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ORIGINAL ARTICLE

Ectopic expression of *ARR1ΔDDK* in tobacco: alteration of cell fate in root tip region and shoot organogenesis in cultured segments

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Abstract A specific deleted version of *ARABIDOPSIS RESPONSE REGULATOR1* (*ARR1*) lacking the signal receiver domain (1–152 amino acids)-coding sequence, referred to as *ARR1ΔDDK*, was amplified using *Arabidopsis thaliana* cDNA prepared from adult leaves and transferred into the genome of *Nicotiana tabacum* cv. Samsun under the transcriptional control of a β-estradiol-inducible expression system. The ectopic expression of *ARR1ΔDDK* affected the morphology of transgenic seedlings and their segments in vitro. In the presence of an inducer, β-estradiol, ectopic expression of *ARR1ΔDDK* induced only the formation of soft, pseudo-bulbous tissue in the root tip region of intact seedlings, which appeared similar to callus generated on a hypocotyl segment in the presence of 2,4-D and 6-benzyladenine (BA), both at 1 μM. Those callus tissues on the root tip region could not generate shoots unless 1 μM BA was supplied. In segment culture, ectopic expression of *ARR1ΔDDK* induced callus-like tissue around the cut-end of cotyledon and hypocotyl segments with occasional shoot formation, suggesting that the expression of *ARR1ΔDDK* could substitute for the effects of cytokinin on these segments. Additionally, treatment with only β-estradiol induced *NtWUS*, a *WUS* ortholog in tobacco, which was detected during the process of callus tissue formation in the root tip region and also in cotyledon or hypocotyl segments. These findings suggest that the *NtWUS* might be associated in the transdifferentiation process caused by the functional regulation of *ARR1ΔDDK* in transgenic tobacco seedlings.

Keywords *ARR1ΔDDK* · Transdifferentiation · Root tip bulbing · *NtWUS* · Type-B ARR · Tobacco

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Introduction

Cytokinins have various effects in the regulation of plant growth and development (reviewed by Mok and Mok 2001). They maintain and promote plant cell division in cultures and are involved in various differentiation processes, including shoot formation (Riou-Khamilichi et al. 1999; Werner et al. 2003; Higuchi et al. 2004; Nishimura et al. 2004). It has been proposed that plants respond to cytokinins via a two-component signaling pathway where histidine (His) kinase acts as an environmental sensor (Kakimoto 1996; Inoue et al. 2001; Ueguchi et al. 2001). The *Arabidopsis* histidine kinases, *AHK2*, *AHK3*, and *AHK4*/*CRE1* (*cytokinin response 1*) act as transmembrane cytokinin receptors, which transfer the signal via phosphorelay to the nucleus, activating two primary classes of *Arabidopsis response regulators* (*ARR*s), named type-A and type-B response regulators (D'Agostino et al. 2000; Ueguchi et al. 2001; Inoue et al. 2001; Hwang and Sheen 2001; Ferreira and Kieber 2005; Riefler et al. 2006). The type-A *ARR*s show a primary response to cytokinin (Brandstatter and Keiber 1998; Sakakibara et al. 1998; D'Agostino et al. 2000), and type-B *ARR*s act as transactivators that control the expression of type-A *ARR*s (Hwang and Sheen 2001; Sakai et al. 2001). The type-A ARR proteins contain a phosphate-accepting receiver domain but, in contrast to type-B ARRs,

they lack a DNA-binding motif in their output domain (D'Agostino and Kieber 1999). Type-B ARRs are positive regulators of the cytokinin response (Ferreira and Kieber 2005; Mason et al. 2005). Among the members of this family, ARR1 has been identified as a primary transcription factor-type response regulator whose nuclear localization could be detected in the presence or absence of its signal receiver domain (DDK), and the DDK region was suggested to partially inhibit the transactivation process (Sakai et al. 2000, 2001). *ARR1* has also been shown to be a regeneration-related gene in *Arabidopsis thaliana* overexpressing *35S::ARR1* and *35S::ARR1ΔDDK*, lacking the signal receiver domain-coding sequence (Sakai et al. 2001). In another study by Leibfried et al. (2005), the homeodomain protein WUSCHEL (WUS), which is required for shoot apical meristem (SAM) maintenance, was detected as a transcriptional repressor of a set of type-A *ARR*s. In that report, a feedback regulatory mechanism was suggested where *WUS* expression suppresses type-A ARRs to upregulate cytokinin signaling, and cytokinin signaling could, in turn, induce type-A *ARR* expression to modulate meristem function.

Recently, ectopic expression of *WUS* and *WUS-RELATED HOMEOBOX5* (*WOX5*) induced bulbous tissues in the tobacco root tip region followed by shoot formation on it (Rashid et al. 2007; Rashid and Kyo 2009a). The *WUS* ortholog in tobacco (*NtWUS*) also generated a similar morphological response in transgenic seedlings, bulbous tissue and adventitious shoot formation in the root tip region (Rashid and Kyo 2009b). In all those studies, the effect of *WUS*, *WOX5*, or *NtWUS* expression was accelerated by the addition of 10 μM 6-benzyladenine (BA). The response of seedling segments, such as inhibition of root growth or normal root elongation, suggested that the upregulation of endogenous cytokinin signaling was induced by ectopic expression of these genes. When transgenic seedlings were cultured only in the presence of cytokinin without the inducer, β-estradiol, however, bulbous tissue formation on the root tip and shoot formation were not observed (Rashid et al. 2007).

Based on these observations, this study was designed to examine the effect of ectopic expression of type-B ARR on the morphology of tobacco seedlings, especially in the root tip region. For this purpose, *ARR1ΔDDK* (lacking the signal receiver domain; Sakai et al. 2000, 2001) was used under the control of a β-estradiol-inducible expression system (XVE system; Zuo et al. 2000). The XVE system was successfully applied by Zuo et al. (2002) to screen for *WUS*, whose ectopic expression caused somatic embryogenesis in *Arabidopsis*. This study describes the effect of ectopic expression of *ARR1ΔDDK*, which caused the induction of pseudo-bulbous tissue formation in the root tip region of transgenic tobacco seedlings. Furthermore, application of exogenous cytokinin markedly promoted profuse shoot formation in this region. Moreover, these results indicate that at least the induction of *NtWUS* expression might be related to the morphological abnormality observed in segment culture or in the root tip region of intact seedlings overexpressing *ARR1ΔDDK*.

Materials and methods

Molecular manipulation and plant materials

Mature *Arabidopsis thaliana* (ecotype Columbia) plants were grown on vermiculite at 22°C in a light incubator (16 h light and 8 h dark). Fresh, mature leaves (collected from 1-month-old plants) were used for total RNA extraction and subsequent gene cloning of *ARR1ΔDDK*. The cDNA clone coding for *ARR1ΔDDK* was obtained by RT-PCR (Prime STAR HS; TaKaRa, Japan). Specific primers for *ARR1ΔDDK* were designed from available sequence data supplied by DDBJ (http://www.ddbj.nig.ac.jp/) as follows: 5′-ctcgagATGGTTAGGAAGAGGAGAAGTGAATG (lower case letters indicate *Xho*I site, the added nucleotides are underlined) and 5′-actagtCAAACCGGAATGTTAT CGATGG (lower case letters indicate *Spe*I site) for *ARR1ΔDDK*. The amplified PCR fragments of *ARR1ΔDDK* (1,622 bp) were first ligated into the *Sma*I (TaKaRa, Japan) digested blunt-end vector pUC18, using LONG DNA ligation kit (TaKaRa). The ligation mixture was used for heat-shock (42°C) transformation of *E. coli* (XL-1 blue) and transformants (white colonies) were selected on LB (Luria-Bertani; Miller 1972) agar (2%) medium supplemented with IPTG (isopropyl-β-D-thiogalactopyranoside, 1 mM; Promega, Japan), X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside, 0.008%; Promega) and ampicillin (100 mg l^{-1}; Wako, Japan). Following bacterial colony PCR, plasmid DNA was extracted (Quick Gene SP kit Plasmid II; Fuji Film, Japan) and purified according to the stipulated protocol. The purified plasmid DNA was digested by *Xho*I (TaKaRa) and *Spe*I (TaKaRa) and the resulting fragment was cloned into the *Xho*I/*Spe*I site in the vector, pER8 (Zuo et al. 2000). The plasmid harboring *ARR1ΔDDK* was used for *Agrobacterium*-mediated transformation of tobacco (*Nicotiana tabacum* cv. Samsun) as previously reported (Rashid et al. 2007). The resulting transgenic tobacco plants were selected in the presence of 50 mg l^{-1} hygromycin B (Wako, Japan) and grown to mature plants to harvest seeds. In this study, those transgenic tobacco plants are referred to as *XVE::ARR1ΔDDK*. Eight transgenic *ARR1ΔDDK* lines were obtained. Among them, five *XVE::ARR1ΔDDK* lines showed higher expression levels of their transgenes in the presence of β-estradiol (Sigma-Aldrich, MI, USA). Each of those plants showing a positive

Fig. 1 Morphological features of the transgenic *XVE::ARR1ΔDDK* seedling in response to *β*-estradiol and/or BA. *Top* The root tip region after 4 days culture on medium without (**a**) and either with 1 μM BA (**b**), 10 μM *β*-estradiol (**c**), or both (**d**). *Bar* 150 μm. *Bottom* The 7-day-old transgenic seedlings were replaced and cultured on LS gel medium without any supplement (**e**), with 1 μM BA (**f**), or with 10 μM *β*-estradiol (**g**). **h** The *β*-estradiol-treated seedling (for 1 week) transferred to LS gel medium containing 1 μM BA and cultured for another 2 weeks. All seedlings were cultured under light condition at 25°C and photographed at 21 days. *Bars* 1 cm

signal in expression analysis was grown to harvest and their selfed seeds were used to examine the morphology of seedlings.

Seedling morphology

Transgenic seeds were surface sterilized, sown on solid LS medium (Linsmaier and Skoog 1965) containing 0.3% gelrite (Wako) and hygromycin B (50 mg l^{-1}) and kept in the dark at 25°C for 1 week. The 7-day-old resistant seedlings were selected and transferred to solid LS gel medium supplemented with or without 10 μM *β*-estradiol to observe any morphological abnormality.

Segment culture of *XVE::ARR1ΔDDK* seedling

Young transgenic seedlings were cut into cotyledons or hypocotyl segments with or without the SAM, root and root tips with the root apical meristem (RAM). These segments were cultured on solid LS gel medium with or without *β*-estradiol (10 μM) and/or BA (1 μM) at 25°C in the dark for 1 week and placed in a light incubator for continuous culture.

Expression analyses

Some transgenic seedlings of the *XVE::ARR1ΔDDK* line were collected after 10 days of culture on solid LS gel medium supplemented with or without *β*-estradiol (10 μM). The harvested seedlings were cut into segments: cotyledons with SAM, hypocotyls and root tips with RAM. These segments were immediately homogenized for total RNA extraction and subsequently RT-PCR was conducted according to the method previously described by Rashid et al. (2007). The sequences of specific primer sets were: 5′-CTCGAGATGGTTAGGAAGAGGAGAAGTGAATG and 5′-ACTAGTCAAACCGGAATGTTATCGATGG for *ARR1ΔDDK*; 5′-GCTCTGGATATGGCCGACTTC and 5′-GTAGCCAGCAGCATGTCGAAG for *XVE*; 5′-ATGG AAGCTGCTCAACAACAAAAC and 5′-TTAAGGGGA ATTAGGAGATCTGCC for *NtWUS*, respectively. As for *Nicotiana tabacum homeobox 15* (*NTH15*), 5′-TCCTC CTCCTATGATGATGCCT and 5′-TTCACATCAACCT CCTCTTCAGA were used according to Tamaoki et al. (1997).

Results

Morphological response of tobacco *XVE::ARR1ΔDDK* seedling

The 7-day-old transgenic *XVE::ARR1ΔDDK* seedlings were transferred onto LS gel medium with or without the inducer, *β*-estradiol, at 10 μM. After transferring to

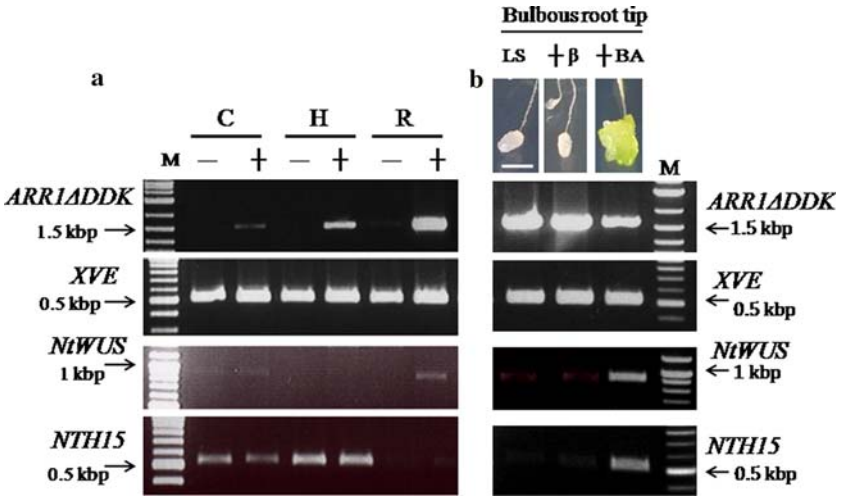

Fig. 2 Expression analyses in *XVE::ARR1ΔDDK* seedling. **a** Transgenic seedlings were cultured on LS gel medium supplemented with (+) or without (−) *β*-estradiol (10 μM) for 10 days. cDNAs were prepared separately from three types of segments; cotyledons with SAM (*C*), hypocotyl (*H*) and root with RAM (*R*). **b** Only the abnormal seedlings (cultured in the presence of *β*-estradiol) showing small bulbing at their root tips were transferred (after 10 days) onto fresh media; LS gel without any supplement (*lane LS*), or, containing either *β*-estradiol (10 μM, *lane β*) or BA (1 μM, *lane BA*). cDNAs were separately prepared from root bulbous tissues after 1 week of second replacement. The approximate lengths of amplified transcripts are indicated on the *left* (**a**) or *right* (**b**). *M* DNA size marker. *Bar* 3 mm (**b**)

medium with *β*-estradiol, only the root tip region of *XVE::ARR1ΔDDK* seedlings started bulbing, within 4 days (Fig. 1a–d). Within 3 weeks, the pseudo-bulbous tissue in the root tip region enlarged to 3–4 mm and inhibited growth of the aerial region was observed (Fig. 1e, g). When placed into the light, those bulbous root tips showed slight greening but no shoots formed (data not shown). However, it was possible to induce adventitious shoots from bulbous root tips within 3 weeks by transferring the *β*-estradiol-treated seedlings to medium containing 1 μM BA and culturing them under light (Fig. 1h).

Gene expression analyses

As shown in Fig. 2, *ARR1ΔDDK* expression (amplified fragment size: 1,622 bp) was detected in all kinds of *β*-estradiol-treated tissues: cotyledons with SAM, hypocotyl and root with RAM, but not in the absence of the inducer. In contrast, *XVE* expression was detected in all parts of treated and untreated seedlings (Fig. 2). To identify the molecular events occurring during the transition to SAM formation from bulbous root tip tissues, expression analyses of *NtWUS* (Rashid and Kyo 2009b) and *NTH15* (Tamaoki et al. 1997) were also performed before and after the addition of BA. *NtWUS* was constitutively expressed in the cotyledon with SAM region, but could not be detected in other parts of *XVE::ARR1ΔDDK* seedlings during the early period of *β*-estradiol treatment (data not shown). Within 1 week of treatment period with the inducer, *NtWUS* expression was faintly detected in the root bulbous tissues (Fig. 2a). However, prolonged treatment with the inducer could not increase the *NtWUS* expression level in those root bulbous tissues (data not shown). *NTH15* was previously identified as a marker gene for the SAM region and the ectopic expression of *NTH15* caused abnormal tobacco leaf morphology accompanied with a decrease and increase of gibberellin and cytokinin levels, respectively (Tamaoki et al. 1997). In the present study, constitutive expressions of *NTH15* were detected in the aerial regions (cotyledon with SAM and hypocotyl) of transgenic seedlings but not in the root (Fig. 2a). These expression patterns were similar to the previous result observed by Tamaoki et al. (1997). *NTH15* expression was not detectable in the root generating bulbous tissues when only *β*-estradiol was present in the culture medium (Fig. 2a). When the *β*-estradiol-treated seedlings were replaced onto the medium containing BA, the expression of both *NtWUS* and *NTH15* were strongly detected in the root bulbous tissue within 1 week (Fig. 2b).

Culture of *XVE::ARR1ΔDDK* seedling segments

Segments from *XVE::ARR1ΔDDK* seedlings were cultured as described in "Materials and methods". Adventitious root formation and elongation of root were normal in case of control cultures but were inhibited in all types of segments when treated in the presence of *β*-estradiol (Fig. 3). At the early stage of only *β*-estradiol treatment, cotyledon segments generated callus-like tissues around their cut-edges (Fig. 3a, b) which occasionally developed

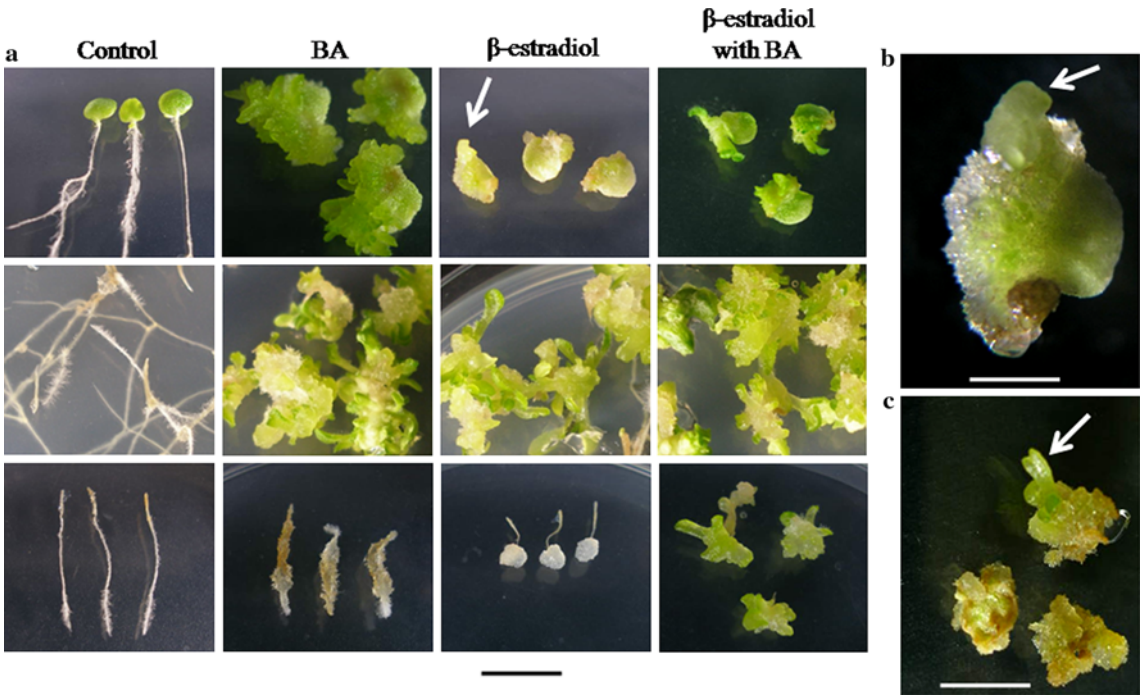

Fig. 3 Segment culture of *XVE::ARR1ΔDDK* seedlings. The 10-day-old transgenic seedlings were cut into segments and cultured on medium without (control) or with BA (1 μM) and/or *β*-estradiol (10 μM). **a** *Top* cotyledon segments without SAM, *middle* hypocotyl segments, and *bottom*, root tip segments. *Arrow* indicates callus growth around the cut-edges of cotyledon segment after treatment with *β*-estradiol. Photographs were taken at 21 days after treatment. *Bar* 1 cm. **b** An enlarged view of greening of callus (*arrow*) developed at the cut-edge of the cotyledon segment (from **a**). *Bar* 5 mm. **c** The 35-day-old culture of cotyledon segments treated with *β*-estradiol. *Arrow* indicates ectopic shoot formation. *Bar* 1 cm. All cultures were conducted in the light at 25°C

into shoots in the following culture (Fig. 3c). In hypocotyl segments, callus-like tissue generation followed by frequent shoot formation was observed in the presence of *β*-estradiol with or without BA, but not in the control (Fig. 3a). Such phenotypic response in hypocotyl segments reveals that only in the presence of the inducer, *β*-estradiol, shoot formation was possible without the requirement of BA. In the root tip segment, treatment in the presence of only *β*-estradiol induced the formation of white bulbous tissues at the RAM region (Fig. 3a). Those white bulbous tissues turned green and subsequently generated ectopic shoots only when BA was supplemented in the culture medium together with the inducer (Fig. 3a).

Expression analyses of segment cultures were also performed during the early period of treatments. *ARR1ΔDDK* expression could be detected in cotyledon segments after 5 days of culture and only when treated with *β*-estradiol (Fig. 4), whereas *NtWUS* expression was induced in the presence of the inducer; similar expression was noted when cotyledon segments were treated with BA alone (Fig. 4). However, *XVE* was constitutively expressed in all types of cultured tissues (Fig. 4). Similar results were also obtained using hypocotyl segments (data not shown).

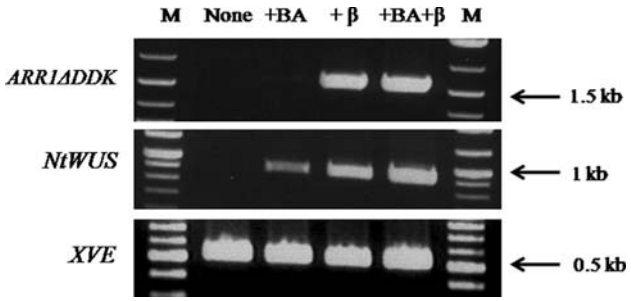

Fig. 4 Expression analysis of different transcripts in cotyledon segments of *XVE::ARR1ΔDDK* seedlings. The cotyledon segments (without SAM) were cultured for 5 days on LS agar medium without any supplement (none), or with either BA (1 μM) or *β*-estradiol (10 μM) and in the presence of both BA and *β*-estradiol (1 and 10 μM, respectively). The approximate lengths of amplified fragments are indicated on the *right*. *M* 100 bp DNA ladder

Discussion

ARR1 was identified as a principal transcription factor-type response regulator that is involved in an early step of cytokinin signal transduction in *Arabidopsis* (Sakai et al. 2001). In this study, the specific deleted (aa 1–152) version of *ARR1* (*ARR1ΔDDK*) was created through sequence-specific

amplification using a cDNA population from *Arabidopsis* and subsequently integrated into the tobacco genome under the control of the XVE system. The gene expression pattern under the control of this system was previously examined in tobacco confirming the ability of this system to localize the transgene in cell nuclei (Rashid et al. 2007; Zuo et al. 2000).

In the previous report, it has been demonstrated that ectopic expression of *WUS* induced bulbous tissue formation at the root tip region followed by SAM development on it, in tobacco seedlings transgenic for *XVE::WUS* (Rashid et al. 2007). Similar phenomenon was also observed using transgenic seedlings for the *WOX5* (Rashid and Kyo 2009a) and *WUS* ortholog in tobacco (*NtWUS*; Rashid and Kyo 2009b). In all three studies, *WUS*, *WOX5*, or *NtWUS* expression resulted in two distinct developmental steps or events: first, bulbous tissue was generated at the root tip region, and later, shoot regeneration occurred on it, which might be related with an upregulation of endogenous (in the case of intact seedlings) or exogenous (in the case of small root tip segments) cytokinins. In this study, however, whole seedlings of the *XVE::ARR1ΔDDK* line, when treated with β-estradiol, generated only 'root tip bulbing' but no shoot formation was observed (Fig. 1g). Such an observation indicates that the functional regulation of *ARR1ΔDDK* is possibly involved in the transdifferentiation process from the RAM to a callus-like novel tissue. Expression analysis data (Fig. 2) indicate that native *NtWUS* might be coordinately induced during the transdifferentiation process (bulbing) in the tobacco root tip region but an optimum level of *NtWUS* expression seems to be critically important to redirect further differentiation for shoot formation in pseudo-bulbous tissue of *XVE::ARR1ΔDDK* seedlings. Additionally, the phenotypic response observed in segment culture (Fig. 3) revealed the predominant expression of *NtWUS* which might indicate its functional association in the transition to adventitious shoots from either cotyledon (Fig. 4) or hypocotyl segments (data not shown). These observations indicate that the functional regulation of *ARR1ΔDDK* in root bulbous tissue might be inadequate to trigger the inclination of the shoot formation process, and at this stage of transdifferentiation (only bulbing), subsequent addition of BA might complement the upregulation of *NtWUS* and subsequently marker gene expression for SAM, *NTH15* (Fig. 2b), in root bulbous tissue. Moreover, ectopic expression of *ARR1ΔDDK* resulted in inhibition of normal growth and development in the aerial region of transgenic seedlings, similar to the orthodox effect of BA (Fig. 1f). This inhibition might result from effector(s) produced from endogenous genes with promoters capable of interacting with the ARRM domain (Sakai et al. 2000).

Until now, different expression profiling experiments on type-B *ARR* loss- and gain-of-function mutants have identified multiple downstream targets, including type-A *ARR*s and other target genes regulating shoot and root development, de-etiolation, leaf expansion, and cytokinin homeostasis (To and Kieber 2008). In the present study, results suggest that the in vivo role of *ARR1ΔDDK* is possibly to maintain a different regulatory mechanism in which at least *NtWUS* might be associated in tobacco tissue. A major future challenge will be to integrate the roles of other players in tobacco while interacting with the ARR1ΔDDK -related network. Such information would allow us to determine the subsequent chain of events that culminates in shoot formation from the tobacco root tip region.

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