

1 New Phytologist Supporting Information

- 2 Article title: Primary restriction of S-RNase cytotoxicity by a stepwise ubiquitination
- 3 and degradation pathway in *Petunia hybrida*
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- 6 Article acceptance date: 20 April 2021
- 7
- 8 The following Supporting Information is available for this article:





Fig. S1. Identification of six ubiquitinated residues of *Petunia hybrida* S₃-RNase 10 11 by LC-MS/MS analysis of cross-pollinated pistils. (a) Aniline blue staining of 12 pollen tubes in a wild-type (PhS_3S_{3L}) pistil cross-pollinated with transgenic pollen containing $PhS_{3L}SLF1$ ($PhS_{3S_{3L}}/PhS_{3L}SLF1$). Bar: 1 mm. (b) A flow chart of 13 LC-MS/MS. *PhS*₃S_{3L} pistils pollinated with cross pollen of *PhS*₃S_{3L}/*PhS*_{3L}SLF1 14 15 containing transgenic *PhS_{3L}SLF1* (T) are collected for protein extraction, digestion, 16 ubiquitinated peptide enrichment and LC-MS/MS. (c) Tandem mass spectrometry (MS/MS) of ubiquitinated PhS₃-RNase peptide. Horizontal and vertical axes 17 18 represent m/z and signal strengths, respectively. I, II and III indicate three 19 ubiquitinated regions of PhS₃-RNase. T, K and C indicate threonine, lysine and 20 cysteine.



27 MS/MS are identical to those of Fig. S1.



		C1	C2	Hva	Hvb
PhS3-RNase	* 20		40 * 60 * ASS Y-PKNK OR-S-N 5 HIGH EK-KR-FRIER	80 * 100 TGDKEKRFLEEDNIINVERHOI	* 120 ORFDETYANTKOPINE : 108
Pis3-RNase	:MEKSQLISALFISLEAFSEVCA	NEDYICLVLTW	ASECYRPKNICREIP-NNEEIHGLWEEKEH-FREEF	DGDK-VSFSLKDRIVND ERHWV	ONKFDEKFAKIK PLWT : 109
PhS3L-RNas	:NFKSOITSALFVVLFFUSETVO	ERELICIVITÀ: VEFVICIVITÀ:	AS CYANHCERIA NN SIHGLWF NVT-IR CY	KPKPTHTTFAGKMLNDEDKHMI	OKYKEAYARRESPTAK : 107
PbrS7-RNas	: MGITGMIYIVTMVFSLIVLILSSTV	GYDYFOFTOOYO	PAVCNSKPTPCKDPPDKLFTVHGLWFSNLNGPHPEN	TNATVNPHRIKNIQAQUKIIMP	NVLDRTNHVGFWN : 110
AAG21384	:NFRLOT LSALFILLFSISTVSA	NEDYFOLVITA	ASECY-PRNFCKER-S-NEFTHGIWEENKH-FRUEF	TGDKISRFKE-DNIINVIERHNI	OWRFDEKYASTKOPLME : 107
AAA33726	:NVKSRIISVFFIFLFSFSEVYC	NEEYLOLVLTW	AS CIGFRDIER, T-V-NETHONNELK-RG-FRIEF AS CFRPKNICK: PA-KNDTHGIWEEITG-FRIEF	TGSPKUETFKD-NNIVDY ERHYV	OKFDENYAKYHOPINS : 109
XP_0042290	:MFKSOI ITALFILFFCLSEIVO	DEDYNOLVLTWI	PSECY-PRGTCKET-S-NEMIHGIWEEKKG-FREEF	SGGKAKKFELHDHIVND DHHMI	KKFTEODAKOKOPIKN : 109
AAA33729	:MFKSHITAVIFILLFSIP:IVG	DEDYNOLVLTN	AS CIPRNICS I - A N SI HGIME NEQ-RR QF	TSTEASLFDGDILDD DRHMI	O KFDKETGMODOPLWH : 108
AAN76454	:MFKSQITSAHFILLFAISPIYC	DEDYNCLVLTME	ATECY-PKGFCCRIPKNFTIHGLWPKER-QRIQF	CAKDYKNVNFEGDIKSSIDHHWI	C RFNKEVGLKY PLAH : 108
BAA76514	:NFKSQITSALFVVLFFLSETVC	EFELBOLVIT	AS CY-ANHOR I-A NN BIHGIWE N-VT-IR CY	CKPKPTHTTFAGKMLND DKHMI	CHRYERAYARRE PTOK : 107
BAC00940	:NVKPQITAALFIVLFAISPAY	DEDSLOLVITE	ASECHMNECVEIAEKNETIHGLWFEK-EG-TVEQN	KPKPNISNFKEKMFNDIDKHWI	C KYDEDYGEKE PLAF : 107
BAC00934	:NPOTTAALLIVLFATSTAT	DEDSLOLVITA	AS CDSNNCKSI-A KNEIHGLWE KEG-TM CC	KPKPDEVNFKDKMFND DKNMI KPKPNEVNFKDKMLNDEDKNMI	C KFDEDYGRDK PLWV : 105
AAA50306	:MFKSLITSTLFIVLFSLSSTYC	DEDKLOLVLTME	PSECHANNCORIVERNSTINGLWPEREGPOLTRY	CKPKLTUNYFSDKMLNDUDKHWI	C KIDQASARKDOPANK : 108
Q40381	:VERSOTTSVEEVLEVLEVISEIVC	AREYNOUVIOR	AT OYAYGOSSERPIENE HELWE NKS-IIENN TA CHTTPCKSIENNE HEGWE NVS-TTENY	AAKENKNIEDDT-KKDD YKRMP	TTAETYCKOH NFWR : 107
AAN76453	:MFESRIMSVLFIFLFALSEVYC	TFEYMOLVLTW	ISECHTKHCERITNETIHGLWEINKN-ALINN	CVPDATNNKITNPE-LLKOVDYRMP	ETSKEIDGKKK®GLNG : 107
BAJ24848 BAJ24847		ABDOLOLVITA:	PTVCHEKHON:IRNS IHGIME NQH-VM NN PS2CHTKDCK2TRND THGIME DCH-VI ND	AKTDCKITNVR-KIKEDDDRMP	DECKSKSEATRT SFRQ : 105
AAK15435	:MLSSOIMSVAFILFLALSEVYC	SEDOLOLVITWE	PSECHEKSCNSI ENETIHGLWP NCH-VMUND	CAKTKKITDVL-KSKE DDRWP	D KYSRNNAIOT SFWR : 105
AAA60465	:NFKLO ASVLOVFLFACS IS	SEDHWOIVITW	AGVCKVKGCPSP-VI N FIHGLWP SIS-VIANN	OPTKTVTITEIN-QITE EKRNP	ETTTACFALTS SFAR : 109
BAC00930	:NFKTCHTLAFFILLCALSDVYC	TENCLOLVIRE	ASECKGKKCEETENNETIHGLWPTIKG-TIENN	CNPDAKMASVTGGKFVKRNKHWP	DILTEAASLNSOGFWA : 106
AAA77040	:MFKTQHTLAFFILLCAIPDVVG	TENCLOLVIRME	ASECKGKKCERTENNETIHGLWPDIKG-TIENN	ONPDAKWASVTGGKFVKRNKHWP	DILTEAASLNSOGFA : 106
ACT35737	:WFQFQHTLAFFIFICAISDVAR	ABDHWOLVITM	AS CDDRROSSTENES THELWE IKG-TVENN AG CKIKCCPET-VI DESTINGINE SVS-VM YN	DPPTRSNKIRETN-IKNE EKRMP	ESTSTACFALKS SFAK : 109
AAD56217	:VFKSLASILUILLHUSEGNC	TREQUOLVETW	TASCHKVNCVBINNESIHGIWPNNKS-RRUNF	CK-STK IKSTDEG-KKAYIEYRMP	NITTEVESKKNOFFNE : 104
AAB40028 CAA53666	:NENLPHTSVFVHFLFAHS: Inc.	ABEYNOL U CM	TTOCHTTPCKNI SND INCIME NVS-TTENE RSSCKTRYCPNPVERNE THCIME KOR-IMPIN	GKEDDWNIIMDGP-EKNGHYVRMP PAKESWKSITDSK-KIKLUEOHMP	D IREKADCMKTENFER : 107
AAA77039	:NFRPQ-IIGFFIMLCAFYHVYG	TEDOLOLVIRWE	TSECNGKNCKSTEKEFEIHGLWPESEA-GEENF	CNPRASTIVRHGTFEKRNKHMP	D MRSKDNSMDN EFWK : 105
P04007	:NSKSOFTSVFFFLLCARSEIVE	AREYNOLULTA	IT CRIKHCERT TNE IHGLWE NHT-TM NY	DRSKPNMFTDGK-KKND DERMP	DITKTKFDSLDKCAFAK : 107
mmillio	m sp yg	5 21v1 5p	C Cr p Ft6HGLWPd	C W	q W
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29 Fig. S3. Locations of six ubiquitinated residues of *Petunia hybrida* S₃-RNase in

- 30 Solanaceous S-RNases. Amino acid sequence alignments of 36 S-RNases in
- 31 Solanaceae. C1-C5, five conserved regions; Hva and Hvb, hypervariable region a
- 32 and b. The black shadow indicates highly conserved sequences and the light less
- 33 conserved ones. The orange rectangles indicate the ubiquitination sites of
- 34 PhS₃-RNase, the green the major lysine residues identified previously (Hua and Kao,
- 35 2008) responsible for the ubiquitination and degradation of *Petunia inflata* S₃-RNase
- 36 and their corresponding amino acids among the 36 S-RNases.
- 37
- 38 Hua, Z., and Kao, T.-H. 2008. Identification of major lysine residues of S₃-RNase of *Petunia inflata* involved in
- 39 ubiquitin-26S proteasome-mediated degradation *in vitro*. *Plant Journal* **54**, 1094-1104.



Fig. S4. Petunia hybrida S₃-RNase with the mutated region (R) I displays largely 41 unaltered biochemical and physical properties. (a) RNase activity detection of 42 His-S₃R and -mutant (M) I expressed by *pCold-TF* vectors. The relative fluorescence 43 unit (RFU) indicating RNase activity during a time course experiment is shown as 44 mean \pm S.D. (n = 3). I: the ubiquitinated region I of PhS₃-RNase. (b) Immunoblot 45 detection of FLAG-tagged S₃R and MI in subcellular fractions of in vitro germinated 46 pollen tubes. EC1, EC2 and WTEC indicate entire cell homogenates of the pistils 47 48 from the transgenic plants containing S_3R -FLAG or MI-FLAG, the pollen tubes of PhS_VS_V treated with EC1 and the pistils from wild-type PhS_3S_{3L} . WTEC was a 49 negative control. S1 and P1, S12 and P12, S160 and P160 indicate supernatant and 50 pellet fractions obtained by centrifugation of EC2 at 1,000 g, 12,000 g and 160,000 g, 51 respectively. cFBP and Sar1 are marker antibodies of cytosol and endoplasmic 52 reticulum (ER), respectively. (c) Physical interactions between $PhS_{3L}SLF1$ and S_3R 53 and MI detected by pull-down assays. Input and pull-down: bait protein 54 SUMO-His-PhS_{3L}SLF1 and prey proteins detected by immunoblots, respectively. 55 MBP and SUMO-His are protein tags. Asterisks indicate bands of target proteins. (d) 56 Split firefly luciferase complementation (SFLC) assay. The numbers on the left side 57 of the color signal bars represent the values of the fluorescent signal. The injection 58 positions of each component on tobacco leaves are indicated in the contour diagram 59 of leaf margin. nLUC and cLUC: transiently expressed N-terminal and C-terminal 60 regions of luciferase. (e) Bimolecular fluorescence complementation (BiFC) assays. 61



- 62 NE and CE: transiently expressed N-terminal and C-terminal regions of YFP by
- *pSPYNE* and *pSPYCE* vectors. YFP, BF and Merge represent the YFP fluorescence,
- bright field and their merged field, respectively. Bars: 20 μ m.





66 Fig. S5. Physical interactions between *Petunia hybrida* S₃R/Mutant (M) and

67 PhS₃SLF1. S₃R/M represents wild-type or a mutant form of PhS₃-RNase. I, II and

68 III indicate three ubiquitinated regions of PhS₃-RNase. K and T indicate lysine and

69 threonine within six ubiquitinated residues of PhS₃-RNase. nLUC and cLUC indicate

70 transiently expressed N-terminal and C-terminal regions of luciferase. The other

annotations of this figure are identical to those of Fig. S4d.





Fig. S6. Predicted three dimensional (3D) structures and surface electrostatic 73 74 potentials of *Petunia hybrida* S₃-RNases with mutated ubiquitinated residues. (a-d) Predicted 3D structures (top) and surface electrostatic potentials (bottom) of 75 PhS₃-RNase (left) and its mutant (M) forms (right). (e,f) Predicted 3D structures of 76 PhS₃-RNase (left) and MIII (right) (e) or PhS₃-RNase (left) and MI/II/III (right) (f). 77 For the predicted 3D structures, blue indicates the hypervariable Hv regions, red T 78 79 (threonine) and its mutant form A (alanine), and green K (lysine) and its mutant form 80 R (arginine). For the predicated surface electrostatic potentials, blue and red indicate



- 81 positive and negative charges, respectively. I, II and III indicate three ubiquitinated
- 82 regions of PhS₃-RNase.





Fig. S7. *Petunia hybrida S₃R* and *S₃R* (*Mutant*) (*M*) transgenes identification by

85 **PCR analysis.** (a) Schematic diagrams of plant expression constructs containing S_3R

86 or $S_{3}R(M)$ driven by a pistil-specific promoter *Chip* between right and left borders

87 of T-DNA. *NPTII*, neomycin phospho-transferase II gene conferring kanamycin

88 resistance. *Nos-pro* and *Nos-ter*, promoter and terminator of Nopaline synthase gene,

respectively. $S_3R(M)$ represents a mutant form of S_3R . (b) Identification of T_0

90 transgenic plants by genomic polymerase chain reaction (PCR). I, II and III: three

91 ubiquitinated regions of PhS₃-RNase. K and T: lysine and threonine within six

ubiquitinated residues of PhS₃-RNase. S_3S_{3L} : wild type and a negative control. H₂O:

93 a negative control.





95 Fig. S8. Identification of *FLAG*-tagged *Petunia hybrida S₃R* and *S₃R* (*Mutant*)

96 (M) transgenes by PCR analysis. (a) Schematic diagrams of plant expression

97 constructs containing S_3R -FLAG or S_3R (M)-FLAG. NPTII, neomycin

98 phospho-transferase II gene. Nos-pro and Nos-ter, promoter and terminator of

99 Nopaline synthase gene. (b) Identification of T_0 transgenic plants by genomic PCR. I,

100 II and III, three ubiquitinated regions of PhS₃-RNase. K and T, lysine and threonine

- 101 within six ubiquitinated residues of PhS₃-RNase. Annotations of this figure are
- 102 identical to those of Fig. S7.





104 Fig. S9. Detection of FLAG-tagged Petunia hybrida S₃R and S₃R (Mutant) (M)

- 105 transgenes expression by immunoblots. The genotypes and numbers of the
- 106 transgenic lines are indicated on top. S_3S_{3L} is wild type used as a negative control. I,
- 107 II and III: three ubiquitinated regions of PhS₃-RNase. K and T: lysine and threonine
- 108 within six ubiquitinated residues of PhS₃-RNase. FLAG and cFBP antibodies were
- 109 used to detect the transgene expression and sample loading, respectively.



111 Fig. S10. *Petunia hybrida* S₃-RNase with the mutated region (R) I slightly

- 112 reduces cross seed sets. (a) Transcripts of transgene (*PhS*₃-*RNase or mutant* (*M*) *I*)
- and native PhS_3 -RNase detected by qRT-PCR. The T₀ transgenic lines are indicated
- below the horizontal axes. S_3S_{3L} is wild type. Data are shown as mean \pm S.D. (n = 3).
- 115 (b) Quantitative analyses of FLAG-tagged S₃R and MI proteins detected by
- 116 immunoblots in Fig. S9. Data are shown as mean \pm S.D. (n = 3). A student's *t*-test
- 117 was used to generate the *p* values. ns (not significant), p > 0.05. (c) Reduced seed set
- 118 per capsule from cross-pollinated T_0 transgenic lines containing *MI* or *MI-FLAG*.
- 119 The transgenic plants containing S_3R or MI and S_3R -FLAG or MI-FLAG were
- pollinated with cross pollen of PhS_VS_V . Numbers below the horizontal lines indicate
- 121 T₀ transgenic line numbers. Bars: 5 mm. (d) Statistical analyses of seed sets per



- 122 capsule from T_0 transgenic lines cross-pollinated with PhS_VS_V pollen. Data are
- shown as mean \pm S.D. (n = 10). A student's *t*-test was used to generate the *p* values.
- 124 **, p < 0.01. (e) and (f) Cell-free degradation of recombinant SUMO-His-tagged S₃R
- or MI by pollen-tube extracts (PTE) of PhS_VS_V (cross) or PhS_3S_3 (self). Left,
- 126 immunoblots of the reaction products incubated with or without MG132 (Mock).
- 127 Start, time point zero in each degradation assay. cFBP antibody was used to detect
- 128 non-degraded loading control. Right, quantitative analyses of the degradation rates.
- 129 Data are shown as mean \pm S.D. (n = 3). The remaining amount at 10 min is indicated.
- 130 (g) Time-course analyses of PhS₃R- and MI-FLAG levels in the cross- (PhS_VS_V) or
- 131 self- (*PhS*₃*S*₃) pollen tubes (PT) incubated with or without MG132 (Mock). Top,
- 132 immunoblots of PhS₃R- or MI-FLAG in the PT using FLAG antibody. cFBP was
- 133 detected as a loading control. The numbers at the bottom indicate the transgenic line
- 134 numbers corresponding to those in **d**. Bottom, quantitative analyses of the
- immunoblots. Data are shown as mean \pm S.D. (n = 3). A student's *t*-test was used to
- 136 generate the *p* values. **, p < 0.01.



138 Fig. S11. Decreased ubiquitination amount of mutant (M) I, II and III mediated

139 by SCF^{S_{3L}SLF1} of *Petunia hybrida*. (a) Immunoblot detection of *in vitro* ubiquitinated

140 recombinant His-tagged S₃R and its mutant forms using anti-ubiquitin (Ub) and -His

141 antibodies. His-S₃R, -MI, -MII and -MIII are labeled as the substrates. I, II and III:

- 142 three ubiquitinated regions of PhS₃-RNase. FLAG: a protein tag. PhS₃R/M indicates
- 143 wild-type or a mutant form of PhS₃-RNase. The vertical lines illustrate the
- 144 ubiquitinated His-S₃R/M. Open and filled arrowheads indicate ubiquitin and
- 145 unubiquitinated His-S₃R/M monomers, respectively. (b) Quantitative analyses of
- 146 immunoblots from **a**. Data are shown as mean \pm S.D. (n = 3).







148 Fig. S12. Ubiquitination of *Petunia hybrida* S₃R and its mutant forms by self-

149 (PhS_3S_3) pollen-tube extracts (PTE). Immunoblots of cell-free ubiquitination

- 150 products of His-tagged S_3R and its mutant (M) forms incubated with self- (*PhS*₃*S*₃)
- 151 PTE using anti-ubiquitin (Ub) and -His antibodies. I, II and III: three ubiquitinated
- 152 regions of PhS₃-RNase. K and T: lysine and threonine within six ubiquitinated
- residues of PhS₃-RNase. Annotations of this figure are identical to those of Fig. S11a.





155 Fig. S13. Reduced seed set per capsule from T₀ transgenic lines with mutated

- 156 region (R) II of *Petunia hybrida* S₃-RNase. The transgenic plants containing S_3R or
- 157 *Mutant* (*M*) II (**a**) and S_3R -FLAG or MII-FLAG (**b**) were pollinated with cross pollen
- 158 of PhS_VS_V . Numbers below the horizontal lines are T₀ transgenic line numbers. FLAG:
- 159 a protein tag. Bars: 5 mm.





Fig. S14. Reduced seed set per capsule from T₀ transgenic lines with mutated
lysine or threonine within the six ubiquitinated residues of *Petunia hybrida*

163 S₃-RNase. (a) and (b) The transgenic plants containing S_3R , mutant (M) K or MT (a)

- and S_3R -, *MK* or *MT*-*FLAG* (b) were pollinated with cross pollen of *PhS_VS_V*. FLAG:
- a protein tag. Bars: 5 mm. (c) Cell-free degradation of recombinant SUMO-His-MK,
- and -MT by self (*PhS*₃*S*₃) pollen-tube extracts (PTE). Data are shown as mean \pm S.D.
- 167 (n = 3). (d) Time-course analyses of MK- and MT-FLAG levels in the cross- (PhS_VS_V)
- 168 or self- (PhS_3S_3) pollen tubes (PT) incubated with or without MG132. The numbers
- 169 at the bottom of the immunoblots and the x-axis indicate the transgenic line numbers
- 170 corresponding to those in **b**. Data are shown as mean \pm S.D. (n = 3). A student's
- 171 *t*-test was used to generate the *p* values. *, p < 0.05; **, p < 0.01; ns (not significant),
- 172 p > 0.05. Annotations of this figure are identical to those of Fig. S10.





Fig. S15. Mutant (M) III largely maintains the biochemical and physical 174 properties of Petunia hybrida S₃-RNase. (a) RNase activity detection of His-S₃R 175 and -MIII expressed by *pCold-TF* vectors. Data are shown as mean \pm S.D. (n = 3). 176 RFU: relative fluorescence unit. III: the ubiquitinated region III of PhS₃-RNase. (b) 177 Immunoblot detection of FLAG-tagged MIII in subcellular fractions of in vitro 178 germinated pollen tubes. EC1, EC2 and WTEC indicate entire cell homogenates of 179 the pistils from the transgenic plants containing *MIII-FLAG*, the pollen tubes of 180 PhS_VS_V treated with EC1 and the pistils from wild-type PhS_3S_{3L} . S1 and P1, S12 and 181 P12, S160 and P160 indicate respective supernatant and pellet fractions obtained by 182 centrifugation of EC2 at 1,000 g, 12,000 g and 160,000 g. (c) The physical 183 interaction between PhS_{3L}SLF1 and MIII detected by pull-down assay. MBP and 184 185 SUMO-His are protein tags. (d) SFLC assay. nLUC and cLUC indicate transiently expressed N-terminal and C-terminal regions of luciferase. (e) BiFC assay. NE and 186 CE: transiently expressed N-terminal and C-terminal regions of YFP. YFP, BF and 187 Merge: YFP fluorescence, bright field and their merged field, respectively. Bars: 20 188 μm. Annotations of this figure are identical to those of Fig. S4. 189





191 Fig. S16. Petunia hybrida S₃-RNase with mutated region (R) III markedly

192 reduces cross seed sets. (a) Transcripts of transgene (*PhS*₃-*RNase or mutant* (*M*) *III*)

- and native *PhS*₃-*RNase* detected by qRT-PCR. Data are shown as mean \pm S.D. (n =
- 194 3). FLAG, a protein tag. (b) Quantitative analyses of S₃R- and MIII-FLAG proteins
- detected by immunoblots in Fig. S9. Data are shown as mean \pm S.D. (n = 3).

196 Student's *t*-test: ns (not significant), p > 0.05. (c) Reduced seed sets per capsule from

- 197 cross-pollinated T₀ transgenic lines containing *MIII* and their statistical analyses.
- 198 Data are shown as mean \pm S.D. (n = 10). Student's *t*-test: **, p < 0.01. Bars: 5 mm.
- 199 (d) Reduced seed sets per capsule from cross-pollinated T₀ transgenic lines
- 200 containing *MIII-FLAG* and their statistical analyses. Data are shown as mean \pm S.D.
- 201 (n = 10). Student's *t*-test: ns (not significant), p > 0.05; **, p < 0.01. Bars: 5 mm. (e)



- 202 Left: immunoblots of recombinant SUMO-His-MIII in the cell-free degradation
- 203 products incubated with or without MG132 (Mock). PTE: pollen-tube extracts. Right:
- quantitative analyses of the immunoblots. Data are shown as mean \pm S.D. (n = 3). (f)
- 205 Top: immunoblots of MIII-FLAG levels in the cross- (PhS_VS_V) or self- (PhS_3S_3)
- 206 pollen tubes (PT) incubated with or without MG132. Bottom: quantitative analyses
- 207 of the immunoblots. The x-axis represents the transgenic line numbers corresponding
- to those in **d**. Data are shown as mean \pm S.D. (n = 3). Student's *t*-test: **, p < 0.01.
- 209 Annotations of this figure are identical to those of Fig. S10.



Fig. S17. Largely unaltered physicochemical properties of *Petunia hybrida*

212 S₃-RNase with mutated region (R) I, II and III. (a) RNase activity detection of

213 His-Mutant (M) I/II/III expressed by *pCold-TF* vectors. RFU: relative fluorescence

- unit. Data are shown as mean \pm S.D. (n = 3). (b) Immunoblot detection of
- 215 FLAG-tagged MI/II/III in subcellular fractions of *in vitro* germinated pollen tubes.
- EC1, EC2 and WTEC indicate entire cell homogenates of the pistils from the
- 217 transgenic plants containing MI/II/III-FLAG, the pollen tubes of PhS_VS_V treated with
- EC1 and the pistils from wild-type *PhS*₃*S*₃*L*. S1 and P1, S12 and P12, S160 and P160
- 219 indicate supernatant and pellet fractions obtained by centrifugation of EC2 at 1,000 g,
- 12,000 g and 160,000 g, respectively. (c) The physical interaction between
- 221 PhS_{3L}SLF1 and MI/II/III detected by pull-down assay. MBP and SUMO-His are
- 222 protein tags. (d) SFLC assay. nLUC and cLUC: transiently expressed N-terminal and
- 223 C-terminal regions of luciferase. (e) BiFC assay. NE and CE: transiently expressed
- 224 N-terminal and C-terminal regions of YFP. YFP, BF and Merge: YFP fluorescence,
- 225 bright field and their merged field. Bars: 20 μm. Annotations of this figure are
- identical to those of Fig. S4.



- Fig. S18. Reduced seed set per capsule from T₀ transgenic lines with mutated
- 229 region (R) I/II/III of *Petunia hybrida* S₃-RNase. The transgenic plants containing
- 230 *mutant (M) I/II/III* (a) or *MI/II/III-FLAG* (b) were pollinated with cross pollen of
- 231 PhS_VS_V . Numbers below the horizontal lines indicate T₀ transgenic line numbers.
- 232 FLAG: a protein tag. Bars: 5 mm.



233 Table S1. List of primer sequences.

Primers	Sequences (5' to 3')	Purposes	
Chip-F	ggtaccCTAGCTAGAAGATCTCTTTTGT	P1	
Chip-R	ctcgagCCTGGAGATCTAATTTTCTTTT	P1	
S₃R-F	ctcgagATGTTTAGATTACAGCTCATAT	P1	
<i>S₃R</i> -R	gageteTCAACCCCGAAACAGAATCTT	P1	
	gageteCTACTTGTCATCGTCGTCCTTG	D1	
<i>S3R-FLA</i> G-K	TAATCACCCCGAAACAGAATC	PI	
<i>S₃R-K217R</i> -R	gageteTCAACCCCGAAACAGAATCTCT	P1	
C D K217D EL 4C D	gageteCTACTTGTCATCGTCGTCCTTG	D1	
<i>S3R-K21/R-FLA</i> G-К	TAATCACCCCGAAACAGAATTC	PI	
	TGACGAAACATACGCCAACGCC	DO	
<i>1102AK103R</i> -F	AGACAACCTCTCTGGGAGC	P2	
<i>T102 (K102D D</i>	TCTGGCGTTGGCGTATGTTTCGT	P2	
1102AK103R-R	CAAACCTCATTTGAATCCA		
	TCATGGAATTACTCCGGGAGCCA	P2	
<i>1153AK154R</i> -F	GACATACATTTGGTGAAAT		
	TCTGGCTCCCGGAGTAATTCCAT	P2	
<i>1153AK154R</i> -R	GAGTTCTGAGAGTTGTCAA		
	TGACGAAACATACGCCAACGCCA	P2	
1102А-Р	AGCAACCTCTCTGGGA		
	GGCGTTGGCGTATGTTTCGTCAA	P2	
1102А-К	ACCTCATTTGAATCCA		
	AAACATACGCCAACACTAGACAA	D 2	
N1U3K-F	CCTCTCTGGGAGCACG	P2	
VIA2D D	TCTAGTGTTGGCGTATGTTTCGTC		
<i>к103К</i> -к	AAACCTCATTTGAAT	P2	
	TCATGGAATTACTCCGGGAGCCA	D 2	
1133A-F	AGCATACATTTGGTGA	P2	



Table S1 (Continued 1)

Primers	Sequences (5' to 3')	Purposes	
<i>T1524</i> D	GGCTCCCGGAGTAATTCCATGAGT	P2	
1155А-К	TCTGAGAGTTGTCAA		
<i>V</i> 154D E	GAATTACTCCGGGAACAAGACATA	P2	
K134K-F	CATTTGGTGAAATCC		
<i>V</i> 154D D	TCTTGTTCCCGGAGTAATTCCATG	P2	
<i>К134К</i> -К	AGTTCTGAGAGTTGT		
	CAATAGGCATGGAATTTGCGCCAA	DO	
СП8А-Р	AAATCTCTACGATCA	P2	
	GGCGCAAATTCCATGCCTATTGTA	DO	
С118А-К	TTCGTGCTCCCAGAG	P2	
18S rRNA-F	CGGCTACCACATCCAAGGAA	P3	
18S rRNA-R	TGTCACTACCTCCCCGTGTCA	Р3	
<i>S</i> ₃ <i>R</i> -F1	TCGTCTTAACATGGCCAGCA	P3	
<i>S</i> ₃ <i>R</i> -R1	CCAGTGGCGCTCTAGAACAT	P3	
<i>S</i> ₃ <i>R</i> -F2	ACCTCTCTGGGAGCACGAAT	P3	
<i>S</i> ₃ <i>R</i> -R2	TCAAACCTATCTGCCGCTGG	P3	
<i>S</i> ₃ <i>R</i> -F3	ACTCCGGGAACAAAGCATACA	P3	
<i>S</i> ₃ <i>R</i> -R3	AGAATCTTCGTGCTGCTCGT	P3	
<i>S</i> ₃ <i>R</i> -F	gaattcAGTGCGAATTTTGACTACTTCC	P4	
<i>S</i> ₃ <i>R</i> -R	gtcgacTCAACCCCGAAACAGAATCTTC	P4	
<i>S₃R-K217R-</i> R	gtcgacTCAACCCCGAAACAGAATCTCT	P4	
CDF	GGGGACAAGTTTGTACAAAAAAGCAG	D <i>5</i>	
S3K-F	CTCCAGTGCGAATTTTGACTACTTCC	P5	
C D D	GGGGACCACTTTGTACAAGAAAGCTG	D <i>5</i>	
<i>J3K-K</i>	GGTCACCCCGAAACAGAATCTTCGTG	r)	
C D V117D D	GGGGACCACTTTGTACAAGAAAGCTG	2.5	
<i>53К-К21/К-</i> К	GGTCACCCCGAAACAGAATTCTCGTG		



235 Table S1 (Continued 2)

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Primers	Sequences (5' to 3')	Purposes	
	GGGGACAAGTTTGTACAAAAAAGCAG	D5	
S3LSLF I-F	CTCCATGGCGAATGGTATTTTAAAGA	P3	
C CLEID	GGGGACCACTTTGTACAAGAAAGCTG	P5	
S3LSLF I-K	GGTCAAATTTTTGTACTTTTGTACTG		
S3R-F	ggatccAGTGCGAATTTTGACTACTTCC	P6	
S ₃ R-R	ctcgagTCAACCCCGAAACAGAATCTTC	P6	
<i>S₃R-K217R-</i> R	ctcgagTCAACCCCGAAACAGAATTCT	P6	
$S_{3L}SLF1$ -F	ggatccATGGCGAATGGTATTTTAAAGA	P6	
S _{3L} SLF1-R	ctcgagCTAAAATTTTTGTACTTTTGTA	P6	
CDE	GGGGACAAGTTTGTACAAAAAAGCAG	D 7	
53K-F	GCTCCAGTGCGAATTTTGACTACTTCC	Ρ/	
C D D	GGGGACCACTTTGTACAAGAAAGCTG	D7	
53 <i>K</i> -K	GGTCACCCCGAAACAGAATCTTCGTG		
	GGGGACCACTTTGTACAAGAAAGCTG		
<i>З3К-К21/К-</i> К	GGTCACCCCGAAACAGAATTCTCGTG	Υ/	

236 F: forward primer; R: reverse primer; P1: *pBI101* construction; P2: *Petunia hybrida*

237 S_3R mutagenesis; T and A: threonine and its mutant form alanine; K and R: lysine

- and its mutant form arginine; P3: qRT-PCR; P4: *pMAL-c2x* construction; P5: BiFC
- or SFLC; P6: *pET30a* construction; P7: *pCold-TF* construction.



Names	Accession numbers ^(a)
Petunia hybrida S ₃ -RNase	U07363
Petunia hybrida S _{3L} -RNase	AJ271065
Petunia hybrida Sv-RNase	AJ271062
Pyrus bretschneideri S7-RNase	XM_009350009
Petunia inflata S ₂ -RNase	AAG21384
Petunia inflata S ₃ -RNase	AAA33727
Petunia inflata Sk1-RNase	BAE73275
Petunia inflata S ₁ -RNase	AAA33726
Solanum lycopersicum S3-RNase	XP_004229063
Nicotiana alata SA2-RNase	AAA87045
Petunia hybrida S _X -RNase	AAA33729
Petunia axillaris S _{C2} -RNase	AAN76454
Solanum tuberosum S2-RNase	Q01796
Petunia hybrida S _{B2} -RNase	BAA76514
Solanum neorickii S-RNase	BAC00940
Solanum habrochaites S2-RNase	AIG62995
Solanum chilense S ₁ -RNase	BAC00934
Solanum chacoense S ₁₁ -RNase	AAA50306
Solanum chacoense S14-RNase	AAF36980
Nicotiana alata S7-RNase	Q40381
Petunia axillaris S _{C1} -RNase	AAN76453
Petunia hybrida S11-RNase	BAJ24848
Petunia hybrida S7-RNase	BAJ24847
Petunia axillaris S ₁ -RNase	AAK15435
Solanum habrochaites S ₄ -RNase	AIG62997
Petunia hybrida S ₁ -RNase	AAA60465
Petunia hybrida S _{B1} -RNase	BAA76513

240 Table S2. Names and accession numbers of S-RNases used in this study.



Table S2 (Continued) 241

 Names	Accession numbers ^(a)
 Solanum peruvianum S22-RNase	BAC00930
Solanum peruvianum S12-RNase	AAA77040
Solanum habrochaites S ₁ -RNase	AIG62994
Petunia hybrida S ₀ -RNase	ACT35737
Solanum chacoense S ₁₂ -RNase	AAD56217
Nicotiana alata S ₆ -RNase	AAB40028
Solanum peruvianum S ₃ -RNase	CAA53666
Solanum peruvianum S ₁₁ -RNase	AAA77039
Nicotiana alata S ₂ -RNase	P04007
Petunia axillaris S ₁₃ -RNase	AAK15436

(a) Accession numbers are from National Center for Biotechnology Information 242

(NCBI) protein database (https://www.ncbi.nlm.nih.gov/). 243



Table S3. Seed sets of S_3S_{3L}/S_3R and S_3S_{3L}/S_3R (*Mutant*) (*M*) T₀ transgenic plants

245 of *Petunia hybrida*.

	Genotype of pollen donor		
T ₀ transgenic lines ^(a)	$S_{3}S_{3L}^{(b)}$	$S_V S_V^{(c)}$	Seed set ^(d)
	(Self)	(Cross)	
S_3S_{3L}/S_3R -60	0/5	10/10	398 ± 37
<i>S</i> ₃ <i>S</i> _{3<i>L</i>} / <i>MI</i> -28	0/5	10/10	332 ± 51
<i>S₃S_{3L}/MI</i> -63	0/5	10/10	268 ± 78
S_3S_{3L}/MI -50	0/5	10/10	259 ± 101
<i>S₃S_{3L}/MII-</i> 61	0/5	10/10	89 ± 19
<i>S₃S_{3L}/MII-</i> 69	0/5	9/10	59 ± 6
S_3S_{3L}/MK -64	0/5	10/10	207 ± 28
$S_{3}S_{3L}/MT$ -53	0/5	10/10	356 ± 16
$S_3S_{3L}/MIII$ -84	0/5	10/10	160 ± 14
S ₃ S _{3L} /MI/II/III-60	0/5	10/10	207 ± 32
<i>S₃S_{3L}/MI/II/III-</i> 45	0/5	10/10	186 ± 17

(a) Numbers of T₀ transgenic plants containing S_3R and its mutant forms (wild type/transgene-line number). I, II and III indicate three ubiquitinated regions of PhS₃-RNase. K and T indicate lysine and threonine within six ubiquitinated residues of PhS₃-RNase. (b) Numbers represent those of mature capsules/pistils pollinated by self pollen of S_3S_{3L} . (c) Numbers represent those of mature capsules/pistils pollinated by cross pollen of S_VS_V . (d) Data are shown as average seed set per capsule \pm S.D., n $252 \ge 9$.



253 Table S4. Seed sets of S₃S_{3L}/S₃R-FLAG and S₃S_{3L}/S₃R (Mutant) (M)-FLAG T₀

254 transgenic plants of *Petunia hybrida*.

	Genotype of pollen donor			
0 transgenic lines ^(a)	$S_{3}S_{3L}$ (b)	$S_V S_V^{(c)}$	Seed sets ^(d)	
	(Self)	(Cross)		
S_3S_{3L}/S_3R -FLAG-34	0/5	10/10	421 ± 25	
S_3S_{3L}/MI -FLAG-24	0/5	10/10	292 ± 49	
S ₃ S _{3L} /MII-FLAG-8	0/5	10/10	143 ± 14	
S_3S_{3L}/MII -FLAG-17	0/5	10/10	104 ± 19	
S_3S_{3L}/MII -FLAG-68	0/5	10/10	93 ± 25	
S_3S_{3L}/MK -FLAG-16	0/5	10/10	113 ± 17	
S_3S_{3L}/MT -FLAG-44	0/5	10/10	334 ± 16	
S ₃ S _{3L} /MIII-FLAG-18	0/5	10/10	261 ± 37	
S ₃ S _{3L} /MI/II/III-FLAG-49	0/5	10/10	93 ± 34	

255 (a) Numbers of T_0 transgenic plants containing *FLAG*-tagged S_3R and its mutant

256 forms (wild type/transgene-line number). (**b**, **c**) Annotations are identical to those of

Table S3. (d) Data are shown as average seed set per capsule \pm S.D., n = 10.