IDENTIFICATION AND FUNCTIONAL ANALYSIS OF ABA-INSENSITIVE3 FROM ROSA CANINA

YANG HUI-FANG1, XU KE-DONG2, KOU YA-PING1, ISHIK ABDURAZAK1, LI JUN-XIANG1 AND ZHAO LIANG-JUN1*

1Department of Ornamental Horticulture and Landscape Architecture, China Agricultural University, 2 Yuanmingsuan West Road, Haidian District, Beijing 100193, People’s Republic of China
2Key Lab of Plant Genetics & Molecular Breeding of Department of Life Science, Zhoukou Normal University, East Wenchang Street, Chaunhua District, Zhoukou, Henan 466001, People’s Republic of China
*Corresponding author’s e-mail: zhaolj5073@sina.com; Tel: 86-010-62733315; Fax: 86-010-62733316

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 PaABI3 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 (Graebert 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, 1994). 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; Shioita 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; Gauget 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; Fuji & 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 & Zeitlinga, 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°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°C for 2 days in the dark and then transferred to a greenhouse at 21°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 ABR51 were used for the first PCR, and then the ABR51 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 RTABIF and RTABIR.

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 *abi3-6* 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 AtUBQIR (Liang et al., 2010).

### Table 1. Primers used in this study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABFS1</td>
<td>TCCCTCDDCDDCDDGATTTCCDDTGGCA</td>
</tr>
<tr>
<td>ABR51</td>
<td>AICYTHACNCHGCKATCDADATTTT</td>
</tr>
<tr>
<td>ABFS2</td>
<td>AACGCTATTCGATGCTTCATCAAC</td>
</tr>
<tr>
<td>GSP1</td>
<td>GAAAGCAGAAACCATTTCTCGAGT</td>
</tr>
<tr>
<td>GSP2</td>
<td>CCTGAGTTCAGGCAAAGGGACGGA</td>
</tr>
<tr>
<td>GSP3</td>
<td>CATACTGTTGATCTCCCAGGTCCTT</td>
</tr>
<tr>
<td>GSP4</td>
<td>GAGATGTTGTTTGGACACCACTCA</td>
</tr>
<tr>
<td>F1</td>
<td>ACCCACCATTCCCCTATCGTTCA</td>
</tr>
<tr>
<td>R1</td>
<td>TCCCAAATTCCAGATAAAATCGAGG</td>
</tr>
<tr>
<td>GFPR</td>
<td>GACGCTCTGATGAGTGAGTGGTAAG</td>
</tr>
<tr>
<td>RTABIF</td>
<td>CAGGCCTCAACGCAAGATGAGAAC</td>
</tr>
<tr>
<td>RTABIR</td>
<td>CTTTTGCGACTTTCCTCGCTTGTG</td>
</tr>
<tr>
<td>18SF</td>
<td>TGCTTAGCAGACGACGCCGAAAC</td>
</tr>
<tr>
<td>18SR</td>
<td>ATCCCTTTGCGAGTGGTCTGAGTCAG</td>
</tr>
<tr>
<td>OEABIF</td>
<td>AGGACCTTAAACAGAAACTCGCGTA</td>
</tr>
<tr>
<td>OEABIR</td>
<td>CCGATCTGCTACAGGAGTGTAAATG</td>
</tr>
<tr>
<td>AuUBQF</td>
<td>AACCCCTTGAGTGAATCATC</td>
</tr>
<tr>
<td>AuUBQR</td>
<td>GTCCCTTCCTTGCTAGAAC</td>
</tr>
<tr>
<td>ABIF</td>
<td>TAAGAAAAACGTAAAGGAGAAAGAA</td>
</tr>
<tr>
<td>ABIR</td>
<td>CTTTGGCACTTTCGCTGTTT</td>
</tr>
</tbody>
</table>

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 SacI (GFPP) and SalI (GFPR) sites of the pSAT6-GFP-N1 vector. This vector contains a modified red-shifted green fluorescent protein (GFP) at NcoI-XbaI 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 BamHI and XbaI sites. The construct was transformed into *A. thaliana* abi3-6 mutant plants via Agrobacterium tumefaciens strain GV3101 by the floral-dip method (Clough & Bent, 1998). Descinated mature seeds were harvested and the putative transormants 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 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 2177bp 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 *Pumus 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* ab3-6 mutant background. The RcABI3 expression level in transgenic lines was checked by PCR analysis using specific primers (Fig. 6A). Seeds derived from ab3-6 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* ab3-6 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 ab3-6, 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 ab3-6 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 gibberelin (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 \textit{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), PaABI3 (Populus trichocarpa cv. Trichobol 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 (Giraudeau 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.
IDENTIFICATION AND FUNCTIONAL ANALYSIS OF ABA-INSENSITIVE3 FROM ROSA CANINA

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.

Acknowledgements

We are grateful to Professor Peter McCourt (Department of Cell and Systems Biology, University of Toronto, Canada) for providing the Arabidopsis abi3-6 mutant seeds. This research was supported by the Surface Project from National Natural Science Foundation of China (Grant No. 30871733).

References


(Received for publication 18 October 2012)