Plant Biotechnology 30, 119–123 (2013)
DOI: 10.5511/plantbiotechnology.13.0109a

Original Paper

Identification of an S-RNase binding protein1 (SBP1) homolog of apple (*Malus*×*domestica*)

Mai F. Minamikawa[1,†], Daisuke Fujii[1,†], Hiroyuki Kakui[1], Nobuhiro Kotoda[2], Hidenori Sassa[1,*]

[1]Graduate School of Horticulture, Chiba University, Matsudo, Chiba 271-8510, Japan; [2]Apple Breeding and Physiology Research Team, National Institute of Fruit Tree Science, Morioka, Iwate 020-0123, Japan
*E-mail: sassa@faculty.chiba-u.jp Tel: +81-47-308-8967 Fax: +81-47-308-8720

Received December 12, 2012; accepted January 9, 2013 (Edited by S. Nakano)

Abstract Many flowering plants exhibit self-incompatibility (SI) to prevent inbreeding and promote outcrossing. This self/non-self discrimination mechanism is controlled by the *S* locus, which contains separate genes for pistil and pollen specificities. In the gametophytic SI (GSI) of Rosaceae, Solanaceae and Plantaginaceae, the pistil *S* determinant, *S-RNase*, encodes extracellular ribonuclease which is thought to act as a cytotoxin to the self pollen tube, while the pollen *S* determinant is the F-box gene called *SLF*/*SFB*/*SFBB*. In *Petunia* (Solanaceae), SLF is reported to be a component of the noncanonical E3 ubiquitin ligase complex with S-RNase binding protein1 (SBP1) and Cullin1 (CUL1), and interact with non-self S-RNases to ubiquitinate them for degradation. Here, we isolated an apple (*Malus*×*domestica*) homolog of SBP1 (MdSBP1) from pollen RNA by RT-PCR. MdSBP1 included a RING-HC domain required for E3 ubiquitin ligase activity, and showed 64.0–68.2% amino acid identities with solanaceous SBP1 proteins. Expression analysis showed that MdSBP1 was expressed in all the organs analyzed. We detected an interaction between recombinant MdSBP1 protein and S-RNase of apple using a pull-down assay.

Key words: Self-incompatibility, S-RNase binding protein, pollen, apple, Rosaceae.

Self-incompatibility (SI) is a mechanism adopted by many flowering plants to prevent inbreeding and promote outcrossing. The S-RNase-based gametophytic self-incompatibility (GSI) of Rosaceae, Solanaceae and Plantaginaceae is controlled by a single multiallelic *S* locus which contains separate genes for pistil and pollen specificities. When the *S* haplotype of a pollen matches one of the two *S* haplotypes of the diploid pistil, the pollen is recognized as self and rejected (de Nettancourt 2001). The specificities of pistil and pollen are controlled by separate but tightly linked genes located at the *S* locus, *S-RNase* and the F-box gene called *SLF*/*SFB*/*SFBB*, respectively (Franklin-Tong 2008; Kao and Tsukamoto 2004; Meng et al. 2011; Sassa et al. 2010; Tao and Iezzoni 2010). The pistil *S* determinant, *S-RNase*, encodes extracellular ribonuclease (Anderson et al. 1986; McClure et al. 1989; Sassa et al. 1996, 1997; Tao et al. 1997; Xue et al. 1996) which is thought to be taken up by pollen tubes and act as a cytotoxin to self pollen (Goldraij et al. 2006; Luu et al. 2000). In *Petunia* of Solanaceae and species of Rosaceae tribe Pyreae, i.e., apples (*Malus*×*domestica*) and pears (*Pyrus* spp.), multiple F-box genes *SLF*s/*SFBB*s are implicated in pollen-part specificity (De Franceschi et al. 2011; Kubo et al. 2010; Kakui et al. 2011; Minamikawa et al. 2010; Saito et al. 2012; Sassa et al. 2007).

SLF has been predicted to act as a component of the E3 ubiquitin ligase complex and interact with non-self S-RNases to ubiquitinate them for degradation (Huang et al. 2006; Qiao et al. 2004a, 2004b; Sijacic et al. 2004). Canonical E3 complex comprises Skp1, Cullin1, F-box protein and Rbx1 (Cardozo and Pagano 2004). In *Petunia inflata*, however, SLF-containing E3 ubiquitin ligase is reported to be a noncanonical SCF-like complex which includes S-RNase binding protein1 (SBP1) in place of Skp1 and Rbx1 (Hua and Kao 2006; Hua et al. 2008). SBP1 contained a RING-HC domain found in Rbx1 (Deshaies and Joazeiro 2009) and was considered to play the roles of Skp1 and Rbx1, binding of F-box protein and ubiquitin activating enzyme E2, respectively (Hua and Kao 2006). SBP1 was first identified as an S-RNase binding protein in *Petunia hybrida* by yeast two-hybrid screening of an anther cDNA library (Sims and Ordanic 2001). The petunia SBP1 included a RING-

Abbreviations: GSI, Gametophytic self-incompatibility; RNase, ribonuclease; SBP1, S-RNase binding protein1; MBP, maltose binding protein.
[†]These authors contributed equally to this work.
This article can be found at http://www.jspcmb.jp/
Published online May 16, 2013

HC domain and interacted with S-RNase, and showed no *S* haplotype-specific sequence polymorphism. SBP1 homologs have been identified in *Solanum chacoense* (O'Brien et al. 2004) and *Nicotiana alata* (Lee et al. 2008); however, SBP1-like protein has not yet been characterized outside Solanaceae. Here, we isolated an apple homolog of *SBP1* from pollen RNA and named it *MdSBP1*. MdSBP1 included a RING-HC domain and was expressed in all the organs analyzed. An *in vitro* binding assay showed interaction between MdSBP1 and S-RNase.

Materials and methods

Plant materials

Leaves and floral organs of an apple cultivar 'Fuji' (S^1S^9) were collected in spring and stored at −80°C until used.

RACE and sequence analysis

RNA was isolated from the leaves and the floral organs of 'Fuji' (S^1S^9) as described by McClure et al. (1990). Total RNA samples were treated with DNaseI (Nippongene, Tokyo, Japan), and used for RACE and RT-PCR as described by Ushijima et al. (2003). A partial EST sequence homologous to solanaceous *SBP1* was selected from apple EST libraries (Moriya et al. 2012). A gene-specific primer MdSBP1-5RACEr1 (5′-CAG GAA ATC AAT GGA CGA TAT T-3′) was designed based on the EST sequence, and used for 5′RACE with pollen cDNA of 'Fuji' (S^1S^9) as a template. A primer MSB5URHS (5′-AGT TGT GCC TTT CAC ACA AGC -3′) was designed from the sequences of the 5′RACE clones, and used to amplify full length cDNA for the apple *SBP1* homolog from pollen cDNA of 'Fuji' (S^1S^9) by 3′RACE.

The amino acid identities among SBP1 proteins were analyzed by GENETYX-MAC (version 16; Genetyx, Tokyo, Japan). The amino acid sequences of SBP1 proteins were aligned using Clustal W (Thompson et al. 1994). A neighbor-joining tree was constructed (Saitou and Nei 1987) based on the alignment using MEGA ver. 5.05. (Tamura et al. 2011).

RT-PCR

Expression of *MdSBP1* was analyzed by RT-PCR with gene-specific primers ctMdSBP1-a (5′-CTA TGG CTG TTC CCC AGC ACC -3′) and MdSBP1Fjr1 (5′-TTT ATA TGA TGT ATG GCT TTG AAT -3′). *Actin* 2 (GenBank accession number GU830959) used as control was amplified using primers PbActin2f1 (5′-ATG GCC GAT GCT GAG GAC ATT CAA CCC CTCG-3′) and PbActin2r1 (5′-ATT GGC ACA GTG TGA CTC ACA CCA TCA CCAG-3′).

Production of antiserum against apple S-RNase

A cDNA clone of *MdS*^{9}*-RNase* (synonym of *S*^{c}*-RNase*; Sassa et al. 1996) was amplified by PCR using FMdSpRNNd (5′-CAT ATG TAC GAT TAT TTT CAA TTT ACG -3′) and RMdS9RNSal (5′-GTC GAC ATA CAG AAT ATT ATT GGT GGG -3′), cloned to the *Eco*RV site of pZErO-2 (Invitrogen, Carlsbad, CA, USA), and sequence-verified. The *Nde*I-*Sal*I fragment of *MdS*^{9}*-RNase* and a *Sal*I–*Bam*HI fragment of the coding sequence of StrepII tag (WSHPQFEK) were cloned into *Nde*I and *Bam*HI sites of pET15b (Novagen, Madison, WI, USA). The construct was introduced into *Escherichia coli* strain SHuffle T7 (New England BioLabs, Beverly, MA, USA). The recombinant MdS^9-RNase protein was expressed in *E. coli*, solubilized from inclusion bodies with a buffer containing 8 M urea, and purified by HisTrap FF Crude column (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The purified MdS^9-RNase protein was dialyzed against a buffer containing 0.5 M Arg-HCl to remove urea, and used to immunize a rabbit to obtain antiserum.

Pull-down assay

The open reading frame of *MdSBP1* was amplified by PCR using primers XbMdSBP1 (5′-CCG CTC GAG GAT CCA TGG CTG TTC CCC AGCACC-3′) and RMdSBP1Xb (5′-GCT CTA GAT TAC AAA TAT ACC TCC ATG CTG ATA AAC -3′). The *MdSBP1* fragment was then cloned into pColdIIMBP (Heang and Sassa 2012) at *Bam*HI and *Xba*I sites for expression of maltose binding protein (MBP)-fused MdSBP1 protein (MBP: MdSBP1). pColdIIMBP is a derivative of pCold II (Takara Bio, Otsu, Japan) and contains the coding sequence of MBP. The construct was introduced into BL21 (DE3) pLysS (Novagen). pColdIIMBP was also transferred to BL21 (DE3) pLysS for expression of MBP as a negative control of the pull-down assay. Expression of MBP: MdSBP1 and MBP proteins were induced by addition of 0.5 mM isopropyl-β-D-thio-galactopyranoside (IPTG) (Wako) and culture at 15°C for 24 h. Crude proteins were extracted from bacteria suspended in a binding buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA and 1 mM DTT) by sonication followed by centrifugation, and reacted with amylose resin (New England BioLabs). Style proteins of the apple cultivar 'Fuji' (S^1S^9) were extracted with an extraction buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, 0.3% β-mercaptoethanol), and incubated with the protein-bound amylose resin at 4°C for 2 h. The beads were then washed five times with a washing buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT and 0.01% Triton X-100). Bound proteins were eluted from the beads by heating at 65°C for 5 min in 30 μl of 2×SDS loading buffer (100 mM Tris-HCl pH 6.8, 4% SDS, 12% β-mercaptoethanol and 20% glycerol), and separated by SDS-PAGE with a 13% gel and detected by anti-apple S^9-RNase antiserum.

Results and discussion

Identification of the apple SBP1 homolog

To isolate the apple *SBP1* homolog, we searched the apple EST libraries (Moriya et al. 2012) and selected a partial cDNA sequence homologous to solanaceous *SBP1*. Based on the sequence, primers were designed for RT-PCR. We obtained an *SBP1* homolog from apple pollen RNA and named it *MdSBP1* (Figures 1, 2). The amino acid identities among SBP1 proteins were 64.0–

```
MdSBP1      1 MAVPQHHFQQHYPPQ-QQQQQSKSFRNLYT-IDGQMSPAVAYYDPGNLHDHPQQQHPPYVPPFHVVGFAPGPVAATDGSDNCADLQCHYG     88
NaSBP1      1 MALPHHHLQLHSIQQ--QQQQSNSFRDIYNNMDGQISTPVAYFNGSNLPEQSQ--HPPYIPAFQVVGLAPGPA-----DEGGLDLQWNYG     81
PiSBP1      1 MALPHHHLQLHIQQQ--PQQQSKSYRDIYNNMDGQISTPVAYFNGSNLPEQSQ--HPPYIPPFQVVGLAPGLV-----DDGGLDLQWNYG     81
PhSBP1      1 ---GTSHLQLHIQQQ--PQQQSKSYRDIYNNMDGQISTPVAYFNCSNLPEQSQ--HPPYIPPFQVVGLAPGLV-----DDGGLDLQWNYG     78
ScSBP1      1 MALPHHHLQLHIQQQQPHQQQSKSYRDLYNNMDGQITTPVVYFNGSNLPEQSQ--HPPYIPPFQVVGLAPGTA-----DDGGLDLQWNYG     83
              ......*.*.*...*   ****.*.*..*...***....*.*....**....*  ****.*.*.***.*** .     .....***..**

MdSBP1     89 FESKRKKLKEQDFLENN-SQISSIDFLQPQSVSTGLGLSLDNTRMASTGDSSLLSLISDDIDHELQRQDAEIDRFLKAQGDRLRQTIMDK    177
NaSBP1     82 LEPKKRRLKEQDFLENNNSQISSVDFLQQRSVSTGLGLSLDNGRLGSCGDSAFLGLVGDDIERELQRQDADIDRYIKVQGDRLRQAILEK    171
PiSBP1     82 LEPKRKRPKEQDFLENNNSQISSIDFLQPRSVSTGLGLSLDNGRLASSGDSAFLGLVGDDIERELQRQDAEIDRYIKVQGDRLRQAILEK    171
PhSBP1     79 LEPKRKRPKEQDFLENNNSQISSIDFLQPRSVSTGLGLSLDNGRLASSGDSAFLGLVGDDIERELQRQDAEIDRYIKVQGDRLRQAILEK    168
ScSBP1     84 LEPKKKRPKEQDFMENNNSQISSVDLFQRRSVSTGLGLSLDNGRLASSCDSAFLGLVGDDIERELQRQDAEIDRYIKVQGDRLRQAVLEK    173
              .*.*....*****.***.*****.*..*..************.*..*..**..*.*..***..*******.***..*.*******....*

MdSBP1    178 VQATQLQTLSAVEDKVHRKLREKEAEVESINEKNMELEERMEQLTVEAGAWQQLARHNENMISTLRFNLQHIYAQSRDSKEGCGDSEVDD    267
NaSBP1    172 VQANQLQTITCVEEKVIQKLREKEAEVEDINKKNMELELRMEQLALEANAWQQRAKYNENLINTLKVNLQHVYAQSRDSKEGCGDSEVDD    261
PiSBP1    172 VQANQLQTVTYVEEKVIQKLREKETEVEDINKKNMELELRTEQLALEANAWQQRAKYNENLINTLKVNLQHVYAQSRDSKEGCGDSEVDD    261
PhSBP1    169 VQANQLQTVTYVEEKVIQKLREKETEVEDINKKNMELELRTEQLALEANAWQQRAKYNENLINTLKVNLEHVYAQSRDSKEGCGDSEVDD    258
ScSBP1    174 VQANQIQAITYVEEKVLQKLRERDTEVDDINKKNMELELRMEQLDLEANAWQQRAKYNENLINTLKVNLQHVYAQSRDSKEGCGDSEVDD    263
              ***.*.*. ..**.**..****...**..**.******.*.***..**.****.*..***.*.**..**.*.*****************

MdSBP1    268 TASCCNGRSINLEMFCKENNDGKEMMTCKACRVNEVCMLLLPCKHLCLCKDCESKLSICPLCQSSKFISMEVYL    341
NaSBP1    262 TASCCNGRATDFHLLCRDSNEMKELMTCKVCRVNEVCMLLLPCKHLCLCKECESKLSLCPLCQSTKYIGMEVYV    335
PiSBP1    262 TASCCNGRATDLHLLCRDSNEMKELMTCKVCRVNEVSMLLLPCKHLCLCKECESKLSLCPLCQSTKYIGMEIYM    335
PhSBP1    259 TASCCNGRATDLHLLCRDSNEMKELMTCKVCRVNEVSMLLLPCKHLCLCKECESKLSLCPLCQSTKYIGMEIYM    332
ScSBP1    264 TASCCNGRATDLHLLCRDSKEMKELMTCRVCRTNEVCMLLLPCKHLCLCKECESKLSLCPLCQSTKYIGMEVYM    337
              ********.......*......**.***..**.***.************.******.******.*.*.**.*.
```

Figure 1. Amino acid sequence alignment of MdSBP1 and other SBP1 homologs of Solanaceae. Amino acid sequences were aligned using Clustal W. Conserved sites and relatively conservative sites are marked with asterisks and dots, respectively. The RING-HC finger motif detected by Pfam (http://pfam.sanger.ac.uk) is denoted by a box. The triangles indicate the cysteine-histidine signature of the motif. The underline indicates the predicted coiled-coil region detected by Pfam. Md, *Malus×domestica*; Na, *Nicotiana alata*; Pi, *Petunia inflata*; Ph, *Petunia hybrida*; Sc, *Solanum chacoense*. Accession numbers: NaSBP1 (EU591514), PiSBP1 (DQ250022), PhSBP1 (AF223395), ScSBP1 (AY545464)

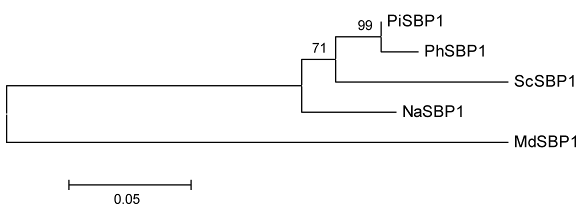

Figure 2. Neighbor-joining tree of SBP1 proteins. The tree was constructed based on the aligned deduced amino acid sequences of SBP1 proteins (Figure 1). The numbers at the nodes are bootstrap values for 100 bootstrap resamplings.

68.2% (Table 1). Sequence analysis showed that MdSBP1 included two probable protein–protein interaction domains, coiled–coil and RING-HC domains (Figure 1), the same as solanaceous SBP1 proteins. The coiled–coil region of petunia SBP1 was reported to be required for binding to SLF (Hua and Kao 2006), while the RING-HC domain is thought to be required for E3 ubiquitin ligase activity (Deshaies and Joazeiro 2009). These features are consistent with the idea that MdSBP1 is a component of noncanonical E3 ligase, and is involved in ubiquitinylation of S-RNase in apple pollen, as hypothesized in *P. inflata* (Hua and Kao 2006).

SBP1 of *Nicotiana alata*, NaSBP1, was reported to interact with the C-terminal domain of pistil arabinogalactan proteins (AGPs), transmitting tract-specific glycoprotein (TTS) and 120-kDa glycoprotein (120K), by yeast two-hybrid assay and *in vitro* binding assay (Lee et al. 2008). An *in vitro* binding assay also showed that the helical and RING domain of NaSBP1 were sufficient for binding to TTS and 120K. Lee et al.

Table 1. Amino acid sequence identities (%) among MdSBP1 and other SBP1 homologs of Solanaceae.

	MdSBP1	NaSBP1	PiSBP1	PhSBP1
NaSBP1	67.6	—		
PiSBP1	68.2	92.5	—	
PhSBP1	68.0	91.8	99.4	—
ScSBP1	64.0	89.0	91.4	90.6

(2008) suggested that binding between NaSBP1 and the pistil AGPs may contribute to signaling and trafficking processes inside pollen tubes. Thus, MdSBP1 may also be involved in the processes inside pollen tubes, although TTS and 120K-like proteins have not yet been identified in styles of apple.

Expression pattern of MdSBP1

RT-PCR analysis revealed that *MdSBP1* was expressed in all the organs analyzed (Figure 3), the same as solanaceous *SBP1* homologs (Hua and Kao 2006; Lee et al. 2008; O'Brien et al. 2004; Sim and Ordanic 2001). The expression pattern suggests that *MdSBP1* is involved in general cellular function besides a possible role in pollination.

Interaction of MdSBP1 with S-RNase

We examined the interaction between MdSBP1 and S-RNase using an *in vitro* binding assay. MBP-fused MdSBP1 (MBP: MdSBP1) and MBP (negative control) proteins were expressed in *E. coli* and reacted with amylose resin. The recombinant protein-bound beads were then incubated with the apple style extract.

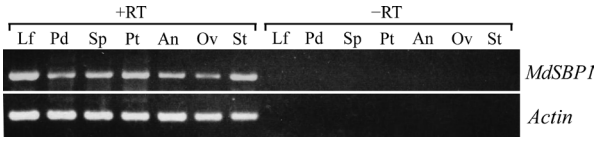

Figure 3. RT-PCR analysis of *MdSBP1* expression. *MdSBP1* was amplified by RT-PCR. Amplification consisted of 35 or 25 cycles for *MdSBP1* and *Actin*, respectively. RT, Reverse Transcriptase; Lf, Leaf; Pd, pedicel; Sp, sepal; Pt, petal; An, Anther; Ov, Ovary; St, style.

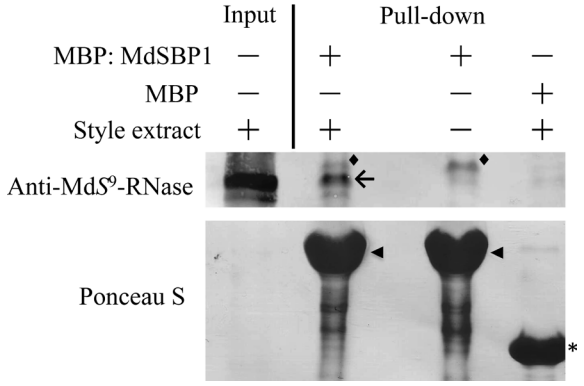

Figure 4. *In vitro* binding assay for MdSBP1 and S-RNase. MBP: MdSBP1 or MBP (negative control) were expressed in *E. coli* and reacted with amylose resin. The recombinant proteins-bound beads were incubated with the style extract. Bound proteins were separated by SDS-PAGE and detected by anti-MdS^9-RNase antiserum (top). Approximately equal recombinant protein loading was checked by Ponceau-S staining of the blot before immunological detection (bottom). The triangle and the asterisk indicate MBP: MdSBP1 and MBP, respectively. The arrow and diamond indicate specific and non-specific signals, respectively.

Bound proteins were separated by SDS-PAGE and detected by anti-apple S^9-RNase antiserum. The result showed that MdSBP1 interacts with S-RNase (Figure 4). Given that MdSBP1 is homologous to solanaceous SBP1, includes RING-HC domain and interacts with S-RNase, biochemical function of MdSBP1 may be similar to that of solanaceous SBP1 proteins. Although the biological significance of the interaction between SBP1 and S-RNase is not clear even in Solanaceae, it was suggested that SBP1 of *Petunia hybrida*, PhSBP1, could be a candidate for the non-allele-specific inhibitor of all S-RNase since it was expressed in pollen and showed no polymorphism in different *S* alleles (Hua et al. 2008; Sims and Ordanic 2001). The RING finger protein was also reported to contribute to ubiquitination specificity (Deshaies and Joazeiro 2009; Noureddine et al. 2002; O'Brien et al. 2004). Biological implication of the interaction between MdSBP1 and S-RNase, and interactors with MdSBP1 other than S-RNase should be experimentally analyzed to clarify the role of MdSBP1.

Acknowledgements

This work was supported by a Grants-in-Aid for Scientific Research (B, 24380004) from the Ministry of Education, Science, Sports and Culture of Japan. M. F. M and H. K. were supported by JSPS Research Fellowship for Young Scientists.

References

Anderson MA, Cornish EC, Mau S-L, Williams EG, Hoggart R, Atkinson A, Bönig I, Grego B, Simpson R, Roche PJ, et al. (1986) Cloning of cDNA for a stylar glycoprotein associated with expression of self-incompatibility in *Nicotiana alata*. *Nature* 321: 38–44

Cardozo T, Pagano M (2004) The SCF ubiquitin ligase: insights into a molecular machine. *Nat Rev Mol Cell Biol* 5: 739–751

De Franceschi P, Pierantoni L, Dondini L, Grandi M, Sanzol J, Sansavini S (2011) Cloning and mapping multiple S-locus F-box genes in European pear (*Pyrus communis* L.). *Tree Genet Genomes* 7: 231–240

de Nettancourt D(2001) Incompatibility and incongruity in wild and cultivated plants. Springer, Berlin

Deshaies RJ, Joazeiro CAP (2009) RING domain E3 ubiquitin ligases. *Annu Rev Biochem* 78: 399–434

Franklin-Tong VE(2008) Self-incompatibility in flowering plants—evolution, diversity, and mechanisms. Springer, Berlin

Goldraij A, Kondo K, Lee CB, Hancock CN, Sivaguru M, Vazquez-Santana S, Kim S, Phillips TE, Cruz-Garcia F, McClure B (2006) Compartmentalization of S-RNase and HT-B degradation in self-incompatible *Nicotiana*. *Nature* 439: 805–810

Heang D, Sassa H (2012) Antagonistic actions of HLH/bHLH proteins are involved in grain length and weight in rice. *PLoS ONE* 7: e31325

Hua Z, Kao TH (2006) Identification and characterization of components of a putative *petunia S*-locus F-box-containing E3 ligase complex involved in S-RNase-based self-incompatibility. *Plant Cell* 18: 2531–2553

Hua Z-H, Fields A, Kao TH (2008) Biochemical models for S-RNase-based self-incompatibility. *Mol Plant* 1: 575–585

Huang J, Zhao L, Yang Q, Xue Y (2006) AhSSK1, a novel SKP1-like protein that interacts with the S-locus F-box protein SLF. *Plant J* 46: 780–793

Kakui H, Kato M, Ushijima K, Kitaguchi M, Kato S, Sassa H (2011) Sequence divergence and loss-of-function phenotypes of *S locus F-box brothers* genes are consistent with non-self recognition by multiple pollen determinants in self-incompatibility of Japanese pear (*Pyrus pyrifolia*). *Plant J* 68: 1028–1038

Kao TH, Tsukamoto T (2004) The molecular and genetic bases of S-RNase-based self-incompatibility. *Plant Cell* 16(Suppl): S72–S83

Kubo K, Entani T, Takara A, Wang N, Fields AM, Hua Z, Toyoda M, Kawashima S, Ando T, Isogai A, et al. (2010) Collaborative non-self recognition system in S-RNase-based self-incompatibility. *Science* 330: 796–799

Lee CB, Swatek KN, McClure B (2008) Pollen proteins bind to the C-terminal domain of *Nicotiana alata* pistil arabinogalactan proteins. *J Biol Chem* 283: 26965–26973

Luu DT, Qin X, Morse D, Cappadocia M (2000) S-RNase uptake by compatible pollen tubes in gametophytic self-incompatibility. *Nature* 407: 649–651

McClure BA, Haring V, Ebert PR, Anderson MA, Simpson RJ, Sakiyama F, Clarke AE (1989) Style self-incompatibility gene products of *Nicotiana alata* are ribonucleases. *Nature* 342: 955–957

McClure BA, Gray JE, Anderson MA, Clarke AE (1990) Self-

incompatibility in *Nicotiana alata* involves degradation of pollen rRNA. *Nature* 347: 757–760

Meng X, Sun P, Kao TH (2011) S-RNase-based self-incompatibility in *Petunia inflata*. *Ann Bot (Lond)* 108: 637–646

Minamikawa M, Kakui H, Wang S, Kotoda N, Kikuchi S, Koba T, Sassa H (2010) Apple *S* locus region represents a large cluster of related, polymorphic and pollen-specific F-box genes. *Plant Mol Biol* 74: 143–154

Moriya S, Iwanami H, Kotoda N, Haji T, Okada K, Terakami S, Mimida N, Yamamoto T, Abe K (2012) Aligned genetic linkage maps of apple rootstock cultivar 'JM7' and *Malus sieboldii* 'Sanashi 63' constructed with novel EST-SSRs. *Tree Genet Genomes* 8: 709–723

Noureddine MA, Donaldson TD, Thacker SA, Duronio RJ (2002) Drosophila Roc1a encodes a RING-H2 protein with a unique function in processing the Hh signal transducer Ci by the SCF E3 ubiquitin ligase. *Dev Cell* 2: 757–770

O'Brien M, Major G, Chantha S, Matton DP (2004) Isolation of S-RNase binding proteins from *Solanum chacoense*: Identification of an SBP1 (RING finger protein) orthologue. *Sex Plant Reprod* 17: 81–88

Qiao H, Wang H, Zhao L, Zhou J, Huang J, Zhang Y, Xue Y (2004a) The F-box protein AhSLF-S2 physically interacts with S-RNases that may be inhibited by the ubiquitin/26S proteasome pathway of protein degradation during compatible pollination in Antirrhinum. *Plant Cell* 16: 582–595

Qiao H, Wang F, Zhao L, Zhou J, Lai Z, Zhang Y, Robbins TP, Xue Y (2004b) The F-box protein AhSLF-S2 controls the pollen function of S-RNase-based self-incompatibility. *Plant Cell* 16: 2307–2322

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4: 406–425

Saito T, Sato Y, Sawamura Y, Shoda M, Takasaki-Yasuda T, Kotobuki K (2012) Dual recognition of S_1 and S_4 pistils by S_4^{sm} pollen in self-incompatibility of Japanese pear (*Pyrus pyrifolia* Nakai). *Tree Genet Genomes* 8: 689–694

Sassa H, Hirano H, Nishino T, Koba T (1997) Style-specific self-compatible mutation caused by deletion of the S-RNase gene in Japanese pear (*Pyrus serotina*). *Plant J* 12: 223–227

Sassa H, Kakui H, Minamikawa M (2010) Pollen-expressed F-box gene family and mechanism of S-RNase-based gametophytic self-incompatibility (GSI) in Rosaceae. *Sex Plant Reprod* 23: 39–43

Sassa H, Kakui H, Miyamoto M, Suzuki Y, Hanada T, Ushijima K, Kusaba M, Hirano H, Koba T (2007) *S locus F-box brothers*: multiple and pollen-specific F-box genes with *S* haplotype-specific polymorphisms in apple and Japanese pear. *Genetics* 175: 1869–1881

Sassa H, Nishio T, Kowyama Y, Hirano H, Koba T, Ikehashi H (1996) Self-incompatibility (*S*) alleles of the Rosaceae encode members of a distinct class of the T2/S ribonuclease superfamily. *Mol Gen Genet* 250: 547–557

Sijacic P, Wang X, Skirpan AL, Wang Y, Dowd PE, McCubbin AG, Huang S, Kao TH (2004) Identification of the pollen determinant of S-RNase-mediated self-incompatibility. *Nature* 429: 302–305

Sims TL, Ordanic M (2001) Identification of a S-ribonuclease-binding protein in *Petunia hybrida*. *Plant Mol Biol* 47: 771–783

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28: 2731–2739

Tao R, Iezzoni AF (2010) The S-RNase-based gametophytic self-incompatibility system in *Prunus* exhibits distinct genetic and molecular features. *Sci Hortic (Amsterdam)* 124: 423–433

Tao R, Yamane H, Sassa H, Mori H, Gradziel TM, Dandekar AM, Sugiura A (1997) Identification of stylar RNases associated with gametophytic self-incompatibility in almond (*Prunus dulcis*). *Plant Cell Physiol* 38: 304–311

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22: 4673–4680

Ushijima K, Sassa H, Dandekar AM, Gradziel TM, Tao R, Hirano H (2003) Structural and transcriptional analysis of the self-incompatibility locus of almond: identification of a pollen-expressed F-box gene with haplotype-specific polymorphism. *Plant Cell* 15: 771–781

Xue Y, Carpenter R, Dickinson HG, Coen ES (1996) Origin of allelic diversity in antirrhinum *S* locus RNases. *Plant Cell* 8: 805–814