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IDENTIFICATION AND FUNCTIONAL ANALYSIS OF *ABA-INSENSITIVE3* FROM *ROSA CANINA*

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Abstract

ABA-insensitive 3 (*ABI3*), initially identified in *Arabidopsis thaliana*, is intermediary in regulating ABA-responsive genes during seed dormancy inception and seed germination developmental program. In order to study whether the ortholog of *ABI3* from *Rosa canina* was functional, we isolated the ortholog by a combination of degenerate polymerase chain reaction (PCR) and rapid amplification of cDNA ends (RACE). It encodes 718 amino acids with a predicted molecular mass of 79.9kDa and a theoretical isoelectric point of 5.78. The predicted amino acid sequence of the RcABI3 is most closely related to the ABI3 orthologs identified in *Prunus avium* (PaABI3 and PaVP1). Expression analysis revealed that *RcABI3* was expressed in seeds and protocorm-like bodies (PLBs), but not in roots, stems, leaves and flowers. On a cellular level, we localized the RcABI3::GFP fusion protein to the nucleus in onion epidermal cells, which was consistent with the nuclear localization of PsABI3 in *Pisum sativum.* The *RcABI3* is able to restore the *Arabidopsis abi3-6* mutant seed dormancy ability and almost completely rescue the ABA sensitivity during seed germination, which suggest that it is a functional ABI3 ortholog. These results suggest that *RcABI3* is appropriate for application in genetic engineering strategies aimed at regulating seed dormancy and germination in *R. canina* or even in *Rosa* plants.

Introduction

Seed dormancy is an important component of plant fitness that causes a delay of germination until the arrival of a favorable growth season, but too deep dormancy will have a significant impact in practical application (Graeber *et al*., 2012). *Rosa* plants are important ornamental plants widely used in the courtyard, flower bed and flower border around the world. Seed as an important introduction and conventional crossbreeding material for *Rosa* plants, the main obstacle is deep dormancy and low germination percentage (Jin *et al*., 1993; Lu *et al*., 2012). Therefore, we need new varieties that have shallow dormancy and high germination percentage. Study on the function of genes involving seed dormancy and germination will supply foundation for resolving this problem. However, there is no report about these genes in *Rosa* plants.

Abscisic acid (ABA) is a plant hormone that plays a significant role in the regulation of many physiological processes, especially in seed dormancy and germination (Nambara *et al.*, 2010; Rehman *et al.*, 2011; Tabur & Öney, 2012). *ABI3* and *VP1* are orthologous genes from *Arabidopsis* and maize respectively, and they encode transcription factor of B3 domain family (McCarty *et al*., 1991; Giraudat *et al*., 1992). ABI3/VP1 proteins act as intermediaries in regulating ABA-responsive genes during seed dormancy inception and seed germination developmental program (Giraudat *et al*., 1994; McCarty, 1995; Bonetta & McCourt, 1998; Zeng & Kermode, 2004). They specially expressed during zygotic embryogenesis (ZE) and somatic embryogenesis (SE) (Giraudat *et al.*, 1992; Shiota *et al.*, 1998; Ikeda-Iwai *et al.*, 2002; Ikeda-Iwai *et al.*, 2003; Suzuki *et al.*, 2003).

All ABI3/VP1 proteins contain four conserved domains: an acidic activation domain A1 and the three basic domains, B1, B2, and B3 (McCarty *et al.*, 1991; Giraudat *et al.*, 1992; Zeng & Kermode, 2004). A number of *ABI3*/*VP1* genes have been isolated from different species. Reports about the function of *ABI3* gene have been found in *Arabidopsis thaliana*, *Zea mays*, *Pisum sativum* and *Chamaecyparis nootkatensis* (Nambara *et al*., 1994; Suzuki *et al*., 2001; Lazarova *et al*., 2002; Gagete *et al*., 2009), but no in *Rosa* plants. Whether the *ABI3* gene from *Rosa* plants has the similar function or not, it needs study.

Rosa canina is an important medicinal plant (Chrubasik *et al.*, 2008; Fujii & Saito, 2009; Kirkeskov *et al.*, 2011; Tayefi-Nasrabadi *et al.*, 2012), ornamental plant (Tian *et al*., 2008; Jiang *et al*., 2010) and rootstock for cut roses (Kroon & Zeilinga, 1974; Vries & Dubois, 1987). The problems in its seeds are just like those of most *Rosa* plants as mentioned above. Our study aims to isolate *ABI3* gene from *R. canina* and to analyze its function. The seeds of *Arabidopsis abi3-6* mutant are green at maturity, lack dormancy and germinated precociously (Nambara *et al*., 1994). It could be an excellent candidate for the functional analysis of *ABI3* orthologous genes. Our analysis suggest that *RcABI3* is able to restore the *Arabidopsis abi3-6* mutant seed dormancy ability and almost completely rescue the ABA sensitivity during seed germination, which show that it is a functional ABI3 ortholog. It may be candidate gene to regulate seed dormancy and germination in *R. canina* or even in *Rosa* plants.

Materials and Methods

Plant material and growth conditions: Tissue culture seedlings of *R. canina* were maintained at 25 ± 2°C under a 16h light/8h dark photoperiods with a light intensity of 110 μmol m^{-2} s^{-1}. Roots, stems and leaves were cut from tissue culture seedlings. Flowers and seeds were got from the *R. canina* planted in Research Garden of China Agricultural University. PLBs were one structure

developing from rhizoid tips through SE (Tian *et al*., 2008; Jiang *et al*., 2010), and were induced as described by Tian *et al*., (2008). All the samples were frozen immediately in liquid nitrogen and stored at -80^{o}C for RNA extraction.

Seeds of *A. thaliana* were surface-sterilized, and planted on 1/2MS medium. To induce synchronous germination, all seeds were stratified at 4^{o}C for 2 days in the dark and then transferred to a greenhouse at 21^{o}C , with 16 light /8 h dark photoperiods.

RNA and DNA isolation, cloning and sequence analysis of *RcABI3* gene: Total RNA was extracted from the seeds of *R. canina* using RN09-EASY spin Kit (Biomed, Beijing, China) according to the manufacturer's instructions. Total RNA preparations were subjected to an on-column DNase digestion to remove the genomic DNA contamination. The first strand cDNA was synthesized using superscriptII reverse transcriptase (Invitrogen, Beijing, China). Genomic DNA was isolated from young leaves using the NuClean PlantGen DNA Kit (ComWin, Beijing, China).

Primers used in this study were listed in Table 1. Nested polymerase chain reaction (PCR) was performed to obtain a partial sequence of *RcABI3* by using the first strand cDNA of *R. canina* as a template. Two degenerate primers ABFS1 and ABRS1 were used for the first PCR, and then the ABRS1 primer and another primer ABFS2 were used for the second PCR. For 3'-rapid amplification of cDNA ends (RACE), two gene-specific primers, GSP1 and GSP2, were used. And the primers GSP3 and GSP4 were used for the 5'-RACE. The RACE reactions were performed with RACE cDNA amplification kit (Invitrogen, Beijing, China) according to the manufacturer's instruction. A full-length cDNA sequence was obtained by combining the 5'-RACE fragment, intermediate fragment and 3'-RACE fragment together. According to the sequences, a forward primer from the 5'-untranslated region (UTR) (F1) and a reverse primer from 3'-UTR (R1) of *RcABI3* were designed to isolate the complete *RcABI3* from both cDNA and genomic DNA. The amplification products were used to determine the sequences of the *R. canina* cDNA and genomic clone, and the positions of introns in the gene.

The sequence alignment of RcABI3 and other ABI3 sequences were compared by DNAMAN (version 6.0) and the phylogenetic tree was constructed by neighbor-joining (NJ) method with MEGA program (version 4.0).

Semi-quantitative reverse transcription PCR (RT-PCR) assay: To investigate the expression of *RcABI3* in *R. canina*, total RNA was extracted from various *R. canina* tissues and the first strand cDNA was synthesized as described above. The primers RTABIF and RTABIR were used to analyze the expression of *RcABI3*. The *18S rRNA* gene (Genbank accession number: FM164424.1) was performed as a normalization control with primers 18SF and 18SR.

To confirm that the transgenic plants were expressing *RcABI3* gene, total RNA was extracted from the leaves of *abi3-6* mutant, lines transformed with empty vector and *RcABI3*, and the first strand cDNA was synthesized as described above. The expression level was checked using *RcABI3* specific primer ABIF and ABIR. *AtUBQ* was used as a normalization control with primers AtUBQF and AtUBQR (Liang *et al*., 2010).

Table 1. Primers used in this study.

Primer name	Primer sequence (5'-3')
ABFS1	TCCCTCCDCTCCCDGATTTCCCDTGCA
ABRS1	ACYTTHACNCCHGCKATCADATATTT
ABFS2	AAGCTGATTCTTGAGTGGGTTCAAAC
GSP1	GAAGCCGAAACCCATCTTCCTGAGT
GSP2	CCTGAGTTAGAGGCAAGGGACGGAA
GSP3	CATACTGTTGTATCCCCGAGTCTTT
GSP4	GAGATGGTTAGTTTGAACCCACTCA
F1	ACCCACCATCCCCTATCTGATTCCA
R1	TCCCACTTGCAGATAAAATCAAGGG
GFPF	ACGAGCTCGATGGATGGAGTGCAAG
GFPR	GACGTCGACCTGTTTCTTAGAT
RTABIF	CAGCCTCACCGGCAATGATGCAAAC
RTABIR	CCTTTGCGACTTTCCTGGCCTTTTG
18SF	TGCCTAGCAGAACGACCCGAGAACA
18SR	ATCCGTTGCCGAGAGTCGTTTAGAC
OEABIF	AGGACCTAACAGAACTCGCCGTA
OEABIR	CCGAATCGTTACGAGAGTAGTAATT
AtUBQF	AACCCTTGAGGTTGAATCATC
AtUBQR	GTCCTTCTTTCTGGTAAACGT
ABIF	TAAGAAACAGTAAAGGAAGAAAGAA
ABIR	CCTTTGCGACTTTCCTGGCCTTTTG

D=G/A/T; K=G/T; H=A/T/C;N=A/T/G/C

All RT-PCR experiments were repeated at least three times.

Subcellular localization: The *RcABI3* open reading frame (ORF) were cloned into the *Sac*I (GFPF) and *Sal*I (GFPR) sites of the pSAT6-GFP-N1 vector. This vector contains a modified red-shifted green fluorescent protein (GFP) at *Nco*I-*Xba*I sites. The RcABI3 fusion products and a control GFP vector were respectively transformed into onion epidermal cells by particle bombardment as described previously (Wang & Fang, 2002). The transient expression of the fusion proteins and control vector were observed using confocal microscopy.

Generation of over-expression transgenic *A. thaliana* plants: The full-length RcABI3 cDNA sequence was amplified with primer OEABIF and OEABIR. The plasmid 35S::RcABI3 was made by ligating the ORF of RcABI3 into the binary vector pCAMBIA2300 using the *BamH*I and *Xba*I sites. The construct was transformed into *A. thaliana abi3-6* mutant plants via Agrobacteriaum tumefaciens strain GV3101 by the floral-dip method (Clough & Bent, 1998). Desiccated mature seeds were harvested and the putative transformants were identified by growth on kanamycin medium. The transformants were verified as mentioned above. Phenotype of abi3-6, WT, and T3 homozygous transgenic generation seeds was photographed.

ABA sensitivity of seeds expressing the *RcABI3* gene: Dry seeds of WT, over expression (OE)-1, OE-2 and OE-6, and immature seeds of *abi3-6*, empty vector were placed on 1/2MS agar plates containing no ABA or (±)-ABA at different concentrations (0.1-10μM) and then subjected to a 2d moist chilling. After 2d moist chilling, the seeds were transferred to germination conditions to monitor germination percentage. Germination percentage was recorded at day 4. The mark of seeds germination is radicle emergence. At least 100 seeds were used in each treatment, and triplicate treatments were carried out for each ABA concentration.

Results

Isolation of *RcABI3* gene from *R. canina* and sequence analysis: To study the function of *ABI3* gene in *R. canina*, we isolated the *ABI3* ortholog from *R. canina* by 5'-and 3'-RACE PCR. As a result, we identified one *RcABI3* cDNA, designated as *RcABI3*. It was 2780bp containing an ORF of 2157bp and encoding a predicted protein of 718 amino acids (Fig. 1) with a predicted molecular mass of 79.9kDa, and a theoretical isoelectric point of 5.78.

The position of Fig. 1: Genomic fragment corresponding to *RcABI3* gene was isolated by PCR using F1 and R1. Comparison of the *RcABI3* cDNA to the genomic sequence revealed that this gene contained six introns, which was different from *AtABI3* which possessed five introns.

The predicted amino acid sequence of *RcABI3* was compared to other ABI3 proteins from *A. thaliana*, *Z. mays*, *Populus trichocarpa* cv. Trichobel and *Prunus avium* by DNAMAN (Fig. 2). The results showed that RcABI3 possessed all the four conserved domains including an acidic activation domain A1 and the three highly conserved basic regions, B1, B2 and B3. The A1 domain shared a much higher degree of homology with *Zea may* (Fig. 2). The RcABI3 displayed a higher degree of amino acid similarity in these four regions than over the entire protein region.

The position of Fig. 2: To investigate the evolutionary relationships among the predicted ABI3 proteins, a phylogenetic tree was constructed using ABI3 proteins from a taxonomically diverse set of species using the MEGA program (Fig. 3). The phylogenetic tree revealed that the putative RcABI3 was placed in one clade with PaABI3 and PaVP1. RcABI3 shares 45.3%, 29.7%, 51.6%, 51.6% with AtABI3, ZmVP1, PtABI3, PaABI3 proteins, respectively.

Tissue specificity of *RcABI3* expression: To gain a better understanding of the tissue specificity of *RcABI3* expression in *R. canina*, the expression profiles of *RcABI3* gene in various *R. canina* tissues were investigated using a semi-quantitative RT-PCR assay. Various tissues were respectively collected as described in materials and methods. *18S rRNA* gene expression was used as a control. *RcABI3* mRNA was detected in seeds and PLBs, but not in roots, stems, leaves and flowers (Fig. 4).

Localization of RcABI3: For analysis of the subcellular localization of RcABI3, a GFP fusion protein construct of RcABI3 driven by the constitutive Cauliflower Mosaic Virus 35S (CaMV 35S) promoter was introduced into onion epidermal cells by particle bombardment. Confocal microscopic examination showed that the RcABI3-GFP fusion protein was targeted into the nucleus, whereas the control GFP alone was distributed throughout the whole cell (Fig. 5). These results suggested that the RcABI3 protein was a nuclear localization protein.

Functional analysis of *RcABI3*: To determine whether *RcABI3* is functionally conserved, the *RcABI3* ORF driven by the CaMV 35S promoter (*35S::RcABI3*) was expressed in the *Arabidopsis abi3-6* mutant background. The *RcABI3* expression level in transgenic lines was checked by PCR analysis using specific primers (Fig. 6A). Seeds derived from *abi3-6* mutant and transformed with empty vector plants were green, had no dormancy and germinated precociously as described by Nambara *et al*., (1994). However, seeds that expressed a functional *RcABI3* gene exhibited WT-like phenotype at maturity (yellow-brown) and obtained the dormancy ability (Fig. 6B). The seeds of *35S::RcABI3* transgenic lines did not germinate precociously, and could germinate after dormancy like WT. These results suggest that *RcABI3* is able to restore the *Arabidopsis abi3-6* mutant seed dormancy ability.

The position of Fig. 6: For studying the ABA sensitivity of seeds expressing *RcABI3* gene, dry seeds of WT, OE-1, OE-2 and OE-6, and immature seeds of *abi3-6*, empty vector were treated as mentioned in materials and methods. All seeds on media with no ABA showed an equal capacity to germinate (96%-100%; Fig. 7). Seeds of the *abi3-6* mutant and empty vector were highly insensitive to ABA and exhibited 96%-100% germination percentage at all of the ABA concentrations tested. However, seeds of WT plants and *35S::RcABI3* transgenic lines were all sensitive to ABA. Their germination was increasingly inhibited along with the rise of ABA concentration. When the ABA concentration rose up to 10μM, there was no seed germination for WT, and very little seed germination for transgenic lines (Fig. 7). And when the ABA concentration was 15μM, for transgenic lines, there was no seed germination (data not shown). These results suggest that The *RcABI3* almost completely rescue the ABA sensitivity during seed germination.

Discussion

ABA and gibberellin (GA) have essential and antagonistic roles in dormancy and germination. ABA promotes dormancy and inhibits germination, while GA is the opposite (Finkelstein *et al.*, 2008; Rehman *et al.*, 2011; Arefi *et al.*, 2012; Graeber *et al.*, 2012; Nadeem *et al.*, 2012). The seeds of *Rosa* plants are mostly in deep dormancy and difficult to germinate, and the high ABA concentration in the seed coat is considered to be the main reason (Lu *et al*., 2012). To regulate their seed dormancy and germination, we should study function of genes responding to ABA during these two processes.

In this report, *R. canina* ortholog of ABI3 was isolated by a combination of degenerate PCR and RACE. Sequence analysis revealed that the deduced amino acids of *RcABI3* gene contained all four regions that are typically conserved: A1, B1, B2 and B3 (Fig. 2). The same domains were also identified in the AtABI3 amino acid sequence (Giraudat *et al*., 1992). A phylogenetic tree based on amino acid sequences was constructed. It suggests that all the ABI3 proteins originated from the same ancestral origin, which subsequently diverged at different phases of evolution. RcABI3 is most closely related to PaABI3 and PaVP1 from *P. avium* (Fig. 3). Furthermore, subcellular localization analysis of RcABI3 revealed that it was present in the nucleus (Fig. 5). This is consistent with the previously identified nuclear localization of PsABI3 in *P. sativum* (Gagete *et al*., 2009). *RcABI3* was expressed in seeds and PLBs, but not in roots, stems, leaves and flowers (Fig. 4), which was in agreement with the previous research that ABI3 specially expressed during ZE and SE (Rohde *et al.*, 1998; Shiota *et al.*, 1998; Lazarova *et al.*, 2002).

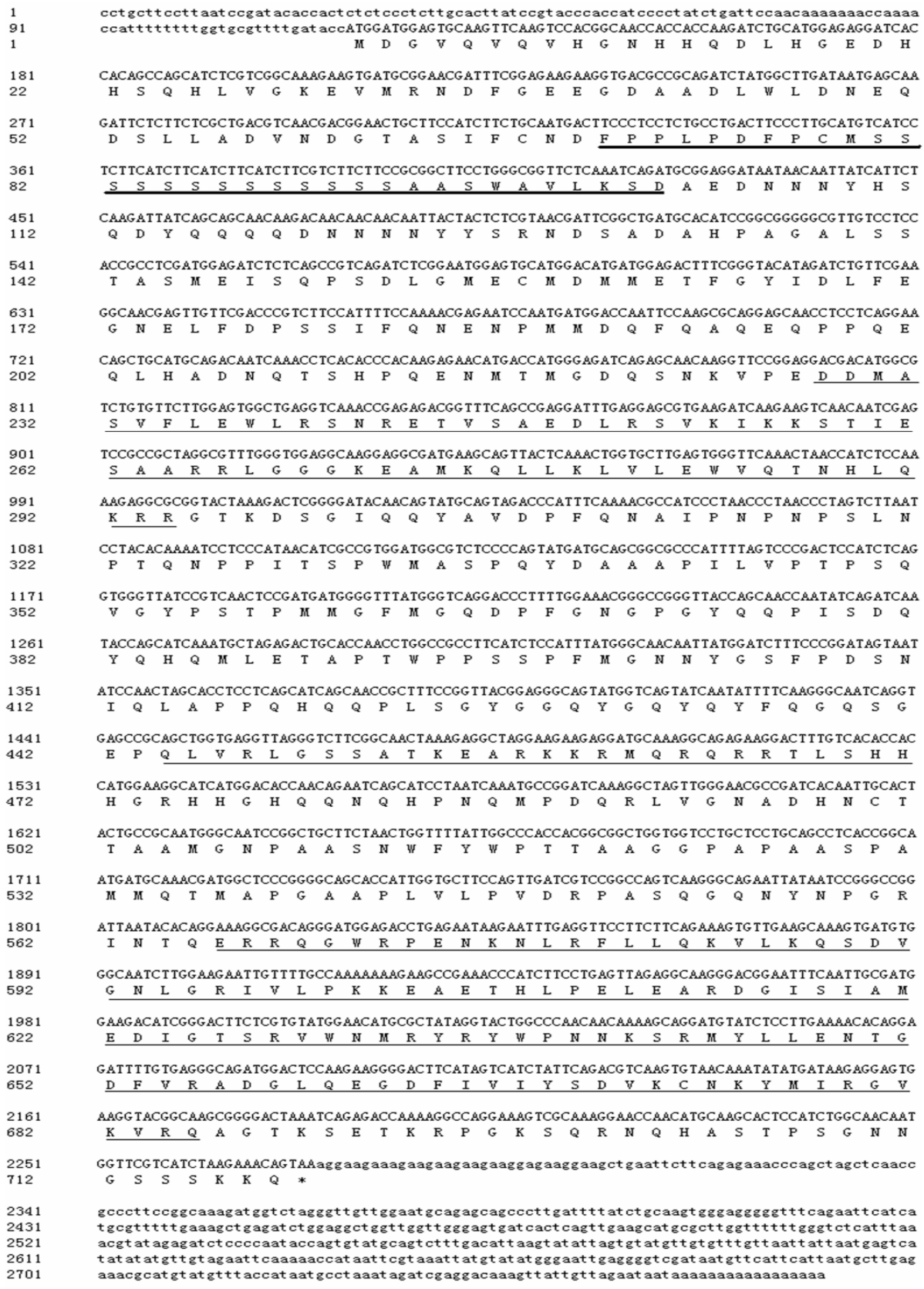

Fig. 1. Nucleotide and deduced amino acid sequence of *RcABI3* (GenBank accession No. JX126487). The A1 domain is underlined in bold black line, and the B1, B2 and B3 domains are underlined in regular black lines.

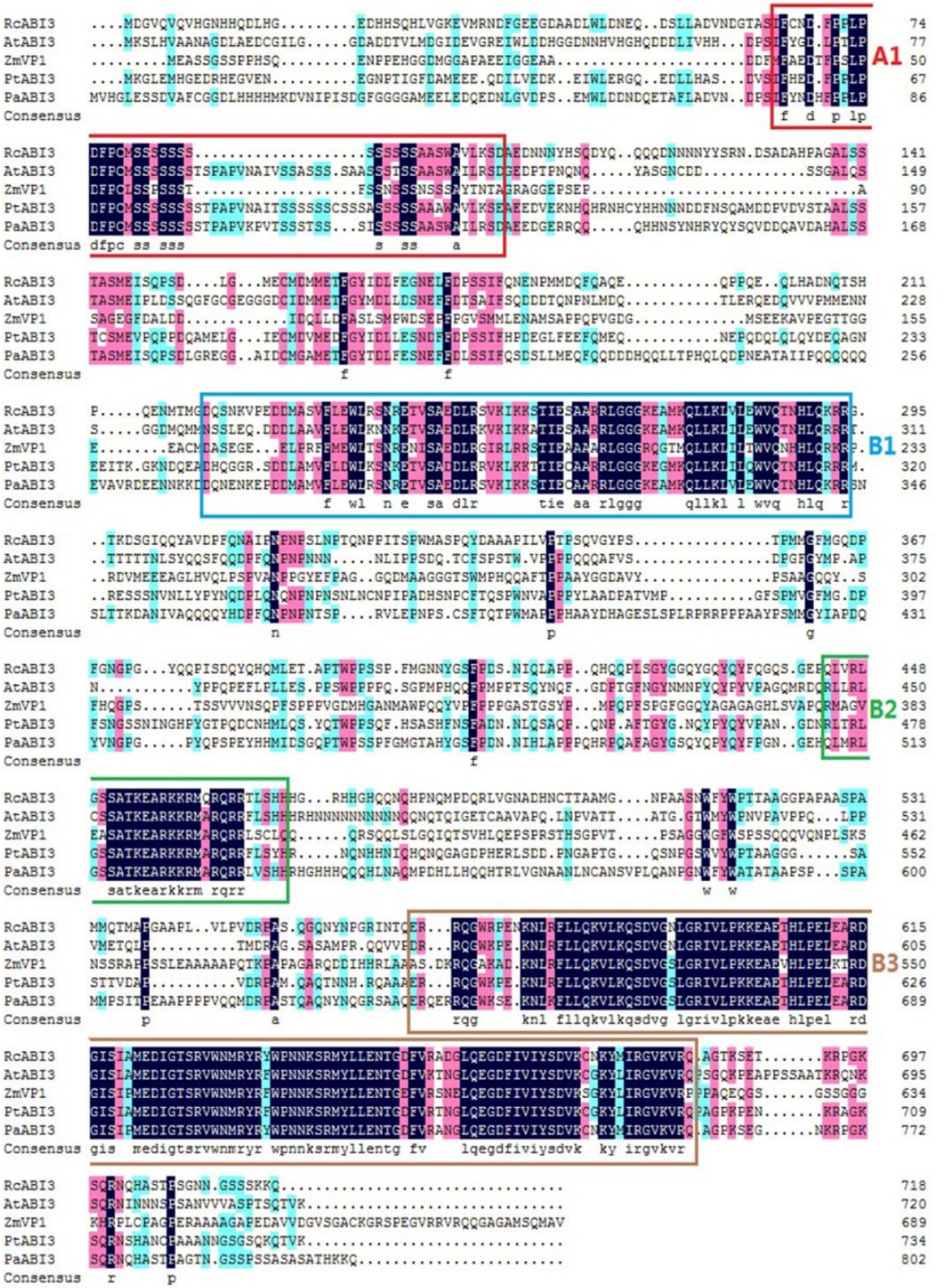

Fig. 2. The alignment of the identified RcABI3 with other ABI3/VP1 proteins. RcABI3, AtABI3 (*Arabidopsis thaliana* ABI3), ZmVP1 (*Zea mays* VP1), PtABI3 (*Populus trichocarpa* cv. Trichobel ABI3), PaABI3 (*Prunus avium* ABI3) were aligned. The four conserved domains correspond to the previously described: the acidic activation domain A1 (red) and the three highly conserved basic regions, B1 (blue), B2 (green) and B3 (brown).

A comparison of the *RcABI3* cDNA sequence with its corresponding genomic DNA showed that this gene contained six introns within the *RcABI3* ORF (data not shown), which indicated that the genomic DNA had not been conserved throughout evolution, as the *RcABI3* gene in *Arabidopsis* contained five introns (Giraudat *et al*., 1992). Interestingly, over-expression of *RcABI3* in *Arabidopsis* was able to restore the *abi3-6* mutant seed dormancy ability and almost completely rescue the sensitivity to ABA during germination, despite having only 45.3% amino acid similarity to AtABI3 (Figs. 6 and 7). These results not only demonstrate that RcABI3 is a functional homologue of AtABI3, but also that the important structural regions of AtABI3 have also been conserved throughout evolution. These regions allow it to interact with other proteins in the ABA signal transduction pathway. The conservation of the *ABI3* gene indicates the importance of the ABA signaling pathway during seed dormancy and germination.

Our results presented here suggest that *RcABI3* is appropriate for application in genetic engineering strategies aimed at regulating seed dormancy and germination in *R. canina or* even in *Rosa* plants through inducible antisense constructs in transgenic plants.

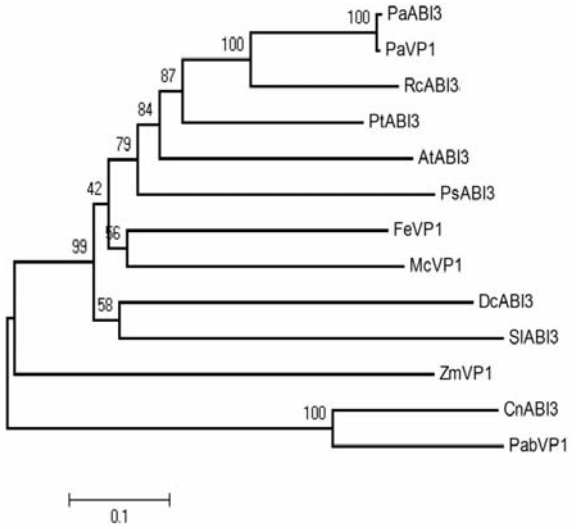

Fig. 3. Phylogenetic tree analysis of RcABI3 and other ABI3/VP1 proteins. The tree was constructed by neighbor-joining (NJ) method with MEGA program. Branch numbers represent percentage of bootstrap values in 1000 sampling replicates and scale indicates branch lengths. The accession numbers are as follows: PaVP1 (*P. avium* VP1, AF411073.1), PaABI3 (*P. avium* ABI3, AF426832.1), PtABI3 (*P. trichocarpa* cv. Trichobel ABI3, AJ003166.1), McVP1 (*Mesembryanthemum crystallinum* VP1, AB015183.1), PsABI3 (*Pisum sativum* ABI3, AB080195.1), AtABI3 (*A. thaliana* ABI3, X68141.1), DcABI3 (*Daucus carota* ABI3, AB005558.1), SlABI3 (*Solanum lycopersicum* ABI3, NM_001247740.1), PabVP1 (*Picea abies* VP1, AF175576.1), ZmVP1 (*Z. mays* VP1, NM_001112070.1), FeVP1 (*Fagopyrum esculentum* VP1, AB099513.1), CnABI3 (*Chamaecyparis nootkatensis* ABI3, AJ131113.1), RcABI3(*R. canina* ABI3, JX126487).

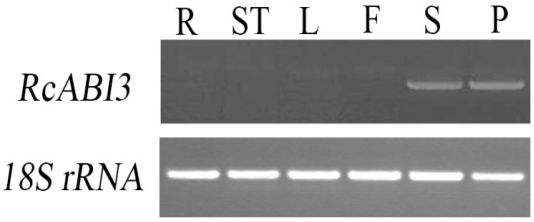

Fig. 4. Expression patterns of *RcABI3* in different tissues. Expression patterns of *RcABI3* in roots, stems, leaves, flowers, seeds and PLBs. *18S rRNA* gene expression was used as a control. The data presented are typical of three independent experiments.

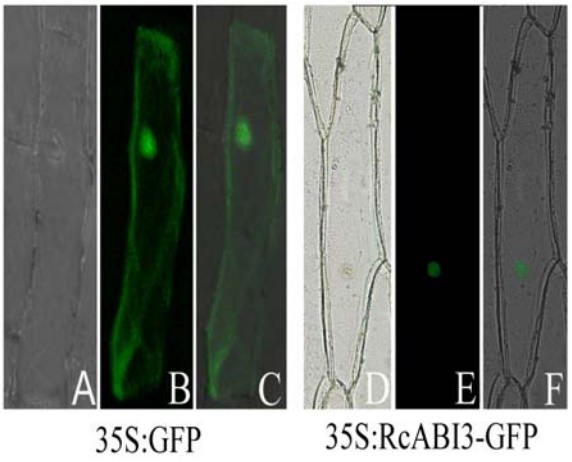

Fig. 5. Subcellular localization of RcABI3 in onion epidermal cells. Bright-field images (A and D), fluorescence images (B and E) and the merged images (C and F) of control and 35S::RcABI3-GFP fusion protein are shown.

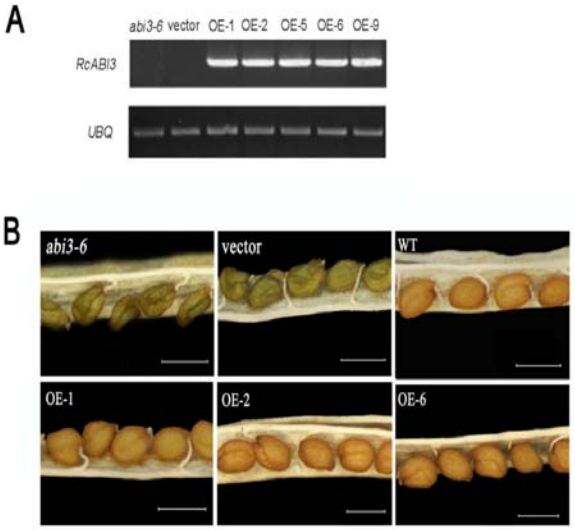

Fig. 6. Phenotype of seeds expressing the *RcABI3* gene. A. The expression level of *RcABI3* in *abi3-6* mutant, lines transformed with empty vector and *RcABI3*. *AtUBQ* gene expression was used as a control. The data presented are typical of three independent experiments. B. Seeds of *abi3-6*, empty vector, WT and T3 generation of *35S::RcABI3* transgenic lines (OE-1, OE-2, OE-6). Bar=500μm.

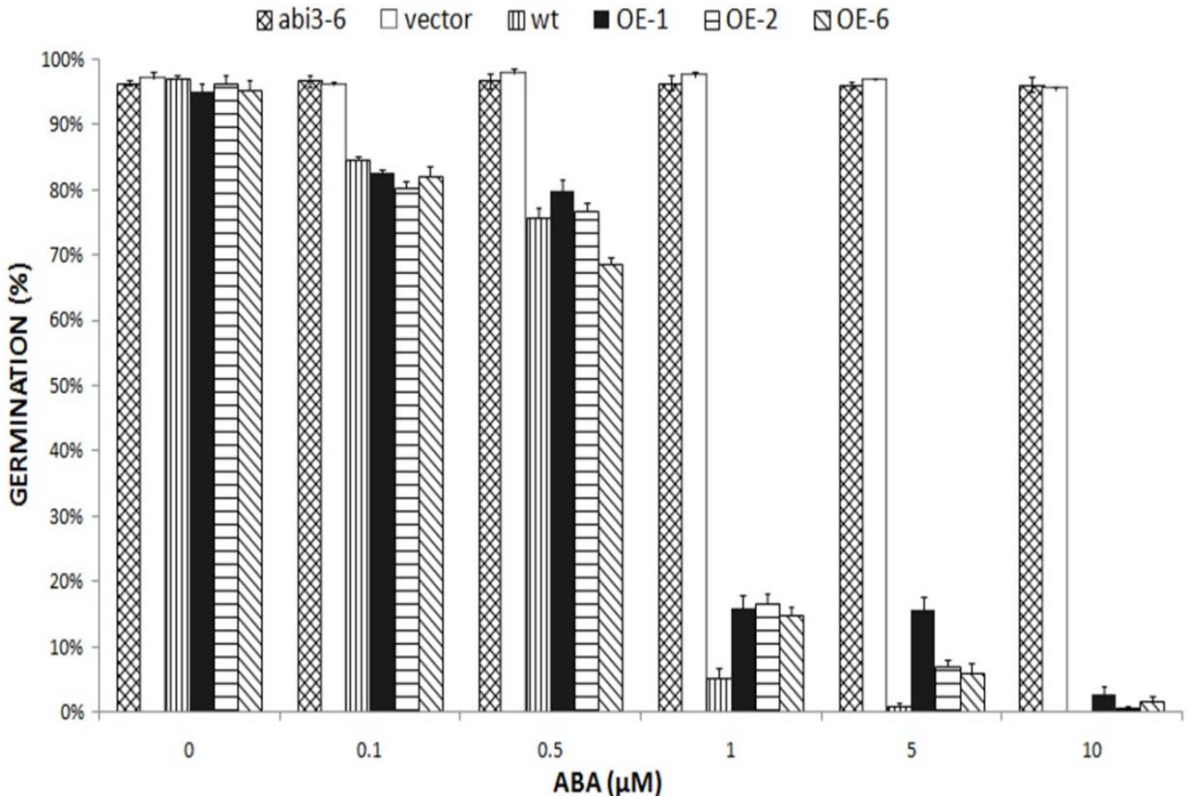

Fig. 7. ABA sensitivity of seeds expressing the *RcABI3* gene. Dry seeds of WT, OE-1, OE2 and OE-6, and immature seeds of *abi3-6*, empty vector were placed on 1/2MS agar plates containing no ABA or (±)-ABA at different concentrations (0.1-10μM) and then subjected to a 2d moist chilling. After 2d moist chilling, the seeds were transferred to germination conditions to monitor germination percentage. The germination percentage was recorded at day 4. At least 100 seeds were used in each treatment, and triplicate treatments were carried out for each ABA concentration.

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