ANALYSING THE FUNCTION AND THE EXPRESSION PATTERN OF AUXIN RESPONSE FACTOR B3 FROM MEDICAGO TRUNCATULA IN THE MODEL PLANT LOTUS JAPONICUS

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Abstract


In plants, Auxin Response Factors (ARFs) regulate gene expression in response to auxin and may act as a transcriptional activators or repressors. ARF proteins bind to auxin response elements (AuxREs) in auxin-responsive gene promoters. Auxin Response Factor B3 from Medicago truncatula (MtARF-B3) was heterologously expressed in the model legume Lotus japonicus. Stable transgenic plants, overexpressing MtARF-B3 and transcriptional reporters were created. In addition, MtARF-B3 ortholog gene of L. japonicus was downregulated and knockdown plants were constructed. Phenotypic and morphological evaluation, quantitative real-time polymerase chain reaction (qRT-PCR) and histochemical GUS assay were used to study the function and expression pattern of MtARF-B3 in the process of somatic embryogenesis and development of tissues and organs. A complex analysis of the obtained results suggests that MtARF-B3 play role in root architecture and in fertility of the model legume L. japonicus.

Key words: Auxin Response Factor B3; gene expression; Lotus japonicus; plant growth; plant development; fertility

Abbreviations: MtARF-B3 – Auxin Response Factor B3 from Medicago truncatula; OE – overexpression; RNAi – RNA interference; WT – wild type

Introduction

One of the most important crops, naturally found in all terrestrial habitats and used as a source of human food and animal feed, biomass for biofuel production and valuable secondary metabolites with an important biomedical and pharmaceutical application, are part of the family Fabaceae (Doyle and Luckow, 2003; Graham and Vance, 2003; Choi et al., 2004; Bonanomi et al., 2009; Gholami et al., 2014). Over the past decade two plant species Medicago truncatula (Cook, 1999) and Lotus japonicus (Stougaard, 2001) were proposed as model systems for molecular genetic studies and analysis related to symbiotic nitrogen fixation process in legumes (Ferguson et al., 2010; Ferguson and Mathesius, 2014). L. japonicus belongs to determinate nodulating legume group and it is self-pollinated diploid plant with small genome, short life cycle, high level of seed production and easy and rapid growth under laboratory conditions (Jiang and Gresshoff, 1997; Szczyglowski et al., 1998). It can be easily transformed by Agrobacterium tumefaciens resulting in stable transformants (Handberg and Stougaard, 1992; Oger et al., 1996; Martiriani et al., 1999; Pacios-Bras et al., 2002; Revalksa et al., 2015). Because of the close relation of L. japonicus to the forage species L. corniculatus (birdsfoot trefoil) (Young and Udvardi, 2009), the knowledge about structure and function of numerous genes, biochemical characteristics and metabolic pathways, could be transferred to legume crop plants but also to non-legume crops such as
sunflower, tomato, corn and rice (Desbrosses et al., 2005; Iantcheva et al., 2015).

In plants, phytohormone auxin is known to regulate different aspects of growth and development (Finet et al., 2010; Lau et al., 2011). Different studies revealed that transcriptional regulation of auxin response genes are regulated by two transcription factor (TF) families called Auxin Response Factors (ARFs) and Aux/IAA (Perez-Rodriquez et al., 2010).

In *L. japonicus* 75 members of ARF gene family are found - 3 times more than the number in *Arabidopsis thaliana* (Sato et al., 2008; Ma et al., 2010). The identified 23 AtARF members in *Arabidopsis* play role in various developmental processes (Yang et al., 2004; Okushima et al., 2005). In soybean (*Glycine max*), 43 typical GmARFs were discovered by phylogenetic analyses (Van Ha et al., 2013). Using hairy roots transformation tool, 30 ARFs, participating in the development of roots and nodules, were identified in the model legume plant *M. truncatula* (Bustos-Sanmamed et al., 2013).

Based on *in silico* analyses another group of researchers characterized 24 MtARFs (Shen et al., 2015).

In our research, the gene of interest, named *MtARF-B3*, was initially identified by using reverse genetic approach (Revalská et al., 2011) in a population of Tnt1 insertional mutants of *M. truncatula* belongs to AgroBioInstitute, part of world-wide collections (d’Erfurth et al., 2003; Tadege et al., 2008; Iantcheva et al., 2009). Based on Blast analyses it was found that the position of Insertion 3 in the mutant line 5928 partially corresponds to a gene encoding Auxin response factor, containing DNA-binding pseudobarrel and B3-binding domains (*Mt5g040880*, PLAZA 3.0 Dicots). It has been found that *MtARF-B3* corresponds to *MtARF17d* (Bustos-Sanmamed et al., 2013) and thereafter *MtARF15* (Shen et al., 2015).

To investigate the function and expression pattern of *MtARF-B3*, the gene was heterologously expressed in *L. japonicus* and assessed by the reporter genes β-glucoronidase and green fluorescent protein (GUS and GFP). Moreover, stable transgenic plants were created by both *MtARF-B3* overexpression (OE) and downregulation of its ortholog gene in the model species, showing its important role in general developmental processes and fertility.

**Materials and Methods**

*Molecular cloning and construction of expression clones for transformation*

GATEWAY cloning (Invitrogen Life Technologies, Inc., http://www.lifetechnologies.com) was used to obtain recombinant plasmids (Karimi et al., 2002). Endogenous promoter was constructed by inserting ~2.0 kb fragment upstream of the start codon of *MtARF-B3* gene (*Mt5g040880*, PLAZA 3.0 Dicots) into pDONRP4P1R donor vector. The Entry clone was recombined into the pEX-KSNFm14GW (promoter-NLS-GUS-GFP) destination vector, possessing neomycin phosphotransferase (*nptII*) gene as a selection marker for transgenic plants to create Expression clone. The

<table>
<thead>
<tr>
<th>Cloning primers</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>attB1</td>
<td>5'-GGGACTGCTTTTTTGTACAAAACCTG-3'</td>
</tr>
<tr>
<td>attB2</td>
<td>5'-GGGCACTTTTGTGTAAGAAAGCTGGT-3'</td>
</tr>
<tr>
<td>attB4</td>
<td>5'-GGGACAACTTTTGTATAGAAAGTTGCT-3'</td>
</tr>
<tr>
<td>F-ARF-B3-OE</td>
<td>5'-ATGTCTTTCAGCAACGCC-3'</td>
</tr>
<tr>
<td>R-ARF-B3-OE</td>
<td>5'-TTTTTGTGCAAGTTGATGCC-3'</td>
</tr>
<tr>
<td>F-ARF-B3-RNAi</td>
<td>5'-GTACAAAAAAGCAGGCTGGGAATGATAAAAAGCAATGG-3'</td>
</tr>
<tr>
<td>R-ARF-B3-RNAi</td>
<td>5'-GTACAAAGAAGGCTGGTGTACGACCGCAAACT-3'</td>
</tr>
<tr>
<td>F-ARF-B3-pro</td>
<td>5'-TGTTCTGAATATATAATCGG-3'</td>
</tr>
<tr>
<td>R-ARF-B3-pro</td>
<td>5'-GGCCGAGAGATACAAAG-3'</td>
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<tr>
<td>Transcript level evaluation primers</td>
<td></td>
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<tr>
<td>F-Mt-actin</td>
<td>5'-TCATATGCTGCTGCATGTATG-3'</td>
</tr>
<tr>
<td>R-Mt-actin</td>
<td>5'-ACTCCAACCGCTACCAGAACCC-3'</td>
</tr>
<tr>
<td>F-Mt-Ubiquitin</td>
<td>5'-GCAATAGAAGCAGGCTGG-3'</td>
</tr>
<tr>
<td>R-Mt-Ubiquitin</td>
<td>5'-TACTCTTGGGCAGGCAAATAA-3'</td>
</tr>
<tr>
<td>F-Mt-ARF-B3 MT5G040880</td>
<td>5'-CAGCAGGTCAAGGTGGT-3'</td>
</tr>
<tr>
<td>R-Mt-ARF-B3 MT5G040880</td>
<td>5'-GGACTGACTCGCTGACGCAAC-3'</td>
</tr>
<tr>
<td>F-Lj- ARF-B3 LJ1G010860</td>
<td>5'-AGGAGGAGGAGGACGAGGAG-3'</td>
</tr>
<tr>
<td>R-Lj- ARF-B3 LJ1G010860</td>
<td>5'-TCAACCTGGACGAATCCA-3'</td>
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full length ORF of the gene was introduced into pDONR221 donor vector and overexpression lines were created. For this purpose pK7WG2 or pK7FWG2 (a C-terminal translational GFP fusion) binary destination vectors were used, as the Entry clone was transferred under the control of CaMV 35S promoter and nptII gene for plant selection (Karimi et al., 2007).

RNA interference (RNAi) strategy was applied for creation of silencing constructs (Limpens et al., 2003). The region of endogenous Lotus gene (Lj1g010860, PLAZA 3.0 Dicots; LjARF-B3) with highest silencing capacity was in silico predict by Xwin Razor software program and pK-7GW1WG2D (II) hairpin RNA expression vector was used. Lj1g010860 was silenced at positions 595-794 bp upstream of the ATG codon within the 5’ untranslated region (5’-UTR), and at positions 1272-1370 bp downstream of the stop codon in the 3’-UTR.

The obtained expression clones were introduced into A. tumefaciens strain C58C1, maintained on 1.5% agar solidified YEB nutrient medium, supplemented with 100 mg/l rifampycin (Rif), 100 mg/l spectinomycin (Sp) and 50 mg/l gentamycine (Gm). The primers for promoter and gene cloning were designed with Primer 3 software program (Table 1).

Plant material, genetic transformation and growth conditions

Seeds of L. japonicus ecotype Gifu B-129, a kind gift of Dr. Hiroshi Kouchi, were surface sterilized using the protocol for M. truncatula seed sterilization (Iantcheva et al., 2015; Revalksa et al., 2015), germinated on Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) and grown in Magenta boxes (60 × 60 × 96 mm, Sigma), at growth chamber (24°C, with a 16-h photoperiod and light intensity of 350 μmol m–2 s–1). Previously described protocol for genetic transformation, based on Agrobacterium-mediated method of leaf discs (Iantcheva et al., 2015; Revalksa et al., 2015), was established to generate plants with ARF-B3 OE, downregulation and transcriptional reporters. After root formation, putative transgenic plants were screened by the hydroponic pots for root inoculation with Mesorhizobium loti MAFF 319090 (2x10⁸ cells/ml) and was changed once or twice a week, depending on the age of plants.

Phenotypic and morphological analysis

Plants with MtARF-B3 OE and LjARF-B3 downregulation were grown under in vitro, in vivo and hydroponic conditions, and assessed for morphological changes compared to Gifu B-129 wild type plants (WT), used as a control. Randomly chosen Km positive T₀ lines, where the level of ARF-B3 transcripts was up-/down-regulated, were characterized for general plant growth and development, size, root system morphology, nodule number, flower development and seed production. The T₁ progeny of selected OE lines with obvious morphological changes and altered transcript levels were further analysed.

Quantitative real-time PCR (qRT-PCR) assays

By using RNeasy Plant Mini Kit (EurEx), total RNA was extracted from lines with MtARF-B3 OE and knockdown of its ortholog Lj1g010860, and WT plants. Primers for gene expression evaluation, created with software pro-
gram Primer 3, were designed to amplify a non-conserved region of the gene (Table 1). First Strand cDNA Synthesis kit (Fermentas) was used to reverse transcribed equal amount of total RNA and relative expression levels were determined with the 7300 Real-Time PCR System (Applied Biosystems). Actin (ACT) and ubiquitin (UBQ10) were used for data normalization, as reference genes. MtARF-B3 transcript level was analysed in in vitro OE lines (T0) and their progeny (T1), while for the RNAi lines, LjARF-B3 expression level was determined only in T0 plants, because downregulation of LjARF-B3 led to sterile plants, unable to produce offspring.

Statistical analysis

Performed experiments were repeated three times and triplicate assays were done for each experimental data set. The data were analysed using OriginPro 8.5.1 and qBase 1.3.5 softwares. Differences were assessed by t-test. Results were expressed as means ± standard deviation (SD). Statistically significant differences were considered when p < 0.05.

Results

The function and expression pattern of Auxin response factor, containing DNA-binding pseudobarrel and B3-binding domains from M. truncatula (Mt5g040880, PLAZA 3.0 Dicots; MtARF-B3) was studied in the model legume plant L. japonicus. MtARF-B3 belongs to the gene subfamily ORTHO03D0200736, which consists of six genes in M. truncatula. The ortholog of MtARF-B3 in L. japonicus is Lj1g010860 (PLAZA 3.0 Dicots) and its function has not been characterised yet. The best ortholog of Lj1g010860 in A. thaliana is Auxin response factor 17 (http://bioinformatics.psb.ugent.be/plaza/versions/plaza_v3_dicots/genes/view/LJ1G010860). In all of the conducted experiments confirmed transgenic T1 OE and transcriptional reporter lines, and T0 RNAi lines from L. japonicus were used.

Analyses of GUS and GFP activity in transcriptional reporter plants of L. japonicus (pMtARF-B3::GUS-GFP)

GUS expression was observed at all stages of indirect somatic embryogenesis (Figure 1A). In young T1 plants, MtARF-B3 signal was detected in vascular system of the roots, leaf epidermal tissue and in the points of active growth, in stems, flower sepalas, stamens, as well as in pollen (Figure 1 B-G). Spotted expression was observed in the seed pods of hydroponic plants (Figure 1 H). Expression of GFP marker gene was found in secondary roots primordia (Figure 1 I), in root meristem and in symbiotic nodules of hydroponic grown plants (Figure 1 J).

Relative expression level of MtARF-B3 and its ortholog gene in OE and RNAi lines of L. japonicus

Transcript level of MtARF-B3 and its ortholog LjARF-B3 was determined in several transgenic lines (Figure 2). The primers, used for gene expression evaluation were designed to amplify non-conserved region of the gene. In addition, we evaluated the expression level of endogenous Lotus gene (Lj1g010860) in plants with MtARF-B3 OE and found that the transcript level of Lj1g010860 correlates with transcript level in the WT plants (data not shown). MtARF-B3 expression profile in the T1 and T0 OE lines of L. japonicus was significantly higher compared to WT control (p < 0.01) (Figure 2 A). The transcript level of LjARF-B3 was slightly decreased in RNAi-3 and RNAi-5 lines (p < 0.5) and up to 7 times lower in RNAi-10 and RNAi-7, than in WT (p < 0.01) (Figure 2 B). The transcript level of MtARF-B3 and its ortholog was also evaluated in the symbiotic nodules of T0 OE and RNAi lines of L. japonicus (Figure 2 C), where MtARF-B3 expression level was higher than in WT (p < 0.001), while the expression level of LjARF-B3 was decreased (p < 0.0001).

Phenotypic and morphological analyses of L. japonicus plants with MtARF-B3 overexpression and knockdown of the gene ortholog

Large pool of L. japonicus T1 OE and RNAi plants from different lines were screened. Confirmed transgenic plants were transferred to greenhouse on soil or to hydroponic system on nutrient solution for T1 seeds production. The obtained RNAi lines were sterile and offspring was not produce. T1 seeds from OE lines were grown on Km selective medium and these with segregation in the classical Mendelian manner (3:1) were grown for T1 progeny. MtARF-B3 and LjARF-B3 expression levels were evaluated in the T1, respectively T0 seedlings (Figure 2 A, B). Independent T1 OE lines were used for further phenotypic alterations and T2 seeds production.

We analysed in vitro T1 OE, in vivo and hydroponically grown T0 OE, and T1 RNAi lines from the model legume plant. Experiment for producing T1 progeny of the OE lines is in progress. Obtained T1 RNAi lines were sterile and even if they produce seed pods, the seeds were defective and not able to germinate. The in vitro lines overexpressing MtARF-B3 did not display any obvious changes in the root morphology and architecture (Figure 3 A row). Plants from four independent T0 lines grown under greenhouse and hydroponic conditions had shorter stems, and
short, thick and branched roots (Figure 3 A row). These plants were early flowering, forming numerous flowers and pods (Figure 3 A row), and showed faster growth than WT. Nevertheless seed number obtained from $T_0$ OE lines was less compare to WT plants (Table 2). The examined OE lines formed fewer nodules than WT (Table 2).

For primary phenotypic screen we analysed four independent $T_0$ RNAi lines from $L. japonicus$. The in vitro $LjARF-B3$ knockdown plants had thick and short stems, and reduced root growth, forming typical “tripod” architecture (Figure 3 B row). Hydroponically cultivated RNAi lines also displayed „tripod“ root system shorter than in WT (Figure 3 C row) with very few nodules ($3.66 \pm 1.52$) (Figure 3 B row; Table 2). The aerial part was poorly developed with curved leaves and abnormal flowers, most of it sterile but some of them formed few small narrow curled pods (Figure 3 B row). The seeds were underdeveloped, very thin and failed to germinate.

Fig. 1. Expression pattern of $pMtARF-B3::GUS:GFP$ in $Lotus japonicus$: (A) different stages of somatic embryogenesis; (B) seedling stage; (C) leaf; (D) leaf epidermis; (E) in vitro roots; (F) flower sepals, stem and stamens; (G) pollen; (H) seed pod; (I) secondary root primordia; (J) vasculature of nodule.
Fig. 2. Relative expression level of MtARF-B3 and its ortholog gene in *Lotus japonicus*. (A) *MtARF-B3* transcript level in *T₀* and *T₁* OE lines and WT plants; (B) *LjARF-B3* transcript level in *T₀* RNAi lines and WT plants; (C) *MtARF-B3* and *LjARF-B3* transcript level in nodules from *T₁* OE and *T₀* RNAi lines and WT plants.

Fig. 3. Morphological evaluation of OE and RNAi lines of *Lotus japonicus*. (A) row – OE lines – *in vitro* roots, roots of hydroponically cultivated plant, roots with nodules, aerial part, seed pod; (B) row – RNAi lines – *in vitro* roots, roots of hydroponically cultivated plant, roots with nodules, aerial part, seed pod; (C) row – WT plants – *in vitro* roots, roots of hydroponically cultivated plant, roots with nodules, aerial part, seed pod.

Table 2

<table>
<thead>
<tr>
<th><em>Lotus japonicus</em> transgenic lines and control</th>
<th>Number of nodules of <em>T₀</em> lines</th>
<th>Number of <em>T₁</em> seeds</th>
</tr>
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<tbody>
<tr>
<td><em>T₀</em> MtARF-B3 OE line 3</td>
<td>40</td>
<td>183</td>
</tr>
<tr>
<td><em>T₀</em> MtARF-B3 OE line 8</td>
<td>20</td>
<td>197</td>
</tr>
<tr>
<td><em>T₀</em> MtARF-B3 OE line 9</td>
<td>35</td>
<td>205</td>
</tr>
<tr>
<td><em>T₀</em> MtARF-B3 OE line 37</td>
<td>50</td>
<td>210</td>
</tr>
<tr>
<td></td>
<td>36.25±12.5</td>
<td>198.75±11.79</td>
</tr>
<tr>
<td><em>T₀</em> LjARF-B3 RNAi line 3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>T₀</em> LjARF-B3 RNAi line 10</td>
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<td>0</td>
</tr>
<tr>
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<td>0</td>
</tr>
<tr>
<td><em>T₀</em> LjARF-B3 RNAi line 7</td>
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</tr>
<tr>
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<td>3.66±1.52</td>
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<tr>
<td><em>L. japonicus</em> control</td>
<td>78</td>
<td>430</td>
</tr>
<tr>
<td><em>L. japonicus</em> control</td>
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<td>74</td>
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<td>73.33±5.03</td>
<td>411.33±22.05</td>
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Discussion

In order to evaluate the function of MtARF-B3, the 35S-MtARF-B3 construct was heterologously expressed in L. japonicus. Besides, MtARF-B3 ortholog gene was downregulated in the model legume plant, applying RNAi strategy for creation of silencing constructs. The expression pattern of MtARF-B3 was studied on the base of generated nuclear-localised transcriptional reporters (pMtARF-B3::GUS-GFP). Gene function was assessed in the OE and RNAi lines and MtARF-B3 essential role in plant growth and development was confirmed. In L. japonicus however knockdown of LjARF-B3 was associated with RNAi binding to the 5'-UTR and 3'-UTR regions of Lj1g010860. Previous studies have shown that a specific gene or genes can be successfully silenced by ds-siRNAs targeting the 3'-UTR (McManus et al., 2002; Mosellin et al., 2004). GUS reporter gene expression was localised at the callus tissue and all stages of somatic embryogenesis, suggesting an involvement of MtARF-B3 in somatic embryogenesis. In A. thaliana several auxin response factors (AtARF10, AtARF16 and AtARF17) play an important role in the control of embryogenesis. Various embryonic defects caused by abnormal cell divisions during embryogenesis are mainly attributed to the altered expression pattern of AtARF16 and AtARF17 (Liu et al., 2010).

Gene reporter activity was detected in different tissues and organs of L. japonicus transcriptional reporter plants during their growth and development. The GUS signal was observed in the vascular system of the roots, as well with GFP expression in the secondary roots, root meristem and nodules. Similar expression pattern was documented for AtARF7, where the expression is observed during the early developmental stages of lateral root primordia and dissipates from the meristematic region after the root primordia emerge from the primary roots (Okushima et al., 2005).

L. japonicus plants with OE and downregulation showed drastic phenotypic alterations in root morphology. RNAi lines had severely reduced root length and tripod architecture. Most likely the knockdown of LjARF-B3 is the reason for these drastic changes in the roots and for decreased nodule number, compared with WT. It has been shown that M. truncatula plants overexpressing miR160, which negatively regulates eight predicted MtARFs, are characterized with reduced root size and nodule number (Pacious-Bras et al., 2013). In L. japonicus, the low-nodulating phenotype of rel3 mutant is due to de-repression of ARF3 and ARF4 by deficiency in TAS3 ta-siRNA biogenesis (Li et al., 2014). Overexpression of miR160 to silence a set of ARFs belonging to the ARF10/ARF16/ARF17 family in soybean, led to production of auxin-hypersensitive plants with significantly fewer nodules compared to control vector (Turner et al., 2013). In L. japonicus T3 OE lines root system remained shortened, even it was well developed. This could be the reason for decreased nodule number in these lines, compared to WT.

In transcriptional reporter plants of L. japonicus, spotted localisation of the GUS signal in the growing regions of leaf petiole was observed. Any expression was not found in the vasculature. It could be proposed that ARF-B3 may contribute to the regulation of leaf blade expansion in L. japonicus. In all of the investigated OE lines the relative transcript level of MtARFB3 was increased, as compared to WT. L. japonicus knockdown lines displayed decreased transcript level of LjARF-B3, as compared to the WT. Furthermore, all of these lines possess similar morphological alterations, as tripod root architecture, small and curved pods and abnormal seeds, without germination capability. Moreover, all RNAi lines of L. japonicus were sterile and displayed underdeveloped plant organs. Analysis of GUS reporter activity in flowers and pollen revealed expression of MtARF-B3 gene of interest. The obtained phenotypic and histochemical results suggest that observed phenotype of L. japonicus RNAi lines is due to knockdown of LjARF-B3 gene. Our results are in an agreement with previously reported results in A. thaliana for expression of AtARF-B3 in flowers and pollen (Liu et al., 2010; Yang et al. 2013). This fact could explain the sterility of the generated LjARF-B3 RNAi legume plants. In addition, AtARF17 from A. thaliana is the closest ortholog of MtARF-B3 in M. truncatula and to LjARF-B3 in L. japonicus and plays a role in pollen wall formation and pollen tube growth, and also affects plant development and fertility (Mallory et al., 2005; Liu et al., 2007).

The investigated MtARF-B3 gene was expressed in different plant tissues and organs, and has specific functions in plant growth and development. Plants with proper expression of MtARF-B3 displayed normal seed development and germination. We assumed that knockdown of LjARF-B3 caused sterility in the generated RNAi plants. Resent progress in molecular biology, genomics, transcriptomics, metabolomics, proteomics and bioinformatics for model legumes provides new opportunities for investigation and analysis of these species (Revalska et al., 2011). Genomes of M. truncatula and L. japonicus share considerable genetic synteny with crop legumes, which facilitates gene discovery and understanding the relationships between genes and phenotypes. Successful translation of accumulated knowledge from models to practical agriculture would lead to new discoveries about developmental process and application of new strategies for crop improvement.
Conclusion

Primary phenotypic screen and expression pattern examination of the GUS and GFP reporter genes, driven by endogenous MtARF-B3 promoter, were conducted in *L. japonicus* plants with *MtARF-B3* overexpression, *LjARF-B3* downregulation and transcriptional reporters. Expression of reporter genes were localized in all stages of somatic embryogenesis, during post-embryonic plant development, in stamens, pollen and growing zone of symbiotic nodules. This study revealed the essential role of *MtARF-B3* in general growth and development of the model legume *L. japonicus*, root system architecture and pods and seeds development. We assumed that knockdown of this gene cause abnormal pod development and seed production, and this is the main reason for sterility in *L. japonicus*.

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