Bulgarian Journal of Agricultural Science, 22 (No 2) 2016, 253–261
Agricultural Academy

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

REVALSKA, M., V. VASSILEVA, G. ZEHIROV and A. IANTCHEVA, 2016. Analysing the function and the expression pattern of Auxin response factor B3 from *Medicago truncatula* in the model plant *Lotus japonicus*. *Bulg. J. Agric. Sci.*, 22: 253–261

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 transfomants (Handberg and Stougaard, 1992; Oger et al., 1996; Martrirani et al., 1999; Pacios-Bras et al., 2002; Revalska 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

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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 (Revalska 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-K7SNFm14GW* (promoter-NLS-GUS-GFP) destination vector, possessing neomycin phosphotransferase (*nptII*) gene as a selection marker for transgenic plants to create Expression clone. The

Table 1

Primers used for cloning and transcript level evaluation

Cloning primers	
attB1	5'-GGGGACTGCTTTTTTGTACAAACTTGC-3'
attB2	5'-GGGGACCACTTTGTACAAGAAAGCTGGGT-3'
attB4	5'-GGGGACAACTTTGTATAGAAAAGTTGCT-3'
F-ARF-B3-OE	5'-ATGTCTTCTCAGCAACGCC-3'
R-ARF-B3-OE	5'-TTTTTGTGCAAGTTTGATGCC-3'
F- ARF-B3-RNAi	5'-GTACAAAAAAGCAGGCTGGGAATGATAAAAGCAATGG-3'
R-ARF-B3-RNAi	5'-GTACAAGAAAGCTGGGTGCTGTCAGTACCGGCAAACT-3'
F-ARF-B3-pro	5'-TGTTCGAATTATATAATCGG-3'
R-ARF-B3-pro	5'-GGCGGAGAGATTAACAAG-3'
Transcript level evaluation primers	
F-Mt-actin	5'-TCAATGTGCCTGCCATGTATGT-3'
R-Mt-actin	5'-ACTCACACCGTCACCAGAATCC-3'
F-Mt-Ubiquitin	5'-GCAGATAGACACGCTGGGA-3'
R-Mt-Ubiquitin	5'-AACTCTTGGGCAGGCAATAA-3'
F-Mt-ARF-B3 MT5G040880	5'-CAACGGCTAAAGGTTGGTGT-3'
R-Mt-ARF-B3 MT5G040880	5'-GGACTGACTCGCTCTGGAAC-3'
F-Lj- ARF-B3 LJ1G010860	5'-AGGAGGAGAGGAGCAAGGAG-3'
R-Lj- ARF-B3 LJ1G010860	5'-TCA ACCTGGACGAATCATCA-3'

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-7GWIWG2D (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; Revalska 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; Revalska 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 PCR for the presence of *nptII* gene for Km resistance. Following gene specific primers were used to amplify a 550 bp fragment: FW 5'-GAACAAGATGGATTGCACGC-3' and REV 5'- GAAGAACTCGTCAAGAAGGC-3'. 30-days old Km positive seedlings were transferred into greenhouse or hydroponic conditions for seed production. The hydroponic containers contained Fahraeus nutrient solution with following composition: 1 g/l $CaCl_2.2H_2O$, 1.2 g/l $MgSO_4.7H_2O$. 1 g/l K_2HPO_4, and 1.5 g/l $Na_2HPO_4.12H_2O$. A starting dose of nitrogen (21 mg/l $Ca(NO_3)_2.4H_2O$), Gibson microelements and ferric citrate (0.005 g/l) were added to the solution. The aeration and pH were kept constant. Plants were grown for 20 to 40 days under light intensity of 200 µmol m^{-2} s^{-1}, with a 16-h photoperiod and day/night temperature around 25ºC/20ºC, respectively. The bacterial suspension was added to the hydroponic pots for root inoculation with *Mesorhizobium loti* MAFF 319090 ($2x10^8$ cells/ml) and was changed once or twice a week, depending on the age of plants.

Histochemical GUS staining, light and confocal microscopy

GUS activity assay was performed as described previously (Gefferson et al., 1987; Willemsen et al., 1998). Different plant tissues or whole seedlings were incubated in 90 % acetone for 30 min at 4 ºC, afterwards washed in phosphate buffer (pH 7.0), follow by incubation in GUS staining solution at 37 ºC. The GUS solution was composed of 5 mg 5-bromo-4-chloro-3-indolyl-*β*-d-glucuronide dissolved in 50 µl formamide, 5 ml 100 mM sodium phosphate buffer (pH 7.0), 200 µl 0.5 M Na_2EDTA, 10 µl Triton X-100, 1 ml 1 mM $K_4Fe(CN)_6x3H_2O$, 1 ml 1 mM $K_3Fe(CN)_6$, 2 ml methanol and adjusted to 10 ml final volume with 740 µl H_2O.

Samples (leaves, roots, pollen) from the examined transcriptional reporter plants were mounted on microscope slides, examined and photographed using Carl Zeiss Axio scope A1 microscope with HIGH CONTRAST DIC (differential interference contrast) coupled to a XC50 digital microscope camera. Fluorescence imaging of roots and nodules was performed by an Axiovert100M confocal laser scanning microscope with software package LSM510 version 3.2 (Zeiss). For excitation of GFP, the 488 nm line of an argon laser was used.

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 T_0 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 T_1 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 (T_0) and their progeny (T_1), while for the RNAi lines, *LjARF-B3* expression level was determined only in T_0 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 T_1 OE and transcriptional reporter lines, and T_0 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 T_1 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 sepals, 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 T_0 and T_1 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 T_0 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* T_0 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 T_1 seeds production. The obtained RNAi lines were sterile and offspring was not produce. T_1 seeds from OE lines were grown on Km selective medium and these with segregation in the classical Mendelian manner (3:1) were grown for T_1 progeny. *MtARF-B3* and *LjARF-B3* expression levels were evaluated in the T_1, respectively T_0 seedlings (Figure 2 A, B). Independent T_1 OE lines were used for further phenotypic alterations and T_2 seeds production.

We analysed *in vitro* T_1 OE, *in vivo* and hydroponically grown T_0 OE, and T_0 RNAi lines from the model legume plant. Experiment for producing T_2 progeny of the OE lines is in progress. Obtained T_0 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 T_0 lines grown under greenhouse and hydroponic conditions had shorter stems, and

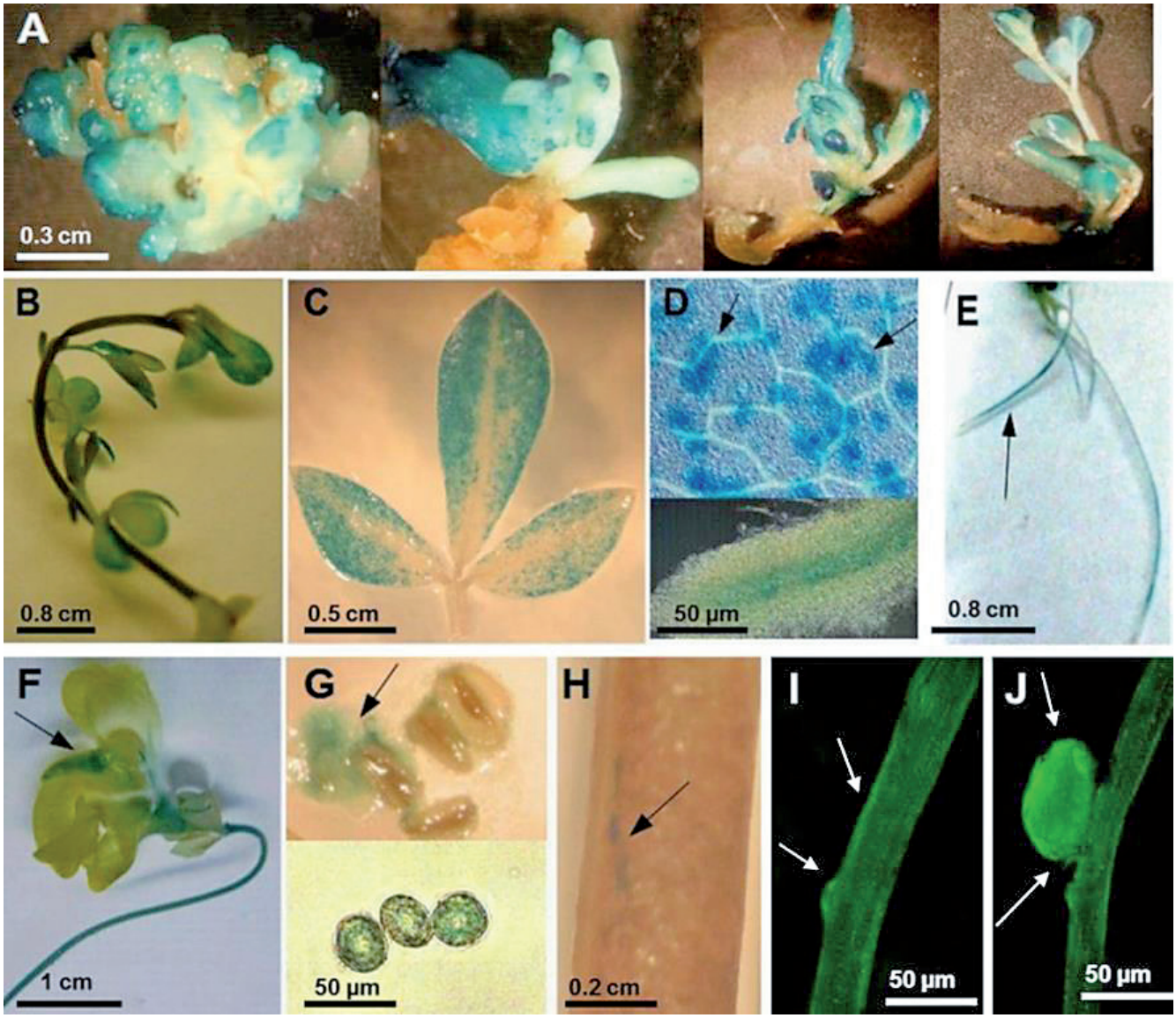

Fig. 1. Expression pattern of *pMtARF-B3::GUS:GFP* in *Lotus japonicus*: (A) different stages of somatic embryogenesis; (B) seedling stage; (C) leaf; (D) leaf epidermis; (E) *in vitro* roots; (F) flower sepals, stem and stamens; (G) pollen; (H) seed pod; (I) secondary root primordia; (J) vasculature of nodule.

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 ± 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.

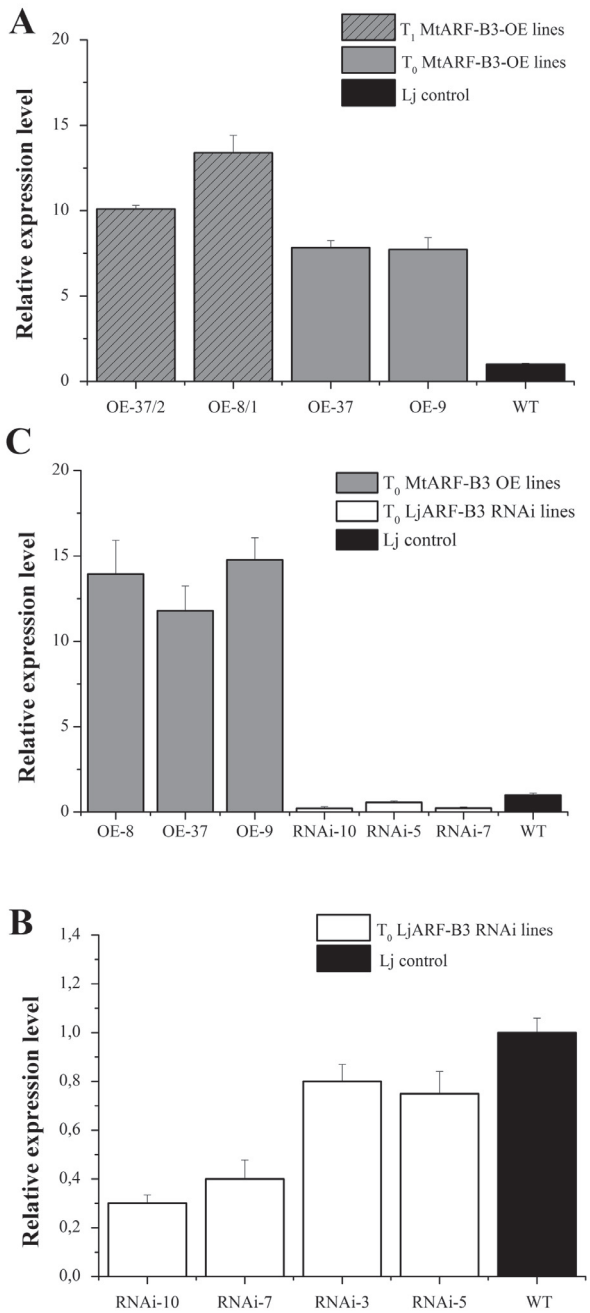

Fig. 2. Relative expression level of *MtARF-B3* and its ortholog gene in *Lotus japonicus*. (A) *MtARF-B3* transcript level in T_0 and T_1 OE lines and WT plants; (B) *LjARF-B3*transcript level in T_0 RNAi lines and WT plants; (C) *MtARF-B3 and LjARF-B3* transcript level in nodules from T_1 OE and T_0 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 hidroponicaly 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

Number of transgenic seeds and nodules in T_0 and T_1 lines from *Lotus japonicus*

Lotus japonicus transgenic lines and control	Number of nodules of T_0 lines	Number of T_1 seeds
T_0 MtARF-B3 OE line 3	40	183
T_0 MtARF-B3 OE line 8	20	197
T_0 MtARF-B3 OE line 9	35	205
T_0 MtARF-B3 OE line 37	50	210
	36.25±12.5	**198.75±11.79**
T_0 LjARF-B3 RNAi line 3	0	0
T_0 LjARF-B3 RNAi line 10	4	0
T_0 LjARF-B3 RNAi line 5	2	0
T_0 LjARF-B3 RNAi line 7	5	0
	3.66±1.52	**0**
L. japonicus control	78	430
L. japonicus control	68	417
L. japonicus control	74	387
	73.33±5.03	**411.33±22.05**

Discussion

I*n 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 embryo development. 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* T_0 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-B*3 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-B*3 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-B*3 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*.

Acknowledgements

This study was supported by a grant from the National Science Found at the Ministry of Education and Science of the Republic of Bulgaria, project Do 02-268 "Integrated functional and comparative genomics studies on the model legumes *Medicago truncatula* and *Lotus japonicus*", Collaboration with Department of Plant Systems Biology, VIB/ Ghent University was one in the frame of mentioned above project. The authors are grateful to Keti Krastanova and Bistra Yuperlieva-Mateeva for valuable technical assistance.

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Received February, 12, 2016; accepted for printing February, 19, 2016