon Bay, WI, USA) PI 458314 (called 314) which carries the S_{11} and S_{12} alleles and PI 230582 (called 582) which carries the S_{13} and S_{14} alleles. Other genotypes used here include plants L25 (S_{11} S_{12}), VF60 (S_{12} S_{12}) and G4 (S_{12} S_{14}, noted for its high regenerability in vitro) all of which have been described previously (Matton et al. 1997; Qin et al. 2001).

Transgenic plants and mutagenesis

The S_{11} genomic DNA was used as template for PCR-based mutagenesis. The mutant $S_{11}$with four amino acid substitutions, the GGGG construct, was generated in several steps. First, one pair of primers 5′-AAATCGGAACGCGAAT CCTCCACCCAAACCA-3′ (C4-A) and 5′-GAGA**CCATGG**TTAAATCAGGCTTACAT-3′ (*Nco*I-Met) was used to amplify a 0.5 kb mutated fragment corresponding to the sequence from the start codon to the C4 region (the mutated sequences are underlined and restriction sites are in bold). A second pair of primers 5′- GGAGGTGGGTTTGGTCTTCTGAG AACTCTCC-3′ (C4-B) and 5′-CTCT**GAATTC**AAGGACATACATTTGATAG-3′ (*Eco*RI-stop) was then used to amplify a 1.2 kb fragment corresponding to the sequence from the C4 to the end of the 3′-UTR. Lastly, the primers *Nco*I-Met and *Eco*RI-stop were used to amplify the 1.7-kb full length mutated S_{11} which was then cloned into pBluescript SK^+ (Strata-

gene). A 1.4-kb *Hin*dIII-*Nco*I promoter fragment from the style-specific tomato chitinase gene (Harikrishna et al. 1996) cloned separately into pBluescript, was merged with the mutated S_{11} at the NcoI site. Following digestion with *Sal*I and *Eco*RI, the 3.1-kb chimeric fragment containing the chitinase promoter and the mutated S_{11} was cloned in the binary transformation vector pBIN19 (Clontech, Palo Alto, CA, USA). The construct was introduced into *Agrobacterium tumefaciens* LBA4404 by electroporation. Plants were transformed by the leaf disc method using the *S. chacoense* G4 genotype as described (Matton et al. 1997).

For the additional constructs, a previous construct containing the chitinase promoter and wild type S_{11}in pBluescript SK^+ was used as a template for PCR-based mutagenesis using a kit (QuikChangeXLsite-Directed Mutagenesis Kit, Stratagene). In the R115G construct, arginine was substituted with glycine using the primers 5′-TTGCGCTTAAAAGAT GGTTTTGATCTTCTGAGA-3′ and 5′-AACGCGAATTTTCTA CCAAAACTA GAAGACTCT-3′. In the K113R construct, lysine was substituted with arginine using the primers 5′-TTGCG CTTA AGAGATAGGTTTGATCTTCTGAGA-3′ and 5′- AACGCGAAT TCTCTATCC AAACTAG AAGAC TCT-3′. All mutant constructs were cloned into vector pBIN19 as described above. All constructs were sequenced before and after transformation into *Agrobacterium tumefaciens* to confirm the sequence at the mutated site.

The presence of a transgene in DNA extracted from regenerated plants was verified by PCR using primers *Nco*I-Met and *Eco*RI-stop. These primers specifically amplify the S_{11} gene from the $S_{12}S_{14}$ background of the transformed host G4.

RNA and protein analysis

To determine the amount of transgene accumulation, total RNA was extracted from the styles of transformed plants as described (Matton et al. 1997). The RNA was electrophoresed on agarose gels, transferred to nylon membranes and hybridized to a radiolabeled probe prepared from the authentic S_{11} genomic DNA.

For measurement of accumulated S_{11}-RNase, total protein of freshly collected styles was extracted and electrophoresed on standard SDS gels. After transfer to nitrocellulose membranes, Western blot analysis was performed with an anti-S_{11} antibody raised against a 15 amino acid peptide corresponding to the HVa region (Matton et al. 1999).

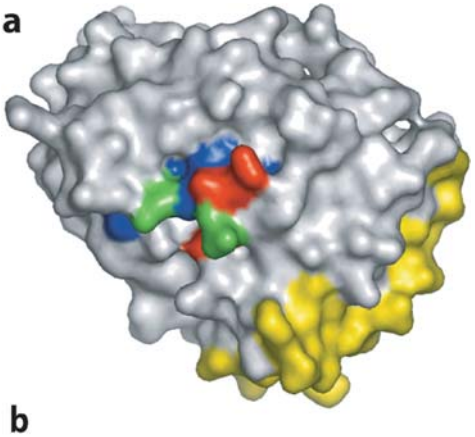
Genetic crosses

Genetic crosses were performed using fresh pollen from plants of known S-allele constitution and were monitored by fruit set. They were classified as fully incompatible if they resulted in no fruit formation after pollination, and compatible if fruits were formed after almost every pollination. Plants were classified as partially compatible when fruit set was between these two extremes. Where appropriate, pollen tube growth inside styles collected about 48 h after pollination, was monitored by epifluorescence microscopy using aniline blue staining (Matton et al. 1997).

Results

The predicted three-dimensional structure of the S_{11}-RNase (Fig. 1) indicates that the C4 region (amino acid sequence: LKDRFDLL) has four charged amino acids directed outwards. The architecture of the C4 region is striking and suggests the possibility that it may bind with high affinity to a protein partner either on the membrane or inside the cytoplasm of the pollen tube. To investigate the role of this highly conserved region, the sequence of the C4 region in the wild type S_{11}-RNase was modified by site-directed mutagenesis to produce a series of various constructs (Fig. 1b) that were introduced into the host genotype G4 (S_{12} S_{14}).

In construct GGGG, all four charged amino acids in C4 (KDR.D) were substituted with glycine (Fig. 1b). A total of 27 transgenic plants harboring the GGGG construct were crossed with S_{11} and S_{12} pollen from either 314 or L25 individuals (producing both S_{11} and S_{12} pollen). All crosses resulted in fruit formation.



Genotype	C4 region sequence
S_{11} RNase	LKDRFDLL
GGGG	L**GGGG**LL
R115G	LKD**G**FDLL
K113R	L**R**DRFDLL

Fig. 1 Three-dimensional structure of the C4 domain in the S_{11}-RNase. **a** Swiss-model structure prediction for the S_{11}-RNase sequence based on the structure of *Nicotiana alata* SF_{11}-RNase. The basic and acidic residues in the C4 domain are in *red* and *green*, respectively, while the hydrophobic residues are *blue*. The HV recognition domains in *yellow* are well separated from the C4 region. Alternate templates for threading, such as S3 from *Pyrus pyrifolia* (Matsuura et al. 2001) produce only slight variations in the predicted structure. **b** Amino acid sequences of the C4 regions in the wild type and mutant sequences used in the present study

Pistils, examined by fluorescence microscopy, revealed the presence of numerous incompatible tubes arrested in the upper third of the style, together with numerous tubes entering the ovary. This semi-compatible response was interpreted as full rejection of S_{12} pollen, an expected behavior of the G4 host (Qin et al. 2001), and full acceptance of S_{11} pollen.

The acceptance of S_{11} pollen, and the resultant compatible phenotype, could have resulted either from inability of the GGGG-S-RNase to enter pollen tubes or from below threshold levels of S_{11}-RNase accumulation. The molecular analyses clearly distinguished between these two possibilities: no S_{11}-RNase could be detected in any transgenic plant with an anti-S_{11}antibody (Fig. 2a). This failure to express the RNase at the protein level was not due to lack of the transgene, as PCR analysis revealed its presence in all regenerants (not shown), or to lack of transcription of the transgene, as Northern blot analysis showed high levels of transgene transcripts in about one third of the plants (Fig. 2b). Our previous transformation studies also show transgene expression in about one third of the plants examined (Matton et al. 1999, 1997). Taken together, these results suggest that the mutated protein might be unable to fold properly and, as a result, is rapidly degraded soon after synthesis.

To avoid this potential problem, a less drastic mutagenesis was performed in the C4 region. In the R115G construct, only the arginine in position 115 was substituted with glycine (LKDGFDLL). In this experiment, a total of 35 transgenic plants were subjected to genetic analysis. The crosses revealed that 19 of them were fully or partially compatible with S_{11} pollen, whereas 16 fully rejected S_{11} pollen (Table 1). The rejection of S_{11} pollen excluded the possibility that the modification in the C4 domain affected entry of the R115G S-RNase into the pollen tubes. Furthermore, all these transgenics accepted S_{13} pollen (the normal compatible response), excluding the possibility that this modification to the C4 region could prevent binding to a general S-RNase inhibitor (Luu et al. 2001). An impairment of the S-RNase to bind to the S-RNase inhibitor should lead, in fact, to full rejection of all pollen types, since the mutant S-RNase would remain fully active.

The western blots confirmed that plants fully incompatible with S_{11} pollen also displayed levels of transgenic S-RNase accumulation close to the values found in wild type plants (Fig. 3a), whereas individuals partially compatible with S_{11} pollen had lower levels of transgenic S-RNase in the styles. This is most evident if the ability of the styles to reject S_{11} pollen is plotted directly against the levels of S_{11}-RNase in the styles as determined by densitometric scans (Fig. 3b). To directly address the potential role of the C4 region in targeting the S-RNase for degradation by ubiquitin-mediated proteolysis, the conserved lysine was substituted with arginine in the K113R construct. A total of 35 transgenic plants were analyzed, of which 17 were fully or partially compatible with S_{11} pollen and nine fully rejected S_{11} pollen (Table 2). The rejection of S_{11} pollen indicates that the modified S-RNase can enter the pollen tubes. Once again, densitometric scans of Western blots confirm the expected relationship between S-RNase levels and the pollen rejection phenotype (Fig. 4). However, crosses with S_{13} pollen revealed full compatibility (Table 2). This compatible response clearly rules out a role of the modified lysine in targeting the S-RNase to the proteasome.

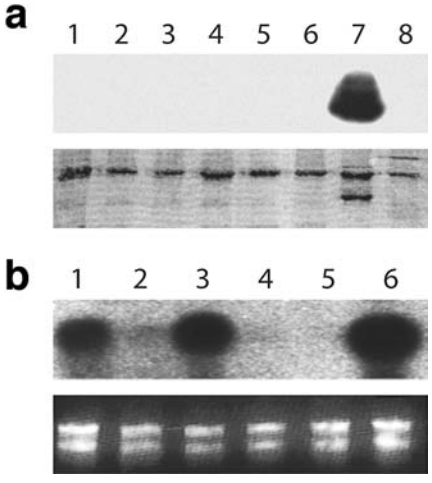


Fig. 2 Expression of the GGGG transgene at the protein and RNA levels. **a** Western blot analysis of stylar protein extracts from six transgenic plants (*lane 1* T16, *lane 2* T21, *lane 3* T24, *lane 4* T25, *lane 5* T26, *lane 6* T34) using the anti-S_{11}-RNase. The plants L25 (*lane 7* $S_{11}S_{12}$) and VF60 (*lane 8* $S_{12}S_{12}$) are shown for comparison. No protein is detectable in the transgenic plant extracts even at long exposure times. *Bottom panel* shows Ponceau-stained membranes as control for protein load. **b** Northern blot analysis of total RNA extracted from styles of five transgenic plants (*lane 1* T21, *lane 2* T24, *lane 3* T25, *lane 4* T26, *lane 5* T34) using a probe prepared from S_{11} genomic sequence. The plant L25 (*lane 6* $S_{11}S_{12}$) is shown for comparison. *Bottom panel* shows the ethidium bromide-stained gel as control for RNA load

Discussion

The analyses described here were undertaken to provide insight into the role played by the C4 domain in S-RNases. The C4 region is highly conserved in the solanaceous S-RNases (Richman et al. 1996; Sassa et al. 1996) and the crystal structure reveals an unusual and rather unique organization (Ida et al. 2001). The four charged amino acids extend outwards from the surface of the protein much like fingers from one hand, while the hydrophobic residues in this region are buried deep in the heart of the protein and in fact constitute an important element defining the RNA-binding fold at the active site of the protein. This organization is observed in the published structure of the *Nicotiana* S-RNase (Ida et al. 2001) and in the predicted structure of our S_{11}-RNase (Fig. 1).

Table 1 Phenotypes of transgenic R115G plants

Pistil genotype $S_{12}S_{14}$	Transgenic plant phenotype			
	× S_{11} S_{12} pollen donor			×$S_{13}S_{14}$ pollen donor
	Compatible	Partially compatible	Incompatible	Compatible
Number of plants	16	3	16	35
Number of fruits per pollinated flowers	61/63	13/21	0/51	68/69

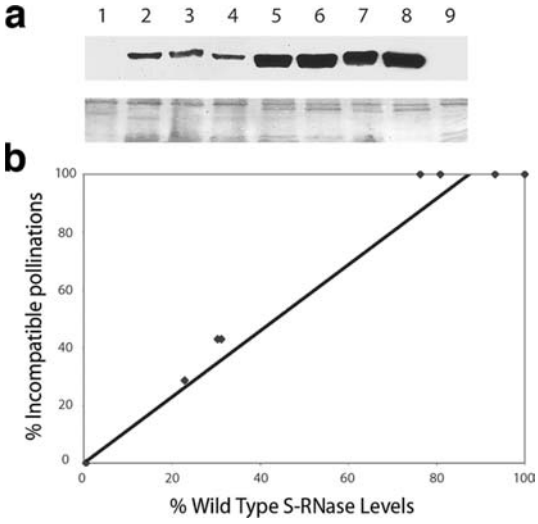


Fig. 3 Expression of the R115G transgene and correlation of phenotype with protein levels. **a** Western blot analysis of stylar protein extracts from seven transgenic plants (*lane 1* T1 *lane 2* T18, *lane 3* T38, *lane 4* T39, *lane 5* T24, *lane 6* T36, *lane 7* T44) using the anti-S_{11}-RNase. The plants L25 (*lane 8* $S_{11}S_{12}$) and VF60 (*lane 9* $S_{12}S_{12}$) are shown for comparison. *Bottom panel* shows Ponceau-stained membranes as control for protein load. **b** The number of incompatible pollinations (calculated as percent of wild type) is plotted as a function of transgene S-RNase levels (shown as percent of L25 wild type levels). RNase levels were quantitated from densitometric scans of the Western blots

One interpretation of this structure is that the necessity of keeping the charged amino acids in the aqueous phase provides a mechanism for pushing the adjacent hydrophobic amino acids into the protein core. However, while this would explain the conserved pattern of charged and non-polar residues, it does not account for the conserved pattern of positively and negatively charged residues. This characteristic suggests instead an alternative role for the C4 region as a high-affinity binding site for other proteins.

The possibility that this region might bind other proteins was intriguing, as there are several candidates potentially binding to a region common to different S-RNases. One candidate is an RNase inhibitor similar to that proposed by the various inhibitor models (Kao et al. 1996; Luu et al. 2001). Alternatively, the C4 (or more specifically a conserved lysine in this region) might be involved in directing the formation of an ubiquitinated intermediate. Lastly, the region might be involved in assuring S-RNase entry into the pollen tubes either alone or as part of a complex. While it is still unclear how many different stylar proteins enter pollen tubes (Lind et al. 1996; Wu et al. 1995) a mechanism clearly exists for non *S*-haplotype-specific uptake of S-RNases (Luu et al. 2000).

The constructs used here were designed to assess all of these possibilities. First, if the charged residues are involved in maintaining the hydrophobic residues of the C4 region in the core of the protein, the GGGG construct should lack this stabilizing force. The position of the hydrophobic residues suggests they are likely to be important for the structure of the protein, so that this mutation might well produce a dramatic change in the three dimensional structure of the protein. Indeed, our results are consistent with this, as none of the transgenics accumulated detectable levels of the S-RNase despite an apparently normal range of mRNA accumulation. It is possible that the modified protein misfolds inside the ER lumen and thus becomes targeted for degradation (Kostova et al. 2003). However, we cannot exclude the possibility that the choice of glycine itself may have influenced the stability of the protein structure. This issue could perhaps be explored by examining protein stability after other alterations (such as to alanines, for example).

Table 2 Phenotypes of transgenic K113R plants

Pistil genotype $S_{12}S_{14}$	Transgenic plant phenotype			
	× $S_{11}S_{12}$ pollen donor			× $S_{13}S_{14}$ pollen donor
	Compatible	Partially compatible	Incompatible	Compatible
Number of plants	17	9	9	35
Number of fruits per pollinated flowers	84/86	35/106	0/52	132/136

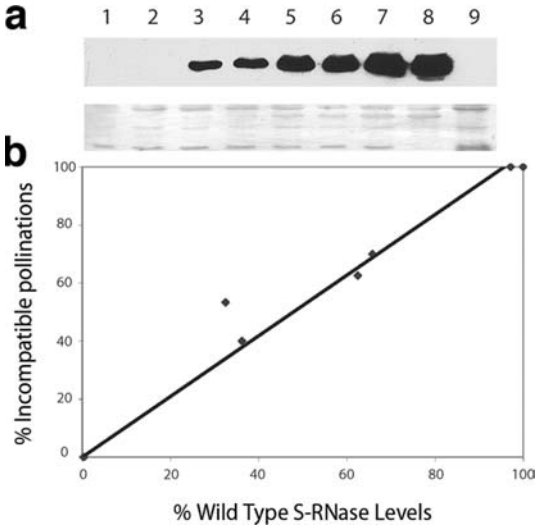


Fig. 4 Expression of the K113R transgene and correlation of phenotype with protein levels. **a** Western blot analysis of stylar protein extracts from seven transgenic plants (*lane 1* T6, *lane 2* T28, *lane 3* T2, *lane 4* T38, *lane 5* T27, *lane 6* T32, *lane 7* T56) using the anti-S11-RNase. The plants L25 (*lane 8* $S_{11}S_{12}$) and VF60 (*lane 9* $S_{12}S_{12}$) are shown for comparison. *Bottom panel* shows Ponceau-stained membranes as control for protein load. **b** The number of incompatible pollinations (calculated as percent of wild type) is plotted as a function of transgene S-RNase levels (shown as percent of L25 wild type levels). RNase levels were quantitated from densitometric scans of the Western blots

The effects of the R115G mutation are expected to be much less drastic than the GGGG mutation, at least with regard to the three dimensional structure of the protein. However, thermodynamic considerations suggest that this single amino acid substitution might have a large effect on the binding affinity with any potential partners. The formation of single ionic bond provides a standard free energy change of about −3 kcal/mol, and thus replacement of this arginine with glycine would be expected to change the equilibrium constant by a factor of 100. Our data indicates, however, that the levels of S-RNase needed for pollen rejection are not different from those observed in wild type or the K113R mutant (compare Figs. 3, 4). This result provides no indication that additional substitutions in the C4 might provoke an effect on the phenotype, and thus suggests that this region is not directly involved in protein–protein interactions. We also note that a single glycine substitution can easily be accommodated without adverse effect on protein stability and RNase activity.

Ubiquitination can occur at specific lysine residues within a polypeptide chain (Batonnet et al. 2004; Galluzzi et al. 2001) presumably by recognition of a particular amino acid context around the targeted lysine. Amino acid sequence alignments of solanaceous S-RNases reveal that in almost all cases two lysines are present in the C4 region (Sassa et al. 1996). In contrast, our S_{11}-RNase has only one, thus allowing a direct test of the potential role of the C4 in protein degradation. In the K113R mutant, lysine was replaced by arginine so that the charge of the protein would remain unchanged, and to thus minimize any effect of the mutation on the three dimensional structure of the protein. However, assuming that ubiquitination is responsible for degradation of S-RNase in Solanum as it is in Anthirrhinum (Qiao et al. 2004a) and that the C4 lysine is used in targeting the protein for ubiquitination, the mutant S-RNase would be expected to reject all pollen types as it could no longer be degraded. Our results clearly demonstrate that the phenotype of K113R transgenics expressing wild type amounts of the mutant RNase are indistinguishable from wild type S_{11}-RNase with respect to its breeding behavior with both S_{11} and S_{13} pollen. It is important to note that our results do not rule out ubiquitination of the S-RNases in mediating compatibility. However, they do clearly eliminate the possibility that the C4 lysine alone is the target. It is still possible that the other conserved lysine (in the C3 region) might play such a role or, alternatively, that other lysines in the protein can be used when the C4 lysine is lost.

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