Genome-Wide Analysis of Almond Skp1 Family Members And Identification of Self-Incompatibility-Related PsdSSK1

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Genome-wide analysis of almond *Skp1* family members and identification of self-incompatibility-related *PsdSSK1*

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Abstract: S-phase kinase-associated protein 1 (*Skp1*) is a component of the SKP1-Cullin1-F-box (SCF) complex, which facilitates ubiquitin-mediated protein degradation in eukaryotes. SLF interacting *Skp1*-like 1 (*SSK1*), which is located outside the S-site, is a newly discovered type of self-incompatibility-related pollen-specific gene whose product interacts with pollen determinants. In this study, we identified 18 members of the *Prunus dulcis Skp1*(*PsdSkp1*) family that were unevenly distributed across six chromosomes of the almond genome. The phylogenetic tree revealed that the *PsdSkp1* members could divided into four groups: I, II, III, and IV. The conserved motifs, conserved domains, and motif structures of the *PsdSSK1* and *PsdSkp1-12* proteins are similar. There were three pairs of fragment duplication genes and one pair of tandem repeat genes, and their functions were highly evolutionarily conserved. Transcriptome data showed that *PsdSkp1* was expressed in almond flower tissues, that its expression was affected by freezing stress, and that its expression changed significantly in cross-pollination. Molecular experiments proved that *PsdSSK1* and *PsdSkp1-12* had the same protein sequence, and the genes were significantly highly expressed mainly in the pollen or throughout pollen cells, with subcellular localization in the cytoplasm. *PsdSkp1-12* is an *ssk1* gene in almond, and it may be involved in the SCF complex to regulate the almond pollen response via SI and cross-compatibility. In conclusion, our research lays a foundation for future functional research on *PsdSkp1* members, especially for exploring the mechanism of almond SI.

Keywords: Almond; *Skp1*; genome-wide identification; expression analyses; *PsdSSK1* clone; Southern blot; Western blot

1. Introduction

S-phase kinase-associated protein 1 (*Skp1*) is a small protein comprising approximately 160 amino acids and constitutes the core component connecting the *Cullin1* and *F-box* proteins. As the core component of SCF-type E3 ubiquitin ligase, *Skp1* mediates the degradation of proteins by the 26S proteasome and plays a key role in eukaryotic cell cycle progression, transcriptional regulation, signal transduction and many other cellular processes[1-3]. Only one *Skp1* gene has been found in protozoa, algae and fungi[4]. In contrast, plants have multiple *Skp1* genes, which is the result of duplication events that led to functional differentiation[5,6]. In plants, *Skp1* genes play an important role in auxin, gibberellin, ethylene, and jasmonate signaling and in photoreactions[7-9], flower development and male meiosis[10,11]. In most cases, *Skp1* functions by forming one of many SCF complexes together with *Cullin1*, *Rbx1* and *F-box* proteins. *Arabidopsis*

thaliana Skp1 (*ASK*) proteins play an important role in the development of flowers and the sterility of male gametophytes[12-14]. *Solanum lycopersicum Skp1* (*SSK*) proteins are involved in the development of tomato fruits[15], and *Triticum aestivum Skp1* (*TSK*) proteins regulate male meiosis in wheat[16]. Overexpression of *Paeonia suffruticosa Skp1* (*PSK1*) was shown to increase the expression of the flowering-related genes *CO*, *LEAFY* and *FT* and promoted flower formation and early flowering. The expression of *PSK1* gradually increases with the development of flower buds and the flowering process[17]. Transgenic *Arabidopsis* plants overexpressing *PSK1* exhibit increased salt tolerance due to increased accumulation of proline and soluble sugars[17]. This effect was similar to that of *ASK1*, which interacts with B-function proteins (B subfamily *MADS-box* gene products) and cooperates with *UFO* and *LEAFY* proteins in flower development and floral organ identity[13]. In *Arabidopsis* flowers expressing mutant *ASK1* genes, increased expression of two proteins was detected: one is a class I heat-shock protein induced in response to stress and provides protection against oxidative damage to cells; the other is an oxygen-evolving complex-related protein. These findings show that mutations in *ASK1* may directly or indirectly cause oxidative stress in floral organs[18]. In upland cotton (*Gossypium hirsutum*), strong expression of *GhSKP1* in the radicle and stamen that comprise dividing cells showed that *GhSKP1* may be involved in the growth and flower development. ABA had a slight inhibitory effect on the expression of *GhSKP1* in cotton leaves[19]. Rice *OSK1* and tomato *SSK1* and *SSK2* are also involved in leaf senescence[20].

A newly discovered *Skp1-like* protein was discovered in *Antirrhinum hispanicum*. SLF-interacting Skp1-like 1 (*SSK1*), located outside the S-site, is a new type of self-incompatibility-related pollen-specific gene that interacts with pollen determinants[21]. Researchers analyzing the composition of the snapdragon (*Antirrhinum hispanicum*) SCF complex found that *AhSSK1* can interact with AhSLF-S2 and CUL-like proteins and that *AhSSK1* can form an SCF complex composed of *SSK1*, *CUL1*, *SLF* and *Rbx1*[21]. In 2010, Zhao Lan et al. [22] also identified and cloned the *AhSSK1* homologous gene from *Petunia hybrida*, *PhSSK1*. The two genes were 48.3% homologous. The same in vitro biochemical experiment proved its interaction with the pollen self-incompatibility (SI) determinants *PhSLF*, *Cullin1* and *S-RNase*. These findings show that *SSK1* acts as a linker within the SCF complex, is responsible for connecting the *F-box* protein, and indicates that *SSK1* is involved in the SI signal response. In addition, transgenic RNA interference (RNAi) experiments showed that downregulation of *PhSSK1* affected the pollen affinity response,

and partial segregation was also detected in a genetic analysis of transgenic offspring. In plantain and Solanaceae species, it has been found that there is a pollen-specific *SSK1* that interacts together with SLF in the SLF-mediated SCF complex and that this *SSK1* is required to degrade S-RNase[22]. In addition, in 2012, Xu and Li [23] identified and cloned two *SSK1* genes from *Pyrus bretschneideri* (Rosaceae), PbSSK1 and PbSSK2. Homologs of the *Skp1-like* gene have also been cloned from apple, pear and sweet cherry; and the products of all these genes can bind to their corresponding *SLF* and *Cullin1* proteins[24,25]. Recently, Zeng et al. [26] cloned SCF complex-related genes from *Prunus tenella* (*PetSSK1* and *PetSBP1*), and using yeast two-hybrid and pulldown experiments, the researchers found that *PetSBP1* mainly connected the Cullin1 protein and *F-box* protein together in wild almond, leading to the formation of a functional SCF complex.

Almond (Rosaceae: *Prunus dulcis*) is an economically important crop tree species that exhibits gametophytic self-incompatibility (GSI)[27]. SI is the most important mechanism controlling plant self-fertilization[28] and has been reported in more than half of all flowering plants and most other Rosaceae species[29,30]. SI refers to the ability of the pistil of a plant to identify and inhibit the germination and growth of its own pollen, in which the E3 ubiquitin ligase SCF^{SFB} (SLF) complex plays a decisive role. The SCF complex has a spherical structure and consists of four main components: *Skp1*, *Cullin1*, *Rbx1*, and *F-box* proteins. The mechanism of S nuclease-based GSI involves tagging a target protein for entry into the ubiquitin proteasome pathway (UPP), thereby causing it to be broken down by the 26S proteasome, which ultimately drives SI[31].

To date, *Skp1* family genes and *SSK1* genes have not been reported in almond. To explore the characteristics of almond *Skp1* family members, we used almond genome-wide data to screen *Skp1* family members. The physicochemical properties, subcellular localization, phylogenetic relationships, conserved protein motifs, conserved domains, gene structure, chromosomal gene distribution, collinear genes and cis-elements were analyzed. Transcriptome data from different tissues of almond, anthers and ovaries under freezing stress, and self- and out-crossed pollen were obtained to calculate the fragments per kilobase per million mapped read (FPKM) values and analyze expression patterns. To determine whether members of the *Skp1* family in almond have *SSK1* function, we cloned the almond *PsdSSK1* gene and assessed the in vitro expression of almond pollen by fluorescence quantification and Southern blot and Western blot analyses. This study provides theoretical data for the functional role of *Skp1* family genes in almond, provides a

theoretical basis and data for determining whether the *SSK1* gene is involved in the SI pathway of almond, and is useful for investigating the SI of almond. The mechanism of action responsible is highly important.

2. Results

2.1 Genome-wide identification of the *Skp1* genes in almond

Eighteen *Skp1* genes were identified and screened in almond. These genes were renamed *PsdSkp1-1~PsdSkp1-17* according to their chromosome and location, and two of them were homologous genes, which were renamed *PsdSkp1-8a* and *PsdSkp1-8b*. The physicochemical properties of the proteins showed that the number of amino acids ranged from 93 (*PsdSkp1-2*) to 367 (*PsdSkp1-8b*) amino acids, and the molecular weight ranged from 10791.13 (*PsdSkp1-2*) to 42582.44 (*PsdSkp1-8b*) Da. The isoelectric point ranged from 4.41 to 5.69, and the hydrophilic and hydrophobic values were all less than 0, indicating that the proteins are hydrophilic. Subcellular mapping showed that *PsdSkp1-8b* was located in the mitochondrion, *PsdSkp1-5* was located in the nucleus, *PsdSkp1-9* and *PsdSkp1-16* were located in the chloroplast, and the other 14 proteins were located in the cytoplasm.

Table 1 Detailed information on the *PsdSkp1* gene family members in almond

Gene name	Gene ID	Chromosome	Start	End	Number of amino acids	Molecular weight/Da	Isoelectric point	GRAVY	Subcellular localization
PsdSkp1-1	Prudul26A001581C1	Pd01	29822524	29823973	156	17767.94	4.53	-0.597	Cytoplasm
PsdSkp1-2	Prudul26A010302C1	Pd02	7447046	7447384	93	10791.13	4.42	-0.674	Mitochondrion
PsdSkp1-3	Prudul26A004902C1	Pd02	11802849	11803382	177	20194.72	4.61	-0.779	Cytoplasm
PsdSkp1-4	Prudul26A003178C1	Pd02	11871398	11871868	156	18027.36	4.46	-0.556	Cytoplasm
PsdSkp1-5	Prudul26A017928C1	Pd02	11878821	11879288	155	17776.12	4.57	-0.483	Nucleus

PsdSkp1-6	Prudul26A022605C1	Pd02	11895200	11895670	156	17882.14	4.41	-0.503	Cytoplasm
PsdSkp1-7	Prudul26A000183C1	Pd02	12798666	12799205	179	20236.17	4.97	-0.375	Cytoplasm
PsdSkp1-8a	Prudul26A002877C1	Pd02	17496362	17500871	340	39478.61	5.33	-0.861	Cytoplasm
PsdSkp1-8b	Prudul26A002877C2	Pd02	17496362	17502012	367	42582.44	5.69	-0.807	Cytoplasm
PsdSkp1-9	Prudul26A026206C1	Pd02	19991933	19992358	141	16037.53	5.07	-0.035	Chloroplast
PsdSkp1-10	Prudul26A016207C1	Pd02	24563273	24568462	356	40766.03	5.45	-0.795	Cytoplasm
PsdSkp1-11	Prudul26A026600C1	Pd04	3293555	3294014	131	15261.61	5.16	-0.463	Cytoplasm
PsdSkp1-12	Prudul26A027568C1	Pd04	7760346	7761334	177	20538.04	4.52	-0.567	Cytoplasm
PsdSkp1-13	Prudul26A018869C1	Pd06	4839281	4841071	98	11291.95	5.12	-0.148	Cytoplasm
PsdSkp1-14	Prudul26A031483C1	Pd07	3083813	3084244	118	13706.56	4.72	-0.469	Cytoplasm
PsdSkp1-15	Prudul26A008750C1	Pd07	16186460	16188084	156	17685.92	4.57	-0.535	Cytoplasm
PsdSkp1-16	Prudul26A016175C1	Pd07	16194299	16195427	157	17936.47	4.77	-0.417	Chloroplast
PsdSkp1-17	Prudul26A019975C1	Pd08	14876840	14878185	157	18049.32	4.54	-0.424	Cytoplasm

129 2.2 Phylogenetic analysis and classification of *PsdSkp1* gene members

130 The phylogenetic tree of almond, *Arabidopsis Skp1* family members, and *SSK1*
131 members of 10 species was divided into four groups on the basis of the results of clustering
132 branching: groups I, II, III, and IV (Figure 1). There was only one *PsdSkp1-13* in group I;
133 in group II, *PsdSkp1-8a*, *PsdSkp1-8b*, and *PsdSkp1-10* were closely related and clustered
134 together with *ASK20* and *ASK21*, suggesting they may be homologous proteins with similar
135 functions. *PsdSkp1-9*, *PsdSkp1-11*, and *PsdSkp1-12* as well as *SSK1* genes from all the
136 species were in group III. Among them, *PsdSkp1-12* is closely related to *FaSSK1*, *Pu* *x*
137 *PcSSK1*, *MdSSK1*, *PaSSK1*, *PtSSK1*, and *PpSSK1* of the Rosaceae family; these are
138 homologous proteins and may be encoded by *SSK1* genes. Group IV contained 19 *ASK*

139 members and 11 *PsdSkp1* members. Among them, *PsdSkp1-1*, *PsdSkp1-15*, and
 140 *PsdSkp1-16* were closely related and clustered together with *ASK2*. The remaining *PsdSkp1*
 141 members clustered relatively closely together with *ASK3* and *ASK4*, while all the other *ASK*
 142 members were clustered together.

143
 144 **Figure 1** Phylogenetic tree of *Skp1* family members and *SSK1* from almond and
 145 *Arabidopsis thaliana*. The evolutionary history was inferred using the maximum likelihood
 146 method and a JTT matrix-based model. This analysis involved 49 amino acid sequences.
 147 The blocks of different colors represent different group members. *ASK* (*Arabidopsis*
 148 *thaliana*:Skp1); *AhSSK1* (*Antirrhinum hispanicum*); *PhSSK1* (*Petunia hybrida*); *JcSSK1*
 149 (*Jatropha curcas*); *PoaSSK1* (*Populus alba*); *FaSSK1* (*Fragaria ananassa*); *PuxPcSSK1*
 150 (*Pyrus ussuriensis* x *Pyrus communis*); *MdSSK1* (*Malus domestica*); *PaSSK1* (*Prunus*
 151 *avium*); *PtSSK1* (*Prunus tenella*); *PpSSK1* (*Prunus persica*).

152 2.3 Motifs, domains and gene structure of *PsdSkp1* gene members

153 The conserved motifs of *PsdSkp1* family members were annotated to 20 conserved
 154 motifs. Motif 1 was considered a Skp1 type, and motif 2, motif 3, and motif 11 were of the
 155 Skp1_POZ type (Figure 2E). Cluster analysis via a phylogenetic tree showed that in
 156 *PsdSkp1-13* in group I, motif 11, motif 12, and motif 20 were conserved. In group II,
 157 *PsdSkp1-11* contained motif 1, while *PsdSkp1-9* and *PsdSkp1-12* contained motif 1 and
 158 motif 11. In group III, *PsdSkp1-8a*, *PsdSkp1-8b*, and *PsdSkp1-10* contained motif 1, motif 2,

motif 4, motif 6, and motif 7, the distributions of which were highly conserved. In group IV, 11 *PsdSkp1* proteins contained motif 1, motif 5 and motif 8, and 8 *PsdSkp1* proteins contained motif 2 (Figure 2B).

The conserved domains of the *PsdSkp1* family members showed that, except in the case of *PsdSkp1-13* and *PsdSkp1-2*, there were no Skp1_POZ types; the other 16 *PsdSkp1* proteins contained a Skp1_POZ domain. Although *PsdSkp1-13* has only one *Skp1* superfamily domain, the other 15 *PsdSkp1* proteins contained 2-3 conserved domains, which are of the Skp1_POZ types, Skp1 and Skp1 superfamilies (Figure 2C). The results were similar to those obtained with respect to the conserved motifs.

Cluster analysis of gene structure and the phylogenetic tree revealed that, in group I, *PsdSkp1-13* had three exons and two introns. In group II, *PsdSkp1-9*, *PsdSkp1-11* and *PsdSkp1-12* had one exon and 0 introns. In group III, *PsdSkp1-8a* and *PsdSkp1-8b* had 8 exons and 7 introns, whereas *PsdSkp1-10* had 9 exons and 8 introns. In group IV, 5 *PsdSkp1* members had 1 exon and 0 introns, and 6 *PsdSkp1* members had 2 exons and 1 intron (Figure 2D).

Figure 2 Phylogenetic clustering of *PsdSkp1* gene members. (A) Phylogenetic tree of *PsdSkp1* family members divided into four groups: I, II, III, and IV. (B) Conserved protein motifs. (C) Conserved protein domains. (D) Gene structure: exon-intron. (E) Motif 1, motif 2, motif 3 and motif 11 conservation according to LOGO.

2.4 Chromosomal distribution and analysis of collinear genes of *PsdSkp1* gene members

PsdSkp1 gene members were distributed across six chromosomes: Pd01, Pd02, Pd04, Pd06, Pd07, and Pd08 (Figure 3A). One *PsdSkp1* gene was found on Pd01, Pd06, and Pd08 each; two *PsdSkp1* genes were distributed on chromosome Pd04; three *PsdSkp1* genes were distributed on chromosome Pd07; and 10 *PsdSkp1* genes were distributed on chromosome Pd02, which may have similar functions.

Chromosomal collinearity analysis revealed that 18 *PsdSkp1* members had three pairs of segmental duplicated genes, *PsdSkp1-1/PsdSkp1-15*, *PsdSkp1-1/PsdSkp1-17*, and *PsdSkp1-15/PsdSkp1-17*, which were distributed on chromosomes Pd01, Pd07, and Pd08. At the same time, a pair of tandem repeat genes, *PsdSkp1-8a/PsdSkp1-8b*, were found on chromosome Pd02. Almond had 7, 18, 21, and 11 pairs of genes that were collinear with those in *Arabidopsis thaliana*, *Malus domestica*, *Prunus persica*, and *Prunus mume*, respectively (Figure 3C). In addition, the Ka/Ks values of three pairs of duplicated fragments, one pair of tandem repeat genes, and one pair of collinear genes among the species were less than 1, indicating that almond was affected mainly by purification and that the gene functions were highly conserved (Supplement Table 1).

Figure 3A Chromosomal distribution of *PsdSkl* genes in almond. The scale on the left shows the chromosome length information, and the chromosome names are listed at the top.

Figure 3B Distribution of duplicated gene pairs in the almond genome. Both genes of the same duplicated gene pair are labeled in red.

Figure 3C Syntenic relations of *Skp1* gene members among almond and four representative plant species. The gray lines in the background indicate collinear blocks within the almond genome and other plant genomes, and the red lines highlight syntenic *Skp1* gene pairs.

2.5 Upstream cis-regulatory elements

The 2000 bp promoter region upstream of 18 PdsKp1 family members in almond was annotated to a large number of core elements (TATA-boxes and CAAT-boxes) and light-responsive elements. In addition, there were various components associated with growth and development, hormone responses, and stress resistance (Figure 4). The GCN4 motif, O2 site, circadian element, CAT-box, Unnamed_1 element, Ry element, MSA-like element, and MBSI element were found to be related to growth and development. Additionally, there were six types of elements related to the hormone response, TGACG

motifs, CGTCA motifs, TATC-boxes, ABREs, TGA elements, and TCA elements, and four types of stress-related components ARE MBSs, AREs, LTRs, and TC-rich repeats.

220

Figure 4 Cis-elements in promoter region of *PsdSkp1* members. The colored boxes indicate the types of cis-elements.

2.6 Expression analysis of *PsdSkp1* gene members

The *PsdSkp1-2*, *PsdSkp1-4*, *PsdSkp1-5*, *PsdSkp1-6*, *PsdSkp1-9*, and *PsdSkp1-14* genes were not expressed in the leaf, flower bud, pistil, or fruit tissues (Figure 5A). In the leaves, all the *PsdSkp1* family members were downregulated. In the flower buds, the *PsdSkp1-8a*, *PsdSkp1-10*, *PsdSkp1-13*, *PsdSkp1-15*, and *PsdSkp1-17* genes were significantly highly expressed, the *PsdSkp1-1* and *PsdSkp1-16* genes were expressed at low levels, and other genes were downregulated. In the pistils, the expression of *PsdSkp1-7*, *PsdSkp1-8a*, *PsdSkp1-11*, *PsdSkp1-12*, and *PsdSkp1-16* was significantly higher than that of *PsdSkp1-1*, *PsdSkp1-10*, and *PsdSkp1-13*. In the fruitlets, the *PsdSkp1-1*, *PsdSkp1-3*, *PsdSkp1-8b*, and *PsdSkp1-17* genes were highly expressed, and the expression of other genes decreased. These results indicate that *PsdSkp1* family members exhibit obvious tissue-specific expression characteristics. Under control and freezing-stress conditions, five genes,

224

235 *PsdSkp1-2*, *PsdSkp1-5*, *PsdSkp1-9*, *PsdSkp1-11*, and *PsdSkp1-14*, were not expressed
 236 (Figure 5B). In control anthers, *PsdSkp1-7* and *PsdSkp1-12* were significantly highly
 237 expressed, while the other genes were downregulated. In the anthers under freezing stress,
 238 the expression of *PsdSkp1-6* increased sharply, and *PsdSkp1-8b* expression increased, but
 239 the expression was low. The expression of *PsdSkp1-12* decreased and was weak; the
 240 expression of other genes decreased. In the control ovaries, the *PsdSkp1-1*, *PsdSkp1-3*,
 241 *PsdSkp1-4*, *PsdSkp1-8a*, *PsdSkp1-8b*, and *PsdSkp1-10* genes were significantly highly
 242 expressed, while the other genes were downregulated. In anthers under freezing stress, the
 243 downregulated expression of *PsdSkp1-1*, *PsdSkp1-3*, *PsdSkp1-4*, *PsdSkp1-8a*, *PsdSkp1-8b*,
 244 and *PsdSkp1-10* was inhibited, while the upregulated expression of *PsdSkp1-13*,
 245 *PsdSkp1-15*, *PsdSkp1-16* and *PsdSkp1-17* was significant. Taken together, these results
 246 indicate that the expression levels of some members of the *PsdSkp1* family change when
 247 anthers and ovaries are subjected to freezing stress to cope with the resulting adverse
 248 effects. An expression heat map of the four types of pistil pollination (A, AXA, I, and IXA)
 249 showed (Figure 5C) that the expression of six genes, *PsdSkp1-2*, *PsdSkp1-5*, *PsdSkp1-7*,
 250 *PsdSkp1-9*, *PsdSkp1-11* and *PsdSkp1-14*, was inhibited. In A, six genes, *PsdSkp1-1*,
 251 *PsdSkp1-3*, *PsdSkp1-4*, *PsdSkp1-6*, *PsdSkp1-8b* and *PsdSkp1-17*, were significantly highly
 252 expressed; three genes, *PsdSkp1-10*, *PsdSkp1-15* and *PsdSkp1-16*, were expressed at low
 253 levels; and the other genes were downregulated. In I, only two genes, *PsdSkp1-16* and
 254 *PsdSkp1-17*, were expressed at low levels, while the other genes were downregulated. In
 255 AXA, the *PsdSkp1-1*, *PsdSkp1-3* and *PsdSkp1-16* genes were expressed, *PsdSkp1-8a* and
 256 *PsdSkp1-10* expression was low, and the expression of the other genes was downregulated.
 257 Compared with that in AXA, the expression of the *PsdSkp1-8A*, *PsdSkp1-12*, *PsdSkp1-13*,
 258 and *PsdSkp1-15* genes in IXA significantly increased.

Figure 5 Expression of PsdSkp1 family members. (A) Expression levels in the leaves, flower buds, pistils, and fruitlets. (B) Expression levels in control anthers, control ovaries, anthers under freezing stress, and ovaries under freezing stress. (C) A: Unpollinated A2-198 pistils, I: Unpollinated ITAP-1 pistils, AxA: Self-pollinated A2-198 pistils, IxA: ITAP-1 pistils pollinated with A2-198. The row normalization method was used to construct the heat map.

2.7 *PsdSSK1* cloning

In accordance with the designed primers, agarose gel electrophoresis was used to detect RNA samples from cultivated almond. PCR was used to clone and amplify the full-length (585 bp) sequence (Figure 6A) of the *PsdSSK1* gene-coding region, which was consistent with the corresponding *PsdSkp1-12* protein sequence. Via analysis of its open reading frame (ORF), the *PsdSSK1* gene, whose conserved domains included a 62 amino acid *Skp1_POZ* domain and a 47 amino acid *Skp1* domain (Figure 6B), was 534 bp in length, encoding 178 amino acids (Figure 6C). Multiple alignments of the *PsdSSK1* protein sequence with the those of 8 other species showed that all of them contained *Skp1_POZ* and *Skp1* domains (Figure 6D), indicating that our results concerning *PsdSSK1* were extremely reliable. The physicochemical properties revealed that the *PsdSSK1* protein was hydrophilic, with a molecular weight of 20538.04 Da, an isoelectric point of 4.52, a

278 molecular formula of $C_{910}H_{1421}N_{233}O_{295}S_6$, a stability index of 41.48, an aliphatic index of
 279 83.11, and a grand average of hydropathy of -0.567.

280
 281 **Figure 6** (A) PCR amplification of the *PdsSSK1* gene. (B) *PdsSSK1* conserved domains. (C)
 282 *PdsSSK1* cDNA sequence and amino acid sequence. (D) Multiple alignment of sequences
 283 of *SSK1* proteins from multiple species.

284 2.8 Expression of the *PdsSSK1* Gene in Different Tissues of Cultivated Almond and Southern and 285 Western blot Detection

286 Almond β -actin was selected as the internal reference gene. The amplification curve
 287 and dissolution curve of the *actin* gene and the *PdsSSK1* gene showed that the dissolution
 288 curves of each showed a single peak, indicating that the specificity of the primers was
 289 strong. During the PCR amplification process, no nonspecific amplification, such as that
 290 reflecting the presence of primer dimers, occurred, which meant the results were suitable
 291 for qPCR analysis. The tissue samples were standardized and analyzed using the $2^{-\Delta\Delta CT}$
 292 method. In the figures, error bars represent the means $\pm$ standard deviations. The expression
 293 level of the *PdsSSK1* gene was highest in pollen tissue, while the expression levels in other
 294 tissues (petals, sepals, styles and leaves) were relatively low (Figure 7B; Figure 7C).

295 Southern blotting of almond cultivated in Xinjiang was performed to determine the
 296 copy numbers of DNA. The Southern blot results of the *PdsSSK1* gene revealed the

following (Figure 7D): *PsdSSK1* gene is present as single copy in the leaves, petals, style, sepals and pollen.

Analysis of the *PsdSSK1* protein expression in the five tissues of almond cultivated in Xinjiang according to Western blot analysis revealed that (Figure 7E) the *PsdSSK1* protein was expressed in the petals, styles, and pollen tissues, and the expression of *PsdSSK1* in the pollen was significantly higher than that in the other tissues.

Figure 7 (A) Cultivated almond flowers; (B, C) Differential expression of the *PsdSSK1* gene in different tissues of cultivated almond tree; (D) Three hours after exposure to CSPD (Lane M: DNA molecular weight marker; PCR products; Lane 1: Leaves; Lane 2: Petals; Lane 3: Styles; Lane 4: Sepals; Lane 5: Pollen); (E) Western blot detection results (Lane M: Positive control; Lane 2, lane 7, lane 8, lane 9: Leaves; Lane 3: Petals; Lane 4: Styles; Lane 5: Sepals; Lane 6: Pollen)

2.9 Subcellular localization analysis

The subcellular location of the *PsdSSK1* protein is the cytoplasm. PGC1-PsdSSK1 was transiently transformed into *Nicotiana benthamiana* epidermal cells. The epidermal cells were removed, and the fluorescence localization was observed under a laser confocal fluorescence microscope; the results are shown in Figure 8. *PsdSSK1* was expressed in the cytoplasm, which is consistent with the prediction results of WoLF PSORT online analysis software.

Figure 8 Subcellular localization of the *PsdSSK1* gene. Green fluorescence (GFP), visible light (BF), merged field (merge), scale bar=50 μ m.

3. Discussion

Skp1 is a component of a SKP1-Cullin1-F-box (SCF) complex that facilitates ubiquitin-mediated protein degradation in eukaryotes. In various plant tissues, the UPP breaks down most intracellular proteins, and the SCF complex is the most common and largest class of E3 family members[32,33]. In this study, we screened 18 *PsdSkp1* family members from the almond genome, all of which were acidic and hydrophilic proteins, and their subcellular location was mainly the cytoplasm. The *ASK1* and *ASK2* proteins in *Arabidopsis thaliana* exhibit a substantial amount of functional overlap and participate in early growth and the abscisic acid (ABA) signaling pathway[34,35]. Moreover, these proteins have the potential to interact with F-box proteins and participate in SCF complexation[36]. Our phylogenetic tree showed that *PsdSkp1-1*, *PsdSkp1-15*, and *PsdSkp1-16* strongly clustered together with *ASK2*. Moreover, the protein interaction results showed that 17 *PsdSkp1* members interacted with Cullin and F-box proteins and participated in the formation of the SCF complex (Supplement Figure 1 and Table 2), which suggested that *PsdSkp1* could regulate the early growth and development of almond and the ABA signaling pathway. *GsSKP21* in soybean is highly homologous to *ASK20* and *ASK21*, and its tissue expression characteristics are similar. Overexpression of *GsSkp21* in *Arabidopsis* significantly increased plant tolerance to alkali stress[37]. *PsdSkp1-8a*,

PsdSkp1-8b, and *PsdSkp1-10* are highly homologous to *ASK20* and *ASK21*, indicating that these three genes may have functions similar to those of *GsSKP21*, may participate in the ABA signaling pathway and may increase the alkali tolerance of almond. *PsdSkp1-12*, which may play a role in almond, is highly homologous to *SSK1* of 10 species. Subsequent cloning and identification of *PsdSSK1* also confirmed this supposition.

The conserved motifs and conserved structures of the proteins revealed that 18 members of *PsdSkp1* proteins had *Skp1* or *Skp1_POZ* domains. Studies have shown that all *Skp1* genes in *Arabidopsis* and rice originate from the same ancestral gene. Phylogenetic analysis revealed that these genes could be divided into three types: type Ia, which have one intron and two exons; type Ib, which not have introns; and type II, which have many introns[38]. The lack of introns in the *Skp1* gene may be caused by reverse transcriptase-mediated rearrangement[38]. Repeated signals and poly(A) extensions of target sites were found in the rice *OSK3* sequence, which may be caused by the rapid evolution of these genes, as shown in both rice and *Arabidopsis*[39]. According to the phylogenetic tree clustering, the exon-intron data showed that the *PsdSkp1* family members were highly conserved, and the exon-intron differences among different groups were substantial. Eight *PsdSkp1* family members did not contain introns, which corresponded to the results obtained in *Arabidopsis* and rice. The *SKP1* gene in dicotyledonous plants and monocotyledonous plants evolved from a single ancestral gene, followed by multiple duplication events[40]. These gene duplication events are essential for the evolution of gene families and provides a good basis for the evolution of new genes as well as for new physiological and morphological changes[41]. In addition, the *PsdSSK1* family members resulting from fragment duplication and tandem repeats may provide new functions for almond *Skp1* members and promote the growth and development of almond. The collinear gene pairs of almond, *Arabidopsis thaliana*, *Malus domestica*, *Prunus persica* and *Prunus mume* were mainly purified, and the gene functions were highly evolutionarily conserved. The promoter region annotations revealed that *PsdSkp1* contains many kinds of elements, such as those that respond to hormones, stress, growth and development, that play an important role in overall plant growth and development.

367 In Arabidopsis, the β -glucuronidase (GUS) activity of *ASKs* occurs mainly in anthers
 368 and stigmas[42], and *ASKs* have been shown to play an important role in flower
 369 development and male gametophyte sterility[11]. Most *PsdSkp1* proteins were expressed in
 370 flower buds and pistils. Interestingly, the genes expressed in the flower buds have two or
 371 more exons and one or more introns; in the pistils, *PsdSkp1-7*, *PsdSkp1-11*, *PsdSkp1-12*
 372 and *PsdSkp1-16* contain only one exon and 0 exons. These results indicate that the number
 373 of introns and exons is closely related to the expression activity of *PsdSkp1* in different
 374 parts of floral organs. *PsdSkp1-7* and *PsdSkp1-12* were significantly highly expressed in the
 375 control anthers, and the expression of *PsdSkp1-6* was significant in anthers under freezing
 376 stress. Compared with *PsdSkp1-7* and *PsdSkp1-12*, *PsdSkp1-6* has one more MYB
 377 transcription factor-binding site (MBSI), which may regulate the production of flavonoids
 378 to make anthers resist freezing stress[43]. In the control ovaries, the expression of
 379 *PsdSkp1-1*, *PsdSkp1-3*, *PsdSkp1-4*, *PsdSkp1-8a*, *PsdSkp1-8b* and *PsdSkp1-10* genes was
 380 significant, which was inhibited after freezing stress, while the expression of *PsdSkp1-13*,
 381 *PsdSkp1-15*, *PsdSkp1-16* and *PsdSkp1-17* genes was significant. In AXA self-pollination,
 382 *PsdSkp1-1*, *PsdSkp1-3*, *PsdSkp1-10* and *PsdSkp1-16* were highly expressed. Notably, the
 383 expression of these four genes in AXA changed little, which may be due to the expression
 384 in the pistil itself. It is suggested these genes may not be expressed or that their products
 385 may not have any activity in pollen. After IXA cross-pollination, the expression of
 386 *PsdSkp1-8a*, *PsdSkp1-12*, *PsdSkp1-13* and *PsdSkp1-15* decreased significantly (or not) in
 387 AXA. Almond is a typical self-incompatible plant species, with abnormal self-pollination
 388 that leads to loss of pollen activity; however, cross-pollination is normal, and pollen
 389 remains active. These results indicate that these four genes regulate pollen expression and
 390 promote cross-pollination. In summary, *PsdSkp1s* and *ASKs* have similar functions and
 391 play an important role in the growth and development of flower organs and male
 392 gametophyte sterility. In addition, the regulation of pollen expression by *PsdSkp1* proteins
 393 is severely affected by freezing stress, and cross-pollination may not be normal under
 394 freezing conditions.

SSK1 can be used as a component to form a functional SCF complex together with Cullin1, *SLF* and *RBX1*. At present, *SSK1* genes have been identified in apple, petunia and other species[44,45]. SSK1s constitute a new class of Skp1-like proteins. The amino acids of *AhSSK1* and *PhSSK1* are 40% homologous with those of other Skp1 proteins, and both have a unique C-terminus comprising 7-9 amino acid residues, which is similar to the conventional WAFE terminal residues found in most plant Skp1 homologs[44]. ORF analysis of the cloned *PsdSSK1* gene revealed that the gene is 534 bp in length, encoding 178 amino acids. Multiple alignment of the sequence of the *SSK1* protein with sequences from other species revealed two distinct domains: Skp1_POZ and Skp1. The quantitative fluorescence expression results showed that *PsdSSK1* was highly expressed in almond pollen, which was consistent with the specific expression of the *SSK1* gene in pollen. Southern blot analysis of the *PsdSSK1* gene revealed present as single copy in the leaves, petals, style, sepals and pollen. The Western blot results showed that the *PsdSSK1* protein was expressed in the petals, styles, and pollen tissues of almond, and the expression of *PsdSSK1* in the pollen was significantly higher than that in the other tissues. The subcellular location of the *PsdSSK1* protein was consistent with the results predicted by WoLF PSORT online analysis software, which was predicted to localize to the cytoplasm. Interestingly, *PsdSSK1* and *PsdSkp1-12* were identical. Combining the phylogenetic tree results, expression patterns and experimental results, we found that *PsdSkp1-12* was an *ssk1* gene in almond that could replace the *Skp1* gene and bind to *SLF* and *cullin1*. *PsdSkp1-12* may form a functional SCF complex together with cullin1, *SLF* and *Rbx1* as components to regulate almond *pollen* activity, SI, and cross-compatibility.

4. Materials and Methods

4.1 Screening of the *Skp1* gene family members in almond and analysis of the physicochemical properties of their proteins

The coding DNA sequence (CDS) of *D. almondi* was downloaded from the Genome Database for Rosaceae (<https://www.rosaceae.org/>), and the hidden Markov model (HMM) profiles of Skp1 and Skp1_POZ (accession numbers PF01466 and PF03931) were downloaded from the Pfam database (<http://pfam.xfam.org/>). The HMMsearch tool was used to search and compare the whole-genome protein data of almond with data of the Skp1

and Skp1_POZ hidden Markov models[46,47]. Domain verification was performed through the Pfam database, and Skp1 protein motifs and redundant genes were discarded. Finally, 18 *Skp1* genes were obtained.

4.2 Sequence alignment, phylogenetic analysis, chromosomal location analysis, gene structure construction, collinearity analysis, and calculation of Ka and Ks values

The protein sequences of the 18 *Skp1* genes from almond were analyzed to determine the number of amino acids, protein molecular weight, isoelectric point and hydrophobicity of the protein, and ExPASy (<http://au.expasy.org/tools>) and WoLF PSORT II (<https://www.genscript.com/wolf-psort.html?src=leftbar>) were used for protein subcellular localization analysis[48,49]. Multiple alignment of the nucleotide and amino acid sequences were performed using ClustalW[50]. Phylogenetic analysis was performed based on the sequences of *SSK1* proteins from *Antirrhinum hispanicum*, *Petunia hybrida*, *Prunus persica*, *Prunus tenella*, *Prunus avium*, *Malus domestica*, *Pyrus ussuriensis* x *Pyrus communis*, *Fragaria ananassa*, *Populus alba* and *Jatropha curcas* via the maximum likelihood method with 1000 bootstrap replicates and visualized with MEGA X software[51]. The chromosomal distribution of all *PsdSkp1* genes was determined based on the identification results, and images of the locations of *PsdSkp1* genes were subsequently drawn with TBtools software. Exon-intron structure analysis of the *PsdSkp1* genes was conducted and the results displayed by comparing CDSs and their corresponding gene sequences from the genome using TBtools[52]. The MEME program was used to statistically identify conserved motifs within the complete amino acid sequences of the *PsdSkp1* proteins[53]. Gene duplication and collinearity analyses were performed using MCScanX via GFF3 files retrieved from the Phytozome database[54]. Genes that were not present on major chromosomes were excluded from the evaluation. Collinearity circle plots were generated using Circos v0.69[55], and the KaKs_Calculator tool was used to calculate the Ka/Ks values of the collinear genes[56].

4.3 Analysis of cis-elements within *Skp1* gene members

The 2000 bp upstream sequences of the identified *Skp1* genes were extracted by TBtools[57]. The extracted sequences were then submitted to the PlantCARE website (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) to predict the cis-elements in

the promoter regions. A diagram of the cis-elements within the *Skp1* genes was constructed by TBtools and modified via Adobe Illustrator CC 2019.

4.4 Analysis of the expression patterns of members of the *Skp1* family in almond

Sequences from following almond tissues were downloaded from the NCBI Sequence Read Archive (SRA) database: leaves (SRR12806975); flower buds (SRR11251344); pistils (SRR5830017); fruitlets (SRR10189226); anthers under normal and freezing-stress conditions (control: SRR2976058; freezing stress: SRR2976061); ovaries under normal and freezing-stress conditions (control: SRR2976060; freezing stress: SRR2976062); and pollen from selfing and outcrossed unpollinated A2-198 pistils (A) (ERR2182599), unpollinated ITAP-1 pistils (I) (ERR2182601), self-pollinated A2-198 pistils (AxA) (ERR2182600), and ITAP-1 pistils pollinated with A2-198 (IxA) (ERR2182602). The transcriptome data were obtained, and FPKM values were subsequently calculated. Afterward, a heat map of the expression patterns of the 18 *Skp1* family members was constructed.

4.5 Cloning of the *PsdSSK1* gene

Tissue samples of leaves, petals, sepals, styles, and pollen of cultivated almond trees were collected and then quickly and completely ground into a powder in a sterile mortar filled with ERR 2182602 liquid nitrogen. Total RNA was extracted using a polysaccharide polyphenol kit from Tiangen, and the integrity of the total RNA was verified via 1% agarose gel electrophoresis[58]. First-strand cDNA was subsequently synthesized using a Fermentas RevertAid First Strand cDNA Synthesis Kit. According to the sequences of the conserved regions of the homologous genes of the target gene available in public databases, a pair of degenerate primers was designed using Primer Premier 5.0 software. PCR amplification of the *PsdSSK1* gene was then performed in which cDNA was as a template. The PCR conditions were as follows: 94 °C predenaturation for 2 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; a 72 °C extension for 10 min; and storage at 4 °C. The PCR products were analyzed via agarose gel electrophoresis and recovered with a spin column-type agarose gel DNA recovery kit. The PCR product was ligated to a pMD-18 vector cloning vector using a T4 ligase kit from Takara. The ligation product was transformed into *Escherichia coli* DH5α competent cells via the heat-shock method (42°C for 45 s). After overnight culture at 37°C, positive monoclonals were selected and sent to the company for sequencing.

4.6 qPCR

Five tissue samples of cultivated almond were ground in a mortar with TRIzol reagent and incubated at room temperature for 5 min. Afterward, chloroform was added for extraction for 3 min, after which the mixture was centrifuged at $12000 \times g$ at 4°C for 15 min. The top colorless liquid was removed and isopropyl alcohol was added to precipitate the RNA. Afterward, the mixture was incubated at room temperature for 10 min. It was then centrifuged at $12000 \times g$ for 10 min at 4°C , after which 75% ethanol in diethyl phosphorocyanidate (DEPC) water was used to clean the RNA. The ethanol was removed by centrifugation, after which the RNA pellet was dried and dissolved in DEPC water. The integrity of the RNA was detected by 1.2% agarose gel electrophoresis[59,60]. A RevertAid First Strand cDNA Synthesis Kit from Thermo Scientific was used to synthesize first-strand cDNA, which was stored at -20°C until use. Using the diluted cDNA as a template and PsdSSK1-F and PsdSSK1-R as upstream and downstream primers, respectively, almond β -actin was selected as an internal reference gene, and actin-F and actin-R were selected as internal reference gene primers. Real-time PCR was subsequently performed. The reaction system (25 μL) consisted of the following: 10 μL of SYBR[®] Green PCR Master Mix, 0.4 μL of forward primer (10 $\mu\text{mol/L}$), 0.4 μL of reverse primer (10 $\mu\text{mol/L}$), 5 μL of diluted cDNA (50 ng/ μL), and 25 μL of RNase-free water[61]. The reaction conditions were as follows: predenaturation at 95°C for 3 min; 40 cycles of 95°C for 10 s, 55°C for 20 s, and 72°C for 20 s; and then 75°C for 5 s. Plate reads were added to collect fluorescence signals (40 cycles). The melting curve analysis temperature range was $65\sim 95^{\circ}\text{C}$, and the temperature was increased by $0.5^{\circ}\text{C}/5\text{ s}$. Three biological replicates were included per sample, three technical replicates were included for each biological replicate, and the program was run after all settings were saved. The obtained cycle threshold (CT) value was quantitatively analyzed via the $2^{-\Delta\Delta\text{CT}}$ method[62].

4.7 Southern blot hybridization

By using a cetyl-trimethylammonium bromide (CTAB) plant genomic DNA rapid extraction kit from Zhongding Company to extract genomic DNA from five tissues of cultivated almond trees, we performed a single digestion of the genome by the use of the restriction enzyme EcoRI. The digested DNA was separated via 0.7% agarose gel electrophoresis at 25 V (constant) and 4°C overnight. The DNA was then transferred to a positively charged HyBond N⁺ nylon membrane via an upward capillary method, after which the membrane was transferred for approximately 20 h, heat, and fixed at 80°C for 2 h[63]. Roche's Digoxin Hybridization Detection Kit II

(chemiluminescence method) was used to synthesize probes and design primers based on the sequence of the *PsdSSK1* gene. PsdSSK1-F (5'-AGGAGGAGAAGAACGACG-3') and PsdSSK1-R (5'-TTCAAAAGCCCAAGCATA-3') were used as the forward and reverse primers, respectively, and the hybridization probe was amplified and synthesized via PCR. The PCR conditions were as follows: 94°C predenaturation for 5 min; 35 cycles of 94°C denaturation for 30 s, 55°C annealing for 30 s, and 72°C extension for 40 s; and 72°C extension for 7 min. The PCR product obtained after labeling with digoxin was analyzed via 1% agarose gel electrophoresis and recovered by an agarose gel purification recovery kit[64]. The cells were subsequently prehybridized at 37°C for 2 h, depleted of the prehybridization solution, added to the hybridization solution containing a digoxin-labeled probe, and hybridized overnight at 37°C[65]. After hybridization, the membrane was washed twice with 20 mL of 2× SSC and 0.1% SDS solution for 5 min each at room temperature. The membrane was then washed twice with 20 mL of 1× SSC and 0.1% SDS solution at 65°C for 15 min each time. The membrane was then equilibrated in 20 mL of washing buffer for 2-5 min, after which it was placed in 10 mL of blocking solution for 30 min. Afterward, 1 mL of CSPD was added to the front side (nucleic acid side) of the membrane, which was then air dried at 15-25°C for 5 min. The excess liquid was removed, after which the membrane was incubated at 37°C for 10 min before machine exposure; the experimental results were recorded[66].

5.8 Western blot hybridization

Samples of leaves, petals, styles, sepals, and pollen tissues of almond trees cultivated in Xinjiang were sampled by the bicinchoninic acid assay (BCA) method. After loading, the polyacrylamide gel was run at 100 V, and the voltage was increased to 160 V until electrophoresis was completed[67]. The gel was removed and transferred to a membrane with a constant current of 280 mA for approximately 1 h. After electroporation was completed, the membrane was removed and washed 4 times with poly(butylene succinate-co-terephthalate) (PBST) for 5 min each. The membrane was blocked in a 5% skim milk powder blocking solution at 37°C for 1 h. A primary antibody was then diluted 1:500 with PBST, and the membrane was incubated at 4°C overnight in the primary antibody dilution[68]. After removing the membrane the next day, the membrane was washed 4 times with PBST for 5 min each time. A secondary antibody was diluted with a blocking solution containing 5% skim milk powder at a ratio of 1:5000. The membrane was allowed to react at 37°C for 1 h with the secondary antibody. After the reaction was

completed, the membrane was removed, and it was washed 4 times in a clean box for 5 min each. Enhanced chemiluminescence (ECL), development, and exposure were then performed[69].

5.9 Construction and subcellular location of the *PsdSSK1* fusion expression vector

The subcellular location of the *PsdSSK1* protein was predicted using the WoLF PSORT II tool. According to the sequencing results and primer design principles of in-fusion construction technology, specific primers (PsdSSK1-GFP-F:GGAATTCATGTCGGCCGAGGAGGAGA and PsdSSK1-GFP-R:CCCAAGCTTGTCTCATCAACTCCTTCAA) with 17 bp vector sequences were designed. The bacterial fluid plasmid containing the correct sequencing results of a pTOPO vector containing the target gene was used as a template, and the reaction system was the same as that used in Section 3.2. Gene sequences containing enzyme restriction sites were amplified via PCR. A PGC-1 vector was digested with the restriction enzymes EcoRI and XhoI to obtain a linearized PGC-1 vector. With in-fusion HD Enzyme Premix, the target gene fragment was inserted into the linearized PGC-1 vector to obtain a PGC1-PsdSSK1 expression vector. The specific operation process was performed according to the instructions of the Novozyme In-Fusion reagent. The plasmid construct was verified via bacterial liquid PCR and sequencing, and the expected positive clones were obtained. The pGC1-PsdSSK1 fusion expression vector construct was transferred into competent *Agrobacterium tumefaciens* GV3101 cells by the freeze-thaw method and then coated in YEB medium that included kanamycin and rifampicin. Monoclones were selected and verified via bacterial liquid PCR, and bacterial liquid containing the correct bands was selected for propagation to an OD600 of 1.0. The samples were subsequently centrifuged at 4000×g for 10 min to collect the thalli. The supernatant was then discarded with resuspended buffer solution (10 mmol/L 2-(N-morpholino)ethanesulfonic acid (MES), 10 mmol/L MgCl₂, 150 μmol/L A), resuspended to an OD600 of 1.0, and incubated at room temperature for 3–4 h. Young and tender tobacco leaves in a good growth state were selected, and the resuspension was carefully injected with a sterile injector into the abaxial side of the leaves for infection. After 2 days of culture, the results were observed by laser confocal microscopy.

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performed the analysis. Jiahui Yang, Wenwen Gao, Yilian Tang and Xintong Ma participated in sample collection and conducted experiments. All authors read and approved the final manuscript.

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Abbreviations

Psd *Prunus dulcis*

GRAVY Grand average of hydropathicity

SI Self-incompatibility

Skp1_POZ S-phase kinase-associated protein 1, tetramerisation domain

UTR Untranslated region

aa Amino acid

bp Base pair

Ka Non-synonymous substitution

Ks Synonymous substitution

ABA Absciscic acid

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