The role of Populus MYB94 transcription factor in seed germination requires the expression of ABA-responsive genes

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ABSTRACT
Plant seed germination is easily influenced by endogenous gene expression, hormones and environmental conditions. Here, we investigated a Populus R2R3-MYB transcription factor (referred to as MYB94) that plays a role in seed germination in close relation to the hormone abscisic acid (ABA). Two independent Arabidopsis transgenic lines overexpressing MYB94 (Oe-lines) and wild-type (WT) were no clear differences in the seed germination rates under normal conditions, but the germination rates of the two Oe-lines were both significantly delayed under ABA treatment. This delay in germination is most likely associated with the endogenous hormone abscisic acid (ABA). We find that the transcript levels of several representative genes in relation to ABA synthesis or signaling in the MYB94 Oe-lines showed significantly increased compared with those of the WT plants. Taken together, our results here reveal the role of the Populus R2R3-MYB transcription factor MYB94 in regulating the ABA-responsive genes in plant seed germination.

Crop plants and their production are easily affected by both internal and external factors, including gene expression, small molecules and environmental stress conditions (Hu et al. 2006; Yang et al. 2012; Todaka et al. 2015). As an important part of the plant life cycle, seed germination is closely related to agricultural production. Therefore, it is of high significance to study the effects of internal and external factors regulating plant seed germination. In the last 20 years, a variety of genetic elements or internal factors in plant cells involved in seed germination have been well characterized, which increases our knowledge in this area (Clerkx et al. 2003; Lara et al. 2003; Lee and Seo 2015). In general, seed germination requires the coordination of a variety of physiological and biochemical processes, including the promotion of receptors to perceive or respond to small molecules such as hormones and environmental signals, the transduction of complex signaling and the control of gene expression to coordinate a variety of intracellular changes in plants (Finkelstein et al. 2002; Monke et al. 2004; Nakashima and Yamaguchi-Shinozaki 2013).

Among the multiple factors involved, the roles of abscisic acid (ABA) have been intensively investigated for years using model plants such as Arabidopsis, which has led to various genes being characterized and our understanding of cellular gene expression being strengthened (Finkelstein et al. 2002; Himmelbach et al. 2003; Cutler et al. 2010). These results are significant for research on plant growth, development and environmental adaptability. ABA content in cells can accumulate quickly when plants are subjected to stress conditions, hence triggering the activation of the expression of numerous genes and changes in cellular activities, such as transcription regulators-controlled ABA- and stress-responsive gene expression, stomatal transpiration in leaves and others (Himmelbach et al. 2003; Wasilewska et al. 2008; Cutler et al. 2010). Seed germination and dormancy are closely related to endogenous ABA. Additionally, the transcription factors such as ABI3, ABI4 and ABI5, which are from different types of gene families, also regulate ABA-responsive gene expression in seeds (Rohde et al. 2000; Lopez-Molina et al. 2002; Haslekas et al. 2003; Park et al. 2011; Feng et al. 2014; Mittal et al. 2014).

The MYB transcription factor is capable of interacting with downstream and upstream target genes via its conserved DNA domains to activate or inhibit the expression of target genes. MYB transcription factor genes of different subgroups in this gene family were divided based on the number of conserved domains (Yanhui et al. 2006; Wilkins et al. 2009; Dubos et al. 2010). In 1982, Klempnauer and colleagues identified the first v-MYB gene in poultry myeloma virus (avian myeloblastosis virus) (1982), subsequently, an increasing number of MYB transcription factor genes were revealed in the plant genome based on sequence analysis and functional identification and were found to be involved in various aspects of plant regulation (Yanhui et al. 2006; Wilkins et al. 2009; Dubos et al. 2010; Xu et al. 2017). The regulation of MYB gene expression provides an improvement basis for the environmental adaptability of crop plants. For example, transgenic Arabidopsis overexpressing the rice MYB3R-2 gene showed a significant improvement in freezing, salt and drought resistance (Dai et al. 2007). In addition, several regulators of MYB family in Arabidopsis play roles that are closely related to plant small molecules and
hormones. For instance, AtMYB2 not only positively regulates plant drought stress, but also can interact Nitric oxide (NO) to modify its DNA-binding activity (Abe et al. 2003; Serpa et al. 2007); the AtMYB96 can regulate abscisic acid signals to mediate seed germination (Lee et al. 2014, 2015; Lee and Seo 2015).

From a global perspective, woody plants are important economic and ecological tree species that are critical as regional and global resources and components of the environment (Brunner et al. 2004; Wilkins et al. 2009). In woody plants, such as Populus spp., most of the functions and working mechanisms of MYB genes remain unknown to date (Mellway et al. 2009; Fang et al. 2017; Xu et al. 2017). Previously, MYB94 was found to be involved in regulating drought stress (Fang et al. 2019). Here, we further studied the function of the transcription factor MYB94 in response to ABA during seed germination.

Results

**MYB94 plays a role in seed germination**

Two independent overexpression lines of the MYB94 gene were created (Oe-lines), L1 and L3, and used for further testing (Figure 1(A)). Under normal conditions, it appeared that there were no large differences in germination between the Oe-lines and Wt plants (Figure 1(B), 0 µM ABA treatment). However, the seed germination of the transformants displayed more inhibition than that of Wt plants under stress conditions. The two Oe-lines L1 and L3 both showed more sensitivity to ABA in seed germination. For instance, upon 0.25 µM ABA treatment, at 4 d, close to 70% of Wt seeds germinated, while only around 41% of L1 and 20% of L3 seeds germinated (Figure 1(B)). Relatively, as shown in Figure 1(B), treatment with higher ABA concentrations (1.0 µM), the two Oe-lines displayed lower germination rates than the Wt plants. Because the changes in responses to exogenous ABA probably reflect endogenous modifications of ABA-related processes in cells, the results indicate that MYB94 plays a role in the ABA-regulated inhibition of seed germination.

**MYB94 regulates the expression of ABA-responsive genes**

Given the effect of ABA-stress on prolonged seed dormancy in association with gene expression activity, we speculate that the MYB transcription factor MYB94 may mediate the endogenous gene expression and hence affect the associated seed germination. This may be dependent on the MYB-responsive gene expression. The expression of MYB94 in Arabidopsis causes significantly increased sensitivity to ABA and is likely involved in regulating ABA-responsive gene or signaling. Semi-qRT-RCR were performed and we found that the expression levels of a variety of stress- and ABA-responsive genes were changed (Figure 2), such as ABA2, a short-chain dehydrogenase/reductase, and ABI1.

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**Figure 1.** Transformsants conferred on overexpression of MYB94 display more sensitivity to exogenous abscisic acid (ABA) during seed germination. (A) Screening of two independent lines (L1 and L3) with well Hygromycin (25 µg/ml) resistance compared with the Arabidopsis wild-type (Wt) plants, Bar = 5 mm; and the two lines were conferred relatively higher transcriptional expression of the Populus MYB94 gene based on RT-PCR results; (B) Germination rates of the testing plants, three independent replications were performed for the mean levels; and more than 30 seeds for the Wt and each transformed lines were performed in each group of the testing assay; *= or **= on the bars showed an significant variation (P < 0.05 or P < 0.01) between Wt and the transformant plants based on the t-test.

**Figure 2.** ABA- and stress- related genes were significantly up-regulated in the transformants conferred on overexpression of MYB94. The gene accession number could be traced according the gene name on NCBI or TAIR.
the type 2C protein phosphatase, which are involved in ABA biosynthesis and signaling in plants, respectively (Gosti et al. 1999; Gonzalez-Guzman et al. 2002). The results showed that MYB94 regulates the expression of ABA-responsive genes in the Oe-lines.

Discussion

For most plants, reproduction initiates from seed germination in their life history. In natural populations, germination means an assurance of plant new location and growth initiation. For agriculture, to some extent, it always influences crop production. In practise, genes, hormones and other molecules plus environmental conditions coordinate seed germination and later growth (Finkelstein et al. 2002; Piskurewicz et al. 2009; Todaka et al. 2015). For woody plants, the processes remain much unknown.

At present, research on the growth and development of Populus is accelerating (Tsai et al. 2006; Jiang et al. 2014; Cho et al. 2016). However, compared with other crop plants, there is still much work needed to further reveal the rules of woody plants. Concentration systematic investigation of the environmental conditions, ABA and other small molecules and the endogenous gene expression and their interactions is required. Here in the study, we demonstrate that the Populus MYB transcription factor MYB94 plays a role in plant seed germination in an ABA-dependent manner (Figure 1B) and Figure 2). The Oe-lines showed more sensitive to exogenous ABA than Wt plants. And it is interestingly found that the critical gene, ARA2 transcript level increased, which probably enhanced ABA biosynthesis in plant by it encoded enzyme activity. Other gene expression, such as the transcript levels of RD29B and ADH1 (Dolferus et al. 1994; Nakashima et al. 2006), could be tightly linked with the ABA level, suggesting an endogenous variation of the stress hormone in the biosynthesis or signaling. There was no doubt that these results from the expression of the Populus MYB transcription factor MYB94.

Transcription factors confer an ability to bind potential DNA site by their conserved repeat domains to play critical roles. MYB94 is in the R2R3 MYB transcription factor family. Changes in the transcript level of the ABA-responsive genes in the two Arabidopsis Oe-lines have provided several clues of MYB94 mediated gene expression. However, how MYB94 regulates the expression of potential target genes and interacts with several sites on the targets in this process through its active domain requires further investigation. For instance, for the role of the MYB transcription factor, it is essential to reveal that the biochemical and physiological detail of MYB94 mediating up-regulation of ABI1 and ABA2 genes in Arabidopsis or Populus in the future.

Materials and methods

Plant materials and growth conditions

The wild-type Arabidopsis thaliana Col-0 and transformants with overexpression of the gene in the Col-0 background were used in this study (Fang et al. 2019). The plant seedlings were grown in the 1/2 MS solid medium supplemented with 1% sucrose. For the treatment of ABA, the Arabidopsis seeds were first sterilized and then plated on the medium supplemented with ABA with different concentrations according to the required testing. Plants were grown in the light-controlled incubator with a cycle of 16 h light and 8 h dark.

Creation of the transformants with overexpressing MYB94

Full-length cDNA was amplified using gene-specific primers (Supplementary Table 1) and inserted into the pCX-SN vector (Chen et al. 2009) in which the target gene was driven by the cauliflower mosaic virus 35S (CaMV35S) promoter. The construct was introduced into Agrobacterium tumefaciens strain EHA105, which was then used for the Wt Arabidopsis transformation by the floral dip method. Homozygous T3 progeny were screened for transformants with hygromycin (30 μg mL-1) added to the 1/2 MS agar medium and primer-specific PCR.

RNA preparation and RT-PCR

For gene transcriptional expression, the Arabidopsis seedlings of 10 d old cultured in 1/2 MS agar medium were separately harvested using liquid nitrogen and then used for RNA extraction with Trizol reagent (Invitrogen). After treatment to remove genomic DNA with DNase I enzyme, total RNA from different lines were subjected to reverse transcription into first-strand cDNA, and then semi-qRT-PCR was performed by using Actin gene as an endogenous control. Three repeats were conducted and the PCR products were subjected to agarose gel electrophoresis to evaluate the transcript level.

Seed germination

Transgenic and Wt mature seeds were harvested and stored at 4°C for more than one month and then were plated on 1/2 MS agar (1.0%) medium after being surface sterilized. After pre-culturing at 4°C for 72 h, the medium plates which supplemented with different concentrations of ABA (0.25, 1.0 μM) were then transported into the light-controlled incubator for germination, and plates without added ABA were set as the control. Then, every 12-/24 h, the number of seeds germinating, which was considered when the primary seeds were just breaking through the seed coats, were counted. For each line in the assay, no fewer than 40 seeds were used for each condition, and three repeats were conducted for an average level.

Statistical analysis

Statistical methods, t-test were performed using SPSS 16.0 (IBM SPSS Statistics). Differences between means were considered significant when the value $P < 0.05$ according to the tests.

Accession numbers

The genes described in this study can be retrieved on line from the Arabidopsis Genome Initiative or GenBank of NCBI database using the accession numbers and gene alias listed in supplementary Table 2.
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H.W. and H.H. conducted the experiments. Q.F. contributed to manuscript preparation. Q.F. wrote the manuscript. Q.F. supervised the project. All the authors contributed to the discussion.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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