

EVALUATION OF THE FUNCTION AND EXPRESSION PATTERN OF *MEDICAGO TRUNCATULA* AUXIN RESPONSE FACTOR B3 AFTER HETEROLOGOUS EXPRESSION IN *ARABIDOPSIS THALIANA*

M. REVALSKA¹, V. VASSILEVA², G. ZEHIROV² and A. IANTCHEVA^{1*}

¹Agricultural Academy, AgroBioInstitute, BG-1164 Sofia, Bulgaria

²Bulgarian Academy of Sciences, Institute of Plant Physiology and Genetics, BG-1113 Sofia, Bulgaria

Abstract

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The phytohormone auxin plays a vital role in almost every aspect of plant growth and development. Expression of auxin-responsive genes is controlled by a family of Auxin Response Factor (ARF) transcription factor family. This study examined the function and expression pattern of a gene encoding Auxin Response Factor B3 from *Medicago truncatula* (*MtARF-B3*) after its heterologous expression in the model plant *Arabidopsis thaliana*. Stable transgenic plants with *ARF-B3* overexpression, downregulation and transcriptional reporters were constructed. Transcriptional and histochemical assays revealed a stable *MtARF-B3* expression in various stages of somatic embryogenesis and during the postembryonic development of *A. thaliana*. Morphological analysis and morphometric measurements confirmed the important role of *MtARF-B3* in general plant growth and development, root growth and seed production.

Key words: *Arabidopsis thaliana*; gene expression; *Medicago truncatula* Auxin Response Factor B3 (*MtARF-B3*); plant development; plant growth

Abbreviations: *ARF* – Auxin Response Factor; *GFP* – green fluorescent protein; *GUS* – β -glucuronidase; *NLS* – nuclear localization signal; *OE* – overexpression; *RNAi* – RNA interference; *TF* – transcription factor; *WT* – wild type

Introduction

The expression of genes involved in plant growth, development and responses to environmental changes is controlled by specific transcription factor (TF) gene families. These families are found in all eukaryotes but in plants they are strongly conserved and act as key regulators of important agronomic traits (Udvardi et al., 2007). TFs are often under hormonal control that may lead to their activation or repression. In plants, TFs that are regulated by the phytohormone auxin through binding to auxin response elements (AuxREs) in the promoters of downstream target genes, are called Auxin Response Factors (ARFs) (Guilfoyle and

Hagen, 2007; Shen et al., 2015). ARFs contain portable domains that can function independently of one another. An example is the amino-terminal DNA binding domain (DBD), a region that functions as an activation or repression domain (AD or RD), based on the amino acid composition, and a carboxy-terminal dimerization domain (CTD) (Guilfoyle and Hagen, 2001). Most ARFs contain two additional C-terminal domains called domain III and domain IV, except for *Arabidopsis thaliana AtARF10*, *AtARF16* and *AtARF17*. These three ARFs have high sequence similarity (Guilfoyle and Hagen, 2001), and specific and overlapping functions in plant growth and development (Liu et al., 2007). The DBD in ARFs is classified as a plant-specific B3-type. The

*Corresponding author: aneliaiancheva@abi.bg

primary structure of B3 domain consists of seven β -strands (β 1- β 7) that form an open β -barrel. Two short α -helices (α 1 and α 2) are present between β -strands 2 and 3, and between β -strands 5 and 6 (Swaminathan et al., 2008). The ARF proteins bind to DNA as dimers (Ulmasov et al., 1999). Formation of heterodimers with Aux/IAA proteins modulates the expression of genes involved in auxin response (Overvoorde and Okushima, 2005).

Recently, *Medicago truncatula* was proposed as a model species for molecular and genetic studies in legumes. Sequencing the genome provides valuable information about relevant gene families (Young and Udvardi, 2009). However, in the model legume plants little is known about the specific functions of the ARF homologs in plant development and environmental interactions (Rammalingam et al., 2003; Udvardi et al., 2007; Libault et al., 2009). Recently, Bustos-Sanmamed et al. (2013) have identified 30 ARFs in the genome of *M. truncatula*. The gene structure, chromosomal location and expression patterns of 24 MtARFs have been characterized by Shen et al. (2015). The genome of *A. thaliana* contains 23 ARF gene family members (Okushima et al., 2005), as some of these members either alone or redundantly control specific developmental processes (Reed, 2001; Hardtke et al., 2004; Li et al., 2004; Tatematsu et al., 2004).

To identify the role of uncharacterised genes from legumes, different strategies for insertional mutagenesis have been developed. Over the last few years, scientists from the Samuel Roberts Noble Foundation and several European groups have regenerated *Tnt1*-insertion mutant populations for *M. truncatula* (d'Erfurth et al., 2003; Tadege et al., 2008; Iantcheva et al., 2009). In addition, a collection of Flanking Sequence Tags (FST) has been generated for many of the mutant lines and deposited for public use (<http://bioinfo4.noble.org/mutant/>). By a reverse genetic approach followed by BLAST analysis confirmation, it has been found that the Insertion 3 in the mutant line So5928 partially corresponds to a gene encoding Auxin Response Factor (Revalska et al., 2011), containing DNA-binding pseudobarrel and B3-binding domains (*Mt5g040880*, PLAZA 3.0 Dicots, http://bioinformatics.psb.ugent.be/plaza/versions/plaza_v3_dicots/). In our study, this gene has been named *MtARF-B3*, whereas in other reports different names (*MtARF17d*, *MtARF15*) have been used (Bustos-Sanmamed et al., 2013; Shen et al., 2015). The function and expression pattern of *MtARF-B3* have been studied in the model legume *Lotus japonicus* (Revalska et al., 2016). Many genes have been cloned in the model species *A. thaliana* because of the short life cycle, small size, fast growth (Koornneef and Meinke, 2010), and susceptibility to genetic manipulation and transformation by *Agrobacterium*

tumefaciens (Clough and Bent, 1998; Zhang et al., 2006). The closest ortholog of *MtARF-B3* in *A. thaliana* is *AtARF17* (*At1g77850*, PLAZA 3.0 Dicots) that is essential for several aspects of plant growth and development (Mallory et al., 2005), and has been a target of post-transcriptional regulation by *microRNA160* (*miR160*) (Rhoades et al., 2002).

In the present paper, the effect of heterologous overexpression of *ARF-B3* from *M. truncatula* in the model plant *A. thaliana* was examined. Besides, the tissue specific expression pattern of this gene was assessed using transgenic plants that carried GUS/GFP reporters (green fluorescent protein/ β -glucuronidase) fused to the *MtARF-B3* promoter sequence. These studies and the RNAi-mediated downregulation of the endogenous *AtARF-B3* (*AtARF17*) gene strongly suggest a crucial role of *ARF-B3* in general plant growth, development and fertility.

Materials and Methods

Cloning and construction of expression clones for transformation

GATEWAY cloning was used to obtain recombinant plasmids (Karimi et al., 2002; Invitrogen Life Technologies, Inc., <http://www.lifetechnologies.com>). Full length open reading frame (ORF) of *MtARF-B3* with length of 1575 bp, was introduced into the *pDONR221* donor vector. The obtained Entry clone was transferred into *pK7WG2* or *pK7F-WG2* (a C-terminal translational GFP fusion) binary destination vectors for overexpression, under the control of CaMV 35S promoter and neomycin phosphotransferase (*nptII*) gene for plant selection (Karimi et al., 2007). For gene silencing, RNA interference (RNAi) method was applied (Limpens et al., 2003; Revalska et al., 2015). *pK7GWIWG2D (II)* hairpin RNA expression vector was used and *At1g77850*, the ortholog gene of *MtARF-B3*, was silenced. For this purpose, fragment of 175 bp from *MtARF-B3* mRNA corresponding to nucleotide positions 1372 - 1549 bp in the ORF of *At1g77850* was used. By using the software program Vector NTI Advance™ (www.lifetechnologies.com), sequence alignment between *MtARF-B3*, its *Arabidopsis* ortholog *AtARF17* and the short fragment of 175 bp from *MtARF-B3* mRNAi used for silencing, was created (Figure 1). *MtARF-B3* endogenous promoter was heterologously expressed in *A. thaliana*. Full length promoter sequence (~2.0 kb upstream of the start codon) of the *MtARF-B3* gene was inserted into *pDONRP-4PIR* donor vector. By recombining the constructed Entry clone into *pEX-K7SNFm14GW* (promoter-NLS-GUS-GFP) (nuclear localization signal) destination vector, possessing *nptII*, was generated. The obtained Expression clones were introduced into *A. tumefaciens* strain C58C1, maintained on

agar solidified YEB (yeast extract broth) nutrient medium (1.5%), supplemented with 100 mg/l rifampicin (Rif), 100 mg/l spectinomycin (Sp) and 50 mg/l gentamicin (Gm). The genetic sequences of primers for promoter and gene cloning were designed with software program Primer 3 (<http://primer3.ut.ee/>) (Table 1).

Plant material, growth conditions and genetic transformation

To obtain lines with overexpression, knockdown and transcriptional reporters, wild type (WT) plants from *A. thaliana* ecotype Columbia-0 (Col-0) were transformed with *A. tumefaciens* using the floral dip method (Clough and Bent, 1998). The selected T₀ plants were grown in a greenhouse on soil at 23°C ± 2°C, aeration 1 m³/s, light intensity of 300 µmol m⁻² s⁻¹, 60% relative humidity, and a 16/8 day/night photoperiod, respectively. Collected seeds were sterilized with 70% (w/v) ethanol for 2 min, followed by 12 min in commercial bleach, then washed several times with sterile distilled water and

plated on square plates containing agar-solidified 0.8% MS culture medium (Murashige and Scoog, 1962) supplemented with 50 mg/l Km for selection. The seeds were grown in dark for 48 h, then moved into light at 21–23°C for 10 days. Km resistant seedlings were transferred into soil in order to obtain homozygous T₃ plants.

Indirect somatic embryogenesis of *A. thaliana*

In order to follow the process of somatic embryogenesis in *A. thaliana*, immature embryos (Gaj, 2001) from *MtARF-B3* transcriptional reporter plants (*pMtARF-B3::GUS-GFP*) and the WT Col-0 were used as initial explants. Siliques with immature seeds were collected from 10-week-old reporter plants and the WT Col-0. The siliques were surface sterilised with 10% (w/v) commercial bleach followed by several brief rinses with distilled water and placed at 4°C for 12–16 h. Siliques were opened with very fine needles and immature zygotic embryos were excised under microscope. At least 20 of these embryos were cultured on B5 solid medium supple-

Table 1

List of primers used for cloning and qRT-PCR

Primers used for cloning	
attB1	5'-GGGGACTGCTTTTGTACAAACTTGC-3'
attB2	5'-GGGGACCACTTTGTACAAGAAAGCTGGGT-3'
attB4	5'-GGGGACAACCTTTGTATAGAAAAGTTGCT-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'-TGTTCTGAATTATATAATCGG-3'
R-ARF-B3-pro	5'-GGCGGAGAGATTAACAAG-3'
Primers used for qRT-PCR	
F-At-actin	5'-TGCCAATCTACGAGGGTTTC-3'
R-At-actin	5'-TTCTCGATGGAAGAGCTGGT-3'
F-Mt-actin	5'-TCAATGTGCCTGCCATGTATGT-3'
R-Mt-actin	5'-ACTCACACCGTCACCAGAATCC-3'
F-At-Ubiquitin	5'-GTCGACCCCTCACTTGGTGT-3'
R-At-Ubiquitin	5'-CCTTGACGTTGTCAATGGTG-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-At-ARF17 AT1G77850	5'-ACTTGCTAACAACGGGATGG-3'
R-At-ARF17 AT1G77850	5'-TTCGGAATCCATCATCTTC-3'

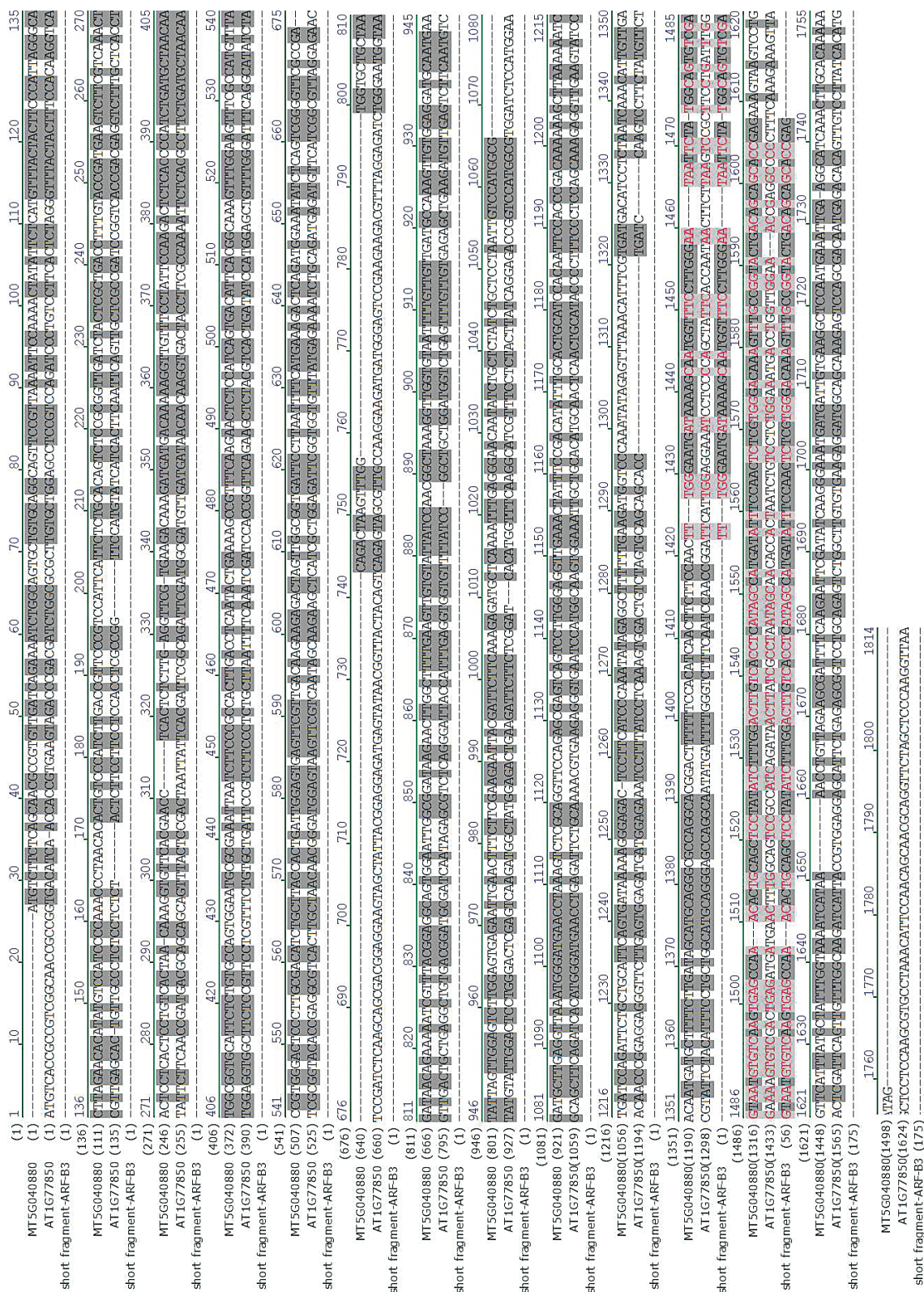


Fig. 1. Sequence alignment between *MtARF-B3*, the *Arabidopsis* ortholog *AtARF17* and the short fragment of 175 bp from *MtARF-B3* mR-NAI used for silencing (Vector NTI Advance™).

Conservative regions are labeled with black letters and gray background; identical nucleotides are labeled with red letters and light gray background

mented with 0.9 mg/l 2,4-D and placed in dark to induce formation of embryogenic callus tissue. After 14 days, the formed callus was plated on ½ MS solid medium supplemented with vitamins for obtaining small plantlets.

GUS activity assay

Histochemical staining for GUS activity was performed essentially as described previously (Jefferson et al., 1987; Willemsen et al., 1998). Whole plants or tissues from the model species were incubated in 90% acetone for 30 min at 4°C, then washed in phosphate buffer (pH 7.0) and incubated overnight in GUS staining solution at 37°C.

Phenotyping analysis

Morphological analysis of different transformants were performed after *in vitro* and *in vivo* cultivation and compared to the WT plants. Assessment of plant growth, flower development and seed production was conducted. Roots, hypocotyls, siliques and leaves were collected for biometric evaluation. Measurement of the primary root length was conducted 5 days after seed germination for 5 consecutive days. Hypocotyl length was measured 10 days after seed germination and siliques length – in 10-week-old plants. Roots, hypocotyls, siliques and leaves of 20 selected plants from different lines with *MtARF-B3* overexpression, knockdown and the WT were measured.

Leaf clearing, light and confocal microscopy

Leaves from mature transgenic and WT plants (about 7 to 8 weeks old) were collected for phenotype assessment. The leaves were incubated in absolute ethanol to clear off chlorophyll (~24–48 h), and transferred into NaOH : EtOH solution (1 volume 1.25 M NaOH and 1 volume absolute EtOH) for 2 h at 60°C. In order to clear starch granules and residual substances from the cells, the leaves were mounted for 2 h in Hoyer's solution, consisting of 8 g chloral hydrate, 1 ml glycerol, 3 ml MQ H₂O, and subsequently cleared for 24 h in lactic acid (Acros Organics) on microscope slides. The samples were examined and photographed using a Carl Zeiss Axio scope A1 microscope with HIGH CONTRAST DIC coupled to a XC50 digital microscope camera. To determine leaf size, and the size and number of epidermal cells in the tip and base leaf regions of the abaxial epidermis, ten individual first rosette leaves were measured by the imaging software ImageJ v1.4.3 (<https://imagej.nih.gov/ij/>). Values for the size and number of epidermal cells from both leaf regions were averaged. Fluorescence imaging of roots 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.

qRT-PCR analysis

Total RNA was extracted from lines with overexpression of *MtARF-B3*, knockdown of the ortholog *At1g77850*, and the WT plants, using RNeasy Plant Mini Kit (EurEx). The primers used to evaluate gene expression (Table 1) amplify a non-conserved region to avoid amplifying homologous genes, as *MtARF-B3* is a member of a multigenic family. Equal amount of total RNA was reverse transcribed with the First Strand cDNA Synthesis kit (Fermentas). Relative expression levels were determined with the 7300 Real-Time PCR System (Applied Biosystems). Two reference genes, actin (ACT) and ubiquitin (UBQ10), were used for data normalization.

Statistical analysis

Three independent experiments were performed, and mean values obtained using triplicate assays. qRT-PCR data were analysed by the qBase v1.3.5 software (Center for Medical genetics, Ghent University Hospital, <http://medgen.ugent.be/qbase>), and the graphs created by the OriginPro v8.5.1 software (<http://www.originlab.com/Origin>). Differences were assessed by t-test. Results are expressed as means ± standard deviation (SD). Statistically significant differences were considered when $p < 0.05$.

Results

In this study, the function and expression pattern of *M. truncatula* Auxin Response Factor, containing DNA-binding pseudobarrel and B3-binding domains (*Mt5g040880*, PLAZA 3.0 Dicots) was investigated. *MtARF-B3* belongs to the gene family *ORTHO03D0200736*, which includes six genes in *M. truncatula* (PLAZA 3.0, Dicots Tree Explorer). Stable transgenic *A. thaliana* plants overexpressing *MtARF-B3*, and lines with knockdown of *AtARF17* were created. Additionally, transgenic lines carrying fusions between the *MtARF-B3* promoter and GUS/GFP reporter genes were constructed. Homozygous transgenic plants (T₃ generation) were obtained by selection of T₁ and T₂ seeds on 50 mg/l Km and the positive seedlings were further cultivated in soil conditions. All experiments were performed with homozygous plants.

Analysis of GUS/GFP activity in transcriptional reporter lines of *A. thaliana*

To assess the heterologous expression of *MtARF-B3* during plant development, the promoter sequence of *MtARF-B3* was fused to the GUS-GFP reporter, and the resulting construct was transformed into the WT Col-0 plants (*pMtARF-B3::GUS-GFP*). Histochemical analysis of the generated transcriptional reporter lines showed GUS activity during

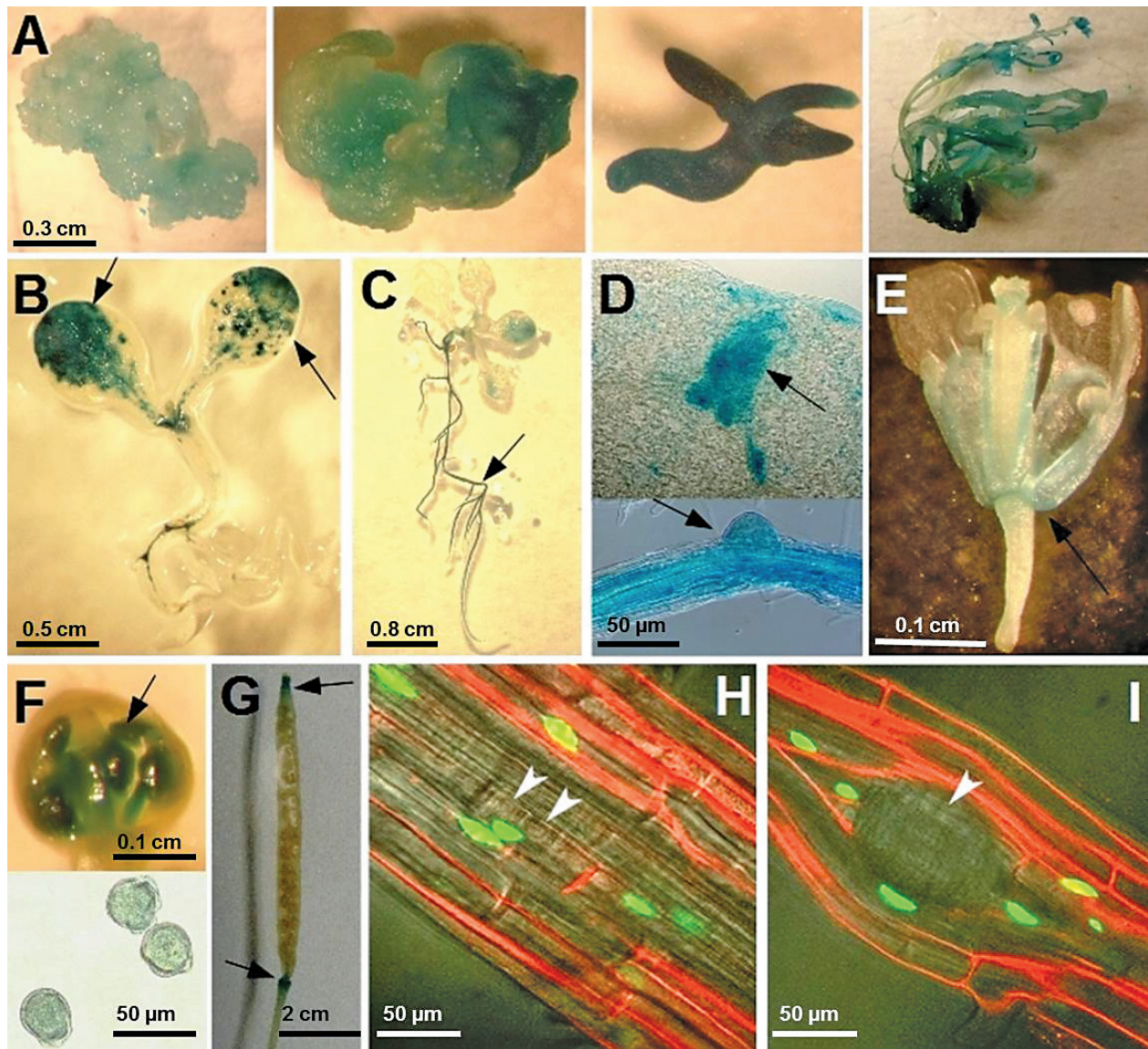


Fig. 2. Expression patterns of *pMtARF-B3::GUS-GFP* in *Arabidopsis thaliana*: (A) different stages of somatic embryogenesis; (B) shoot apical meristem and cotyledon of 5-day-old seedling; (C) 20 day-old-seedling with expression in roots and leaves; (D) zones of leaf growth and emerging lateral root; (E) flower sepals, filaments and stigma; (F) floral buds with stamens and pollen; (G) growth zones of siliques; (H) early-stage lateral root epidermal cells; (I) emerging lateral root epidermal cells

different developmental stages of somatic embryogenesis (Figure 2 A). In young plants, *MtARF-B3* expression was seen in shoot apical meristem and cotyledon of 5-day-old seedling (Figure 2 B), leaf lamina, primary root and secondary root primordia, and in the growing leaf regions (Figure 2 C, D). *MtARF-B3* gene was also expressed in sepals and

pistil of flowers, flower buds, stamens and pollen (Figure 2 E, F), as well as in the growing regions of siliques (Figure 2 G). Notably, *MtARF-B3::GFP* expression was not seen in the early-stage of emerging lateral root primordia and in vascular tissue, but it was detected in the root epidermal cells (Figure 2 H, I).

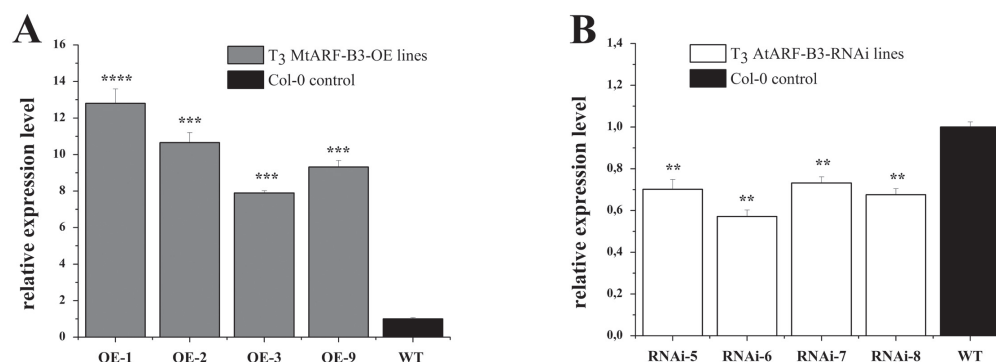


Fig. 3. Relative expression level of *MtARF-B3* and the ortholog gene *At1g77850* in *Arabidopsis thaliana*: (A) *MtARF-B3* transcript levels in homozygous OE lines and WT plants; (B) *At1g77850* transcript levels in homozygous RNAi lines and WT plants. Statistically significant differences were considered when $p < 0.05$. **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$, compared with the WT control

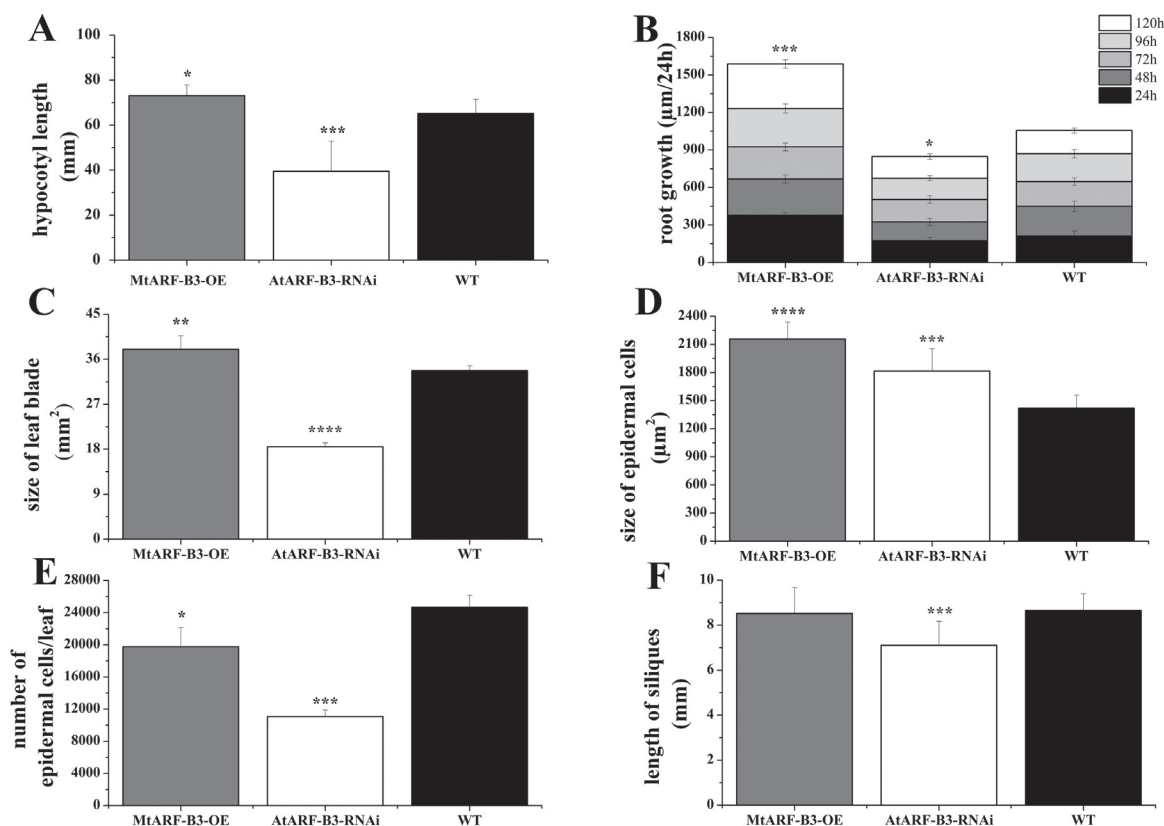


Fig. 4. Quantification of leaf and root morphology in OE and RNAi lines of *Arabidopsis thaliana*: (A) hypocotyl length in *in vitro* cultivated 10-day-old plants; (B) dynamics of root growth in *in vitro* cultivated 5-day-old plants; (C) size of leaf blade in soil cultivated 7 to 8-week-old plants; (D) size of epidermal cells in soil cultivated 7 to 8-week-old plants; (E) number of epidermal cells/leaf in soil cultivated 7 to 8-week-old plants; (F) length of siliques in soil cultivated 10-week-old plants. Statistically significant differences were considered when $p < 0.05$. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$, compared with the WT control

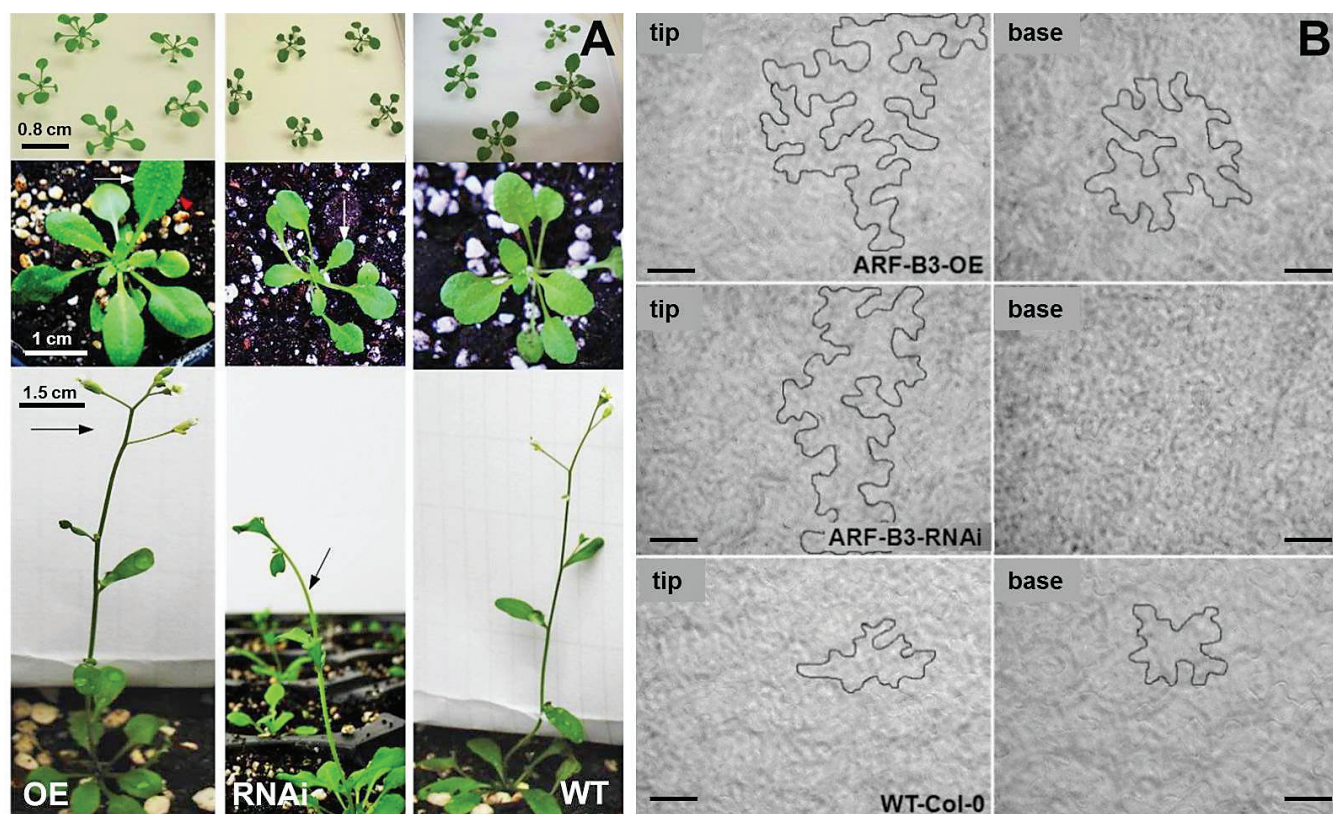


Fig. 5. Morphological overview of OE and RNAi lines of *Arabidopsis thaliana*: (A) phenotype of rosettes at day 25th and day 35th after germination, and inflorescence at day 45th after germination of OE and RNAi lines, and the WT Col-0; (B) representative images of giant cells occurrence in the abaxial leaf epidermis at the tip (75%) and the base (25%) of the longitudinal leaf axis of OE and RNAi lines, and the WT Col-0. Labels: black lines delineate giant and regular-sized cells. Scale bar = 50 μm

Expression profile of MtARF-B3 and its ortholog gene in overexpression and knockdown lines of A. thaliana

Expression levels of *MtARF-B3* in *A. thaliana* lines with overexpression (OE) were determined by examining the abundance of *MtARF-B3* transcripts, whereas in the lines with RNAi-mediated knockdown, transcript levels of *AtARF17* were measured. Two to four individual plants from each line were randomly selected and the *MtARF-B3/AtARF17* expression level was measured and compared to the WT plants. In OE lines, *MtARF-B3* transcript level was increased up to 13 times (Figure 3 A), while the expression of *AtARF-B3* was decreased up to 0.3 – 0.4 times in knockdown lines compared to WT (Figure 3 B).

Phenotypic and morphometric analysis of A. thaliana lines with MtARF-B3 overexpression and knockdown of the ortholog gene

To identify the function of *MtARF-B3* gene and its role in plant development, morphological and morphometric analy-

sis of the studied model plant, cultivated under *in vitro* and *in vivo* conditions, were performed. At least twenty plants per line were screened for morphological variations and then analysed. The following parameters were evaluated in *in vitro* conditions: hypocotyl length and dynamics of root growth. The size of leaf blade, number and size of epidermal cells and length of siliques was measured in soil-cultivated plants.

The OE lines had hypocotyls with a significantly greater length compared to the WT plants, whereas in the RNAi lines the hypocotyl length was considerably reduced (Figure 4 A). Five days after germination, root growth dynamics of seedlings was monitored during five consecutive days. The OE lines had significantly faster growth of the main root than the WT (Figure 4 B). In the OE lines, most intense root growth was observed during the first and the last 24 hours from the experiment. The root growth of the RNAi lines was slower than in the WT (Figure 4 B), but at a constant growth rate for all time intervals.

We further analysed the effect of *ARF-B3* overexpression and knockdown on *A. thaliana* leaf morphology. *MtARF-B3* overexpression led to a 1.13-fold increase in the leaf size, as compared to the WT plants (Figure 4 C). Leaf size of the RNAi lines was significantly reduced (Figure 4 C). Microscopic examination of the leaves of OE lines revealed larger epidermal cells compared to the WT (Figure 4 D). The leaves of RNAi lines also had significantly larger size of epidermal cells than that of the WT, however this size was smaller compared to the OE lines. In the WT Col-0 plants, the total number of epidermal cells was higher compared to OE and RNAi lines (Figure 4 E). Siliques length in the OE lines was not significantly different compared to the WT plants ($p > 0.5$), however, the length of siliques in RNAi lines was shorter than in the WT plants (Figure 4 F).

Next, we investigated whether the growth conditions could affect the observed phenotypes of the generated *A. thaliana* transgenic lines. Twenty-five-day-old *ARF-B3* OE and *ARF-B3* RNAi plants were transferred from selective media to soil, and ten days after soil adaptation screened for morphological changes. The OE lines showed bigger size, larger serrated leaves and similar flowering time to the WT (Figure 5 A). Some of the OE lines had many well developed trichomes on the leaf surface. Approximately 60% of the knockdown mutants couldn't survive in soil conditions. These plants had a shortened root system and serrated leaves. Most of the survived RNAi plants were smaller and grew slower than the WT (Figure 5 A). Mature plants flowered later, had shorter stem, reduced fertility and decreased seed production, compared to the WT (Figure 5 A). Using differential interference contrast (DIC) microscopy, the abaxial epidermis was examined at the tip and the base of the longitudinal leaf axis corresponding to 75% and 25%, respectively (Figure 5 B). In OE lines, the presence of giant pavement cells in the tip and the base leaf regions was noted, while in the RNAi lines, only single giant cells in the tip region of leaf epidermis was observed (Figure 5 B).

Discussion

To analyse/estimate the quantitative effect of *MtARF-B3* from the model legume plant *M. truncatula*, the gene was heterologously overexpressed in *A. thaliana* using the 35SCaMV promoter. Besides, the endogenous *ARF-B3* was downregulated, employing RNAi-mediated gene silencing technology. Additionally, nuclear-localised transcriptional reporters (*pMtARF-B3::GUS-GFP*) were generated, and the expression pattern of *MtARF-B3* was examined. Expression of the GUS reporter gene driven by the endogenous *MtARF-B3* promoter was observed at all stages of somatic

embryogenesis in *A. thaliana*, suggesting an involvement of this gene in somatic embryo development. Liu et al. (2010) have shown that *AtARF10*, *AtARF16* and *AtARF17* play an important role in controlling embryogenesis. Various embryonic defects caused by abnormal cell divisions during embryogenesis are mainly attributed to the altered expression pattern of *AtARF16* and *AtARF17*.

During postembryonic development, gene reporter activity was restricted to the epidermal layer of the primary root and the base of emerging lateral root primordia, but was not detected in the meristematic zone of these primordia. Similar expression pattern is 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). Investigation of root growth dynamics in *A. thaliana* showed faster growth in OE lines, resulting in well-developed final root architecture. The important role of *AtARF17* in primary root growth, lateral and adventitious root formation has been previously reported by Okushima et al. (2005) and Sorin et al. (2005). The poorly developed root system of the *AtARF-B3* knockdown mutants might be one of the reasons for their decreased ability to survive in soil conditions. It has been shown that plants expressing miRNA-targeted version of *ARF17* have severe developmental defects, including abnormalities in embryonic, root, vegetative and floral development. The frequency of early death is greater in the mutant plants, as some die at the seedling stage, other before transition to flowering (Mallory et al., 2005). Furthermore, knockdown of *ARF-B3* in *L. japonicus* led to morphological alterations in the root system, as tripod root architecture and shortened root length (Revalska et al., 2016).

The *pMtARF-B3::GUS* expression had spotted localisation in the growing regions of leaf petiole. Same expression pattern is observed in *L. japonicus* transcriptional reporter plants after heterologous expression of *MtARF-B3* promoter sequence (Revalska et al., 2016). Our results suggested the contribution of *ARF-B3* to the regulation of leaf blade expansion in *A. thaliana*. Phenotypic and morphometric analysis revealed an increased leaf size in *A. thaliana* OE lines. Giant epidermal cells were observed not only in OE lines but also in RNAi lines. It is very likely that both *ARF-B3* overexpression and downregulation caused abnormal division patterns in the leaf cells, resulting in changed expression of a subset of target genes controlling leaf cell division and expansion. The changed hypocotyl length in the lines with altered expression of *ARF-B3* was another indication of its role in plant growth and development. The hypocotyl length in OE lines was increased 2.0-fold, com-

pared to RNAi lines, which complemented the observed leaf and root phenotypes in *A. thaliana* plants with gain or loss of *ARF-B3* function. The RNAi lines showed developmental defects, such as small leaves and serrated leaf margins. Similar serrated-leaf phenotype has been reported for transgenic *A. thaliana* plants expressing a miR160-resistant form of *AtARF10*, carrying silent mutations in the miRNA target site (Liu et al., 2007).

Analysis of the transcriptional GUS reporter activity revealed the expression of *MtARF-B3* in flowers and pollen. We also observed that the OE lines produced more seed pods than the WT, which suggest that *MtARF-B3* is involved in seed production, which has been reported before for *AtARF17* (Liu et al., 2007). Knockdown of *AtARF17* in *A. thaliana* led to reduced fertility and low seed production in the survived plants. These observations confirmed the previously reported expression of *AtARF17* in flowers, particularly in pollen grains (Liu et al., 2010). It has been shown that the *A. thaliana AtARF17* gene that is the ortholog of *MtARF-B3* in *M. truncatula*, plays a role in pollen wall formation and pollen tube growth (Yang et al., 2013), and also affects plant development and fertility (Mallory et al., 2005). Another study revealed that ARF-B3-RNAi lines of *L. japonicus* possess small and curved pods and abnormal seeds, without germination capability, which led to sterile plants (Revalska et al., 2016). Thus, the investigated *MtARF-B3* gene was expressed in different plant tissues and organs, and has specific functions in the proper seed development and germination. The previous reports about the important role of *AtARF17* and *LjARF-B3* for maintaining normal development of roots, leaves and flowers (Mallory et al., 2005; Liu et al., 2007; Revalska et al., 2016) were confirmed by our results. Taken together, these results suggest that *MtARF-B3* plays an important role in plant growth and development.

Conclusion

The constructed *A. thaliana* lines with *ARF-B3* overexpression, downregulation and transcriptional reporters allowed performing detailed phenotypic analysis and characterising the expression patterns of the reporter genes GUS and GFP, after heterologous expression of *MtARF-B3* promoter. We could detect that *MtARF-B3* was expressed at various stages of somatic embryogenesis and during the formation of plant organs and tissues. The analysis performed revealed the important role of *MtARF-B3* in general plant growth and development, particularly in the modeling of root architecture and development of seeds. Knockdown of *AtARF-B3* had often a lethal effect on plants, associated with partially or completely sterile flowers and low seed production.

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