Short Communication

Overexpression of *OsAP2* and *OsWRKY24* in *Arabidopsis* results in reduction of plant size

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Abstract   Recently, two rice genes, *OsAPETALA2* (*OsAP2*) and *OsWRKY24* have been reported to be positive regulators involved in increased lamina inclination and grain size through cell elongation. Here, we found that the two genes have tightly linked expression patterns and functional convergence in rice, and are also likely to play an opposite role in *Arabidopsis*. Overexpression of the two rice transcription factors in *Arabidopsis* caused smaller plant size with reduced cell size, and the expression of a series of genes encoding expansins and xyloglucan endotransglucosylase/hydrolases (XTHs) involved in cell elongation was reduced. However, transgenic *Arabidopsis* expressing OsWRKY24-SRDX as a synthetic chimeric repressor displayed indistinguishable phenotypes from wild-type plants. Moreover, the subcellular localization pattern of OsWRKY24 in *Arabidopsis* was different from that in rice. Thus, we demonstrate an example of transcription factors from one species playing distinct roles in different plant species.

Key words: APETALA2 transcription factor, cell size, RNA expression, transgenic *Arabidopsis*, WRKY transcription factor.

Transcription factors (TFs) are master regulators that control the expression of many other genes. Recently, two rice TFs, *Oryza sativa* APETALA 2 (OsAP2) and OsWRKY24 have been isolated as positive regulators in cell elongation in rice (Jang and Li 2017). The AP2 family TFs characterized by the AP2/ERF DNA-binding domain are plant-specific (Riechmann and Meyerowitz 1998) and known to be involved in regulation of diverse processes in plant development and stress responses (Nakano et al. 2006; Sharoni et al. 2011; Zhao et al. 2015).

In the progression of plant growth and development, *AINTEGUMENTA* and *AINTEGUMENTA-LIKE6* are related to flower organ growth and ovule development in *Arabidopsis* (Elliott et al. 1996; Jofuku et al. 1994; Klucher et al. 1996; Krizek 2009; Mizukami and Fischer 2000). Moreover, mutations in *AP2* increased the seed size because of increased cell number and size in the *Arabidopsis* embryo (Jofuku et al. 2005; Ohto et al. 2005). Other *AP2* TFs, *AtERF1*, *AtDREB1*, and *TINY* were able to dwarf plant height of *Arabidopsis* (Liu et al. 1998; Solano et al. 1998; Wilson et al. 1996).

In rice, several *AP2* genes including *SUBMERGENCE1A* (*SUB1A*), *SNORKEL1* (*SK1*), *SK2*, *Oryza sativa ERF protein associated with tillering and branching* (*OsEATB*) and *REDUCED LEAF ANGLE1* (*RLA1*)/*SMALL ORGAN SIZE1* (*SMOS1*) have been reported to play roles in regulating internode elongation, which is primarily post-mitotic expansion of differentiating cells (Aya et al. 2014; Hattori et al. 2009; Qi et al. 2011; Xu et al. 2006). In particular, *RLA1*/*SMOS1* was revealed to be a positive regulator in the brassinosteroid (BR) signaling pathway and also an auxin-dependent regulator of cell expansion (Hirano et al. 2014; Qiao et al. 2017).

The WRKY family TFs are only found in higher plants and the family is one of the largest TF families in plants. Since the first member was isolated in sweet potato, many WRKY TFs have been identified, including 74 in *Arabidopsis* and 102 in rice (Rushton et al. 2010). WRKY TFs are defined by their WRKY domain, a conserved DNA-binding domain containing 60 amino acid residues, which binds to a W-box (TTG ACC/T) in the regulatory DNA sequences. Additionally, WRKY TFs possess a zinc-finger motif at their C-terminus, either Cx4–5Cx22–23HxH (CCHH) or Cx7Cx23HxC (CCHC). Members of the WRKY family TFs are involved in a wide range
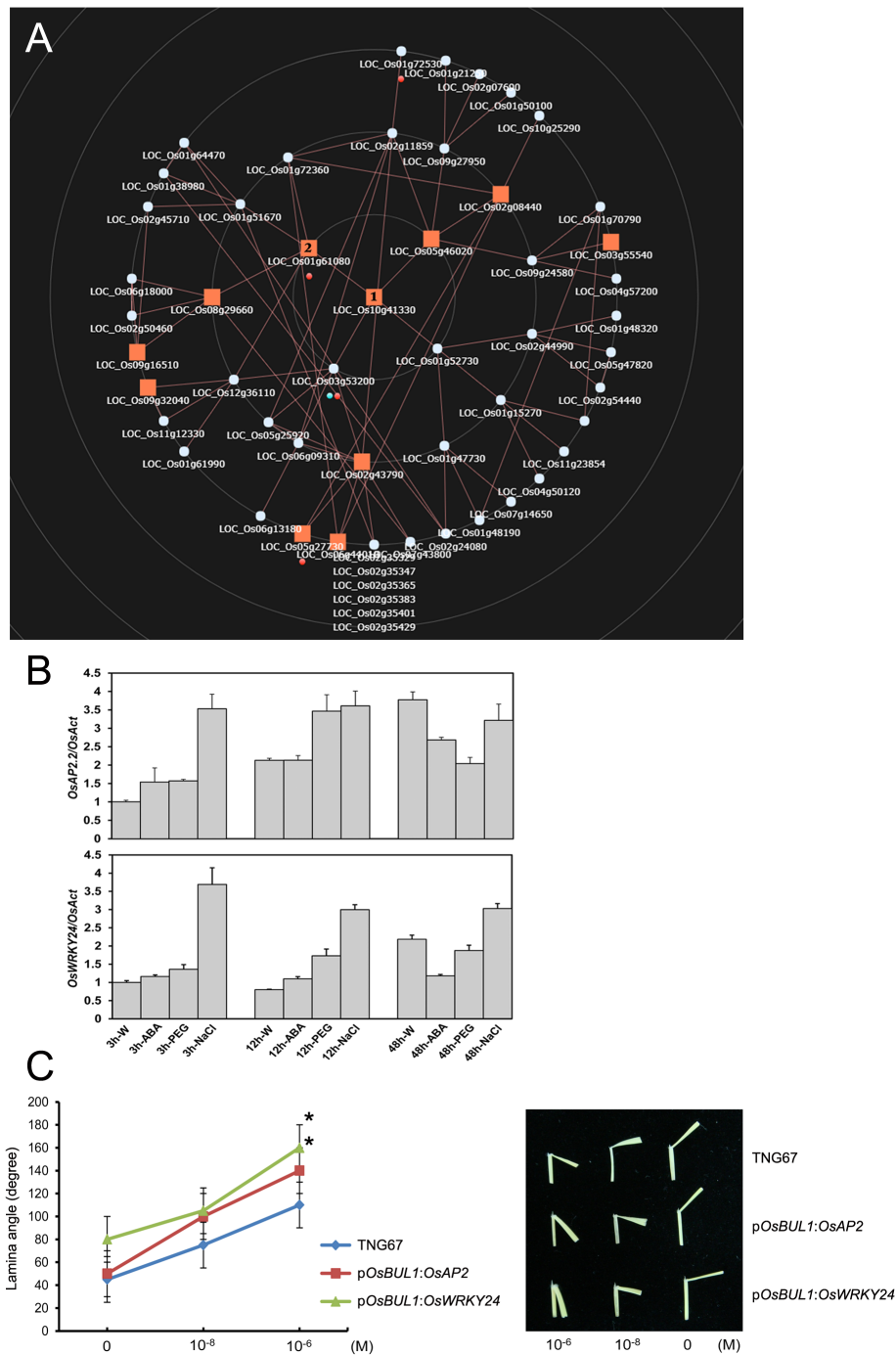
Figure 1. Expression patterns with functional convergence of *OsAP2* and *OsWRKY24* in rice. (A) In the RiceFREND database, *OsAP2* (Os10g41330) marked as 1 is presented to be co-expressed with *OsWRKY24* (Os01g61080) marked as 2. The red and blue circles represent plant-pathogen interaction and the phosphatidylinositol signaling system, respectively (in KEGG pathway). (B) Expression of *OsAP2* and *OsWRKY24* under different abiotic stress conditions. Rice seedlings were germinated and incubated on 1/2 MS media for 10 days and transferred to test tubes containing water (mock), ABA (60 μM; Sigma-A4906), PEG6000 (15%; Alfa Aesar-A17541) and NaCl (150 mM; Amresco-0241) solution, respectively. The plants were grown for 2 days under 12 h-light photoperiod at a constant temperature of 28°C, and each sample was harvested at 3 h, 12 h and 48 h time points. *Actin* (*Act*) was used as standard for quantification of gene expression. Data are represented as mean±SD of three biological replicates. W, water control; ABA, Abscisic acid 60 μM; PEG, Polyethylene glycol 6000 15%; NaCl, NaCl 150 mM. (C) Lamina inclination assay (Li et al. 2017) with exogenous brassinolide treatment (10^{-8} and 10^{-6} M; Sigma-E1641). Leaves of transgenic rice plants expressing *OsAP2* and *OsWRKY24* under the control of *OsBUL1* promoter (Jang and Li 2017) were used together with those of WT control, TNG67. Values represent mean±SD ($n{\geq}15$). Data that is significantly different from the corresponding control is indicated (*$p{<}0.05$; students' *t*-test).
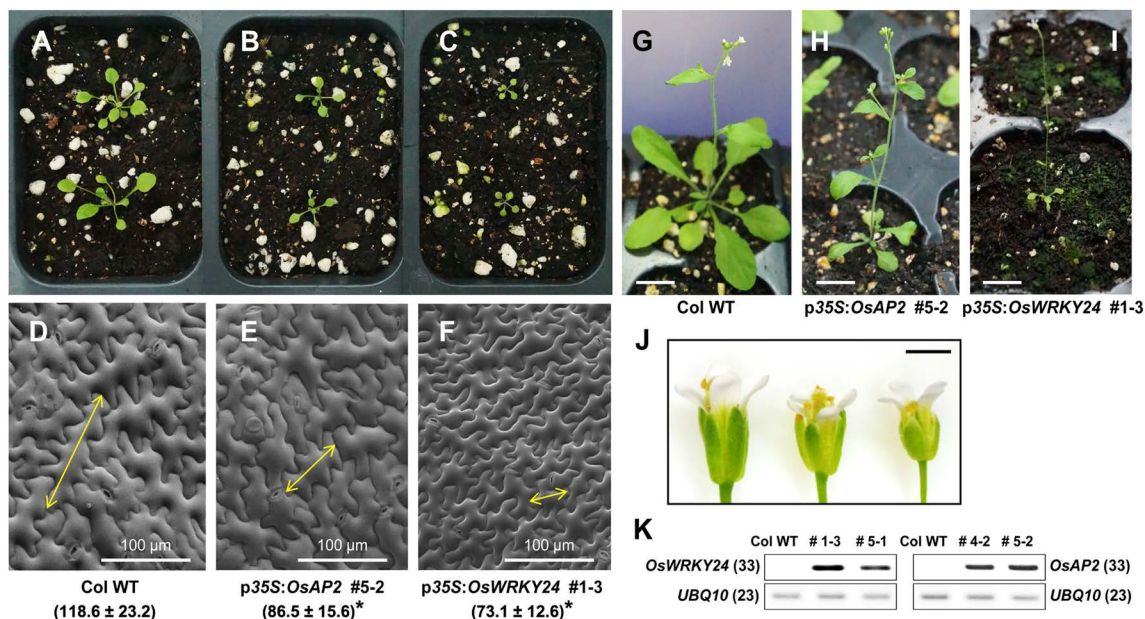
Figure 2.   Transgenic *Arabidopsis* plants overexpressing *OsAP2* and *OsWRKY24*. Twenty-four-day-old seedlings of wild-type Columbia (A, D), p*35S:OsAP2* (B, E) and p*35S:OsWRKY24* (C, F) plants. Epidermal cells of the first leaves from each plant are shown in D, E and F. Yellow opposing arrows on D, E and F indicate the length of a representative epidermal cell from each genotype. The cell length of each genotype is shown as mean±SD (*μm*, *n*≥15). Student's *t*-test was used for statistical analysis (**p*<0.05). The cell length was measured with ImageJ software (https:// imagej.nih.gov/ij/). At the flowering stage, the tiny body size of transgenic *Arabidopsis* plants (H, I) can be observed compared to wild-type plant (G). Bar=1 cm (G–I). (J) Flower size is also reduced in transgenic plants for p*35S:OsAP2* (middle) and p*35S:OsWRKY24* (right) compared to that of wild-type plants (left). Bar=1 mm. (K) Expression of transgenes, *OsAP2* and *OsWRKY24* in transgenic plants. The numbers of cycles used in the RT-PCR are in parenthesis.

of developmental and physiological processes including trichome and seed development, senescence, biotic and abiotic stress response (Jiang et al. 2017). In terms of cell elongation/enlargement, a loss-of-function mutant of the maternally expressed *WRKY* gene, *ttg-2* produced small seeds displaying reduced cell elongation in integuments, and precocious endosperm cellularization (Garcia et al. 2005). Recently, new roles of WRKY TFs in BR signaling have been revealed (Chen et al. 2017) and BR regulates a wide range of plant growth and development, such as cell elongation, cell division, xylem differentiation and stress responses. In rice, *OsWRKY78* is an example of a WRKY TF that functions in regulating cell elongation (Zhang et al. 2011). Both RNA interference (RNAi) and T-DNA insertion lines provided evidence showing that *OsWRKY78* may act as a regulator in stem elongation and seed development of rice. Recently, a foxtail millet mutant, *loose-panicle 1* (*lp1*) impaired in a *WRKY* gene, has been reported to have an interesting phenotype, reduced internode length but increased panicle length (Xiang et al. 2017).

Previously, we reported that two rice TFs, OsAP2 (Os10g41330) and OsWRKY24 (Os01g61080) are positive regulators for cell elongation acting downstream of *Oryza sativa BUL1 Downstream Gene1* (*OsBDG1*) in rice (Jang and Li 2017). Increased expression of *OsAP2* and *OsWRKY24* in lamina joint and panicle of rice caused increased lamina inclination and grain

size through the cell elongation in those parts. In this study, we tested whether the functional roles of the two rice TFs are conserved in a heterologous system, *Arabidopsis*. To our surprise, transgenic *Arabidopsis* plants overexpressing the two rice TFs exhibited smaller plant body size with reduced epidermal cell size, which is an opposite phenotype to that of transgenic rice (Jang and Li 2017). Thus, we show that TFs, master regulators that control gene expression in one plant species may produce distinct phenotypes in heterologous systems. This information may provide more possibilities for application of genetic modification (GM) technology in plants.

First, we conducted co-expression analysis of the two rice genes, *OsAP2* and *OsWRKY24* by using RiceFREND database (Sato et al. 2013; http://ricefrend.dna.affrc. go.jp). The database includes 815 pieces of microarray data from various tissues at different developmental stages and phyto-hormone treatment conditions with single or multiple guide gene searches. The expression of the two genes, *OsAP2* and *OsWRKY24* are closely linked based on the analysis result through the database (Figure 1A) supporting our previous spatiotemporal expression analyses (Jang and Li 2017). Moreover, the two genes are also involved in stress response in rice: *OsAP2* transcript is known to be more abundant in the submergence tolerant rice, M202 (*Sub1*) related to control M202 at 1 day after submergence (Jung et al. 2010) and *OsWRKY24*

was previously shown to enhance resistance to salt and drought through the cross-talk of gibberellic acid and abscisic acid signaling pathways under stress conditions (Zhang et al. 2009). As shown in Figure 1B, the expression patterns of the two genes by treatment of
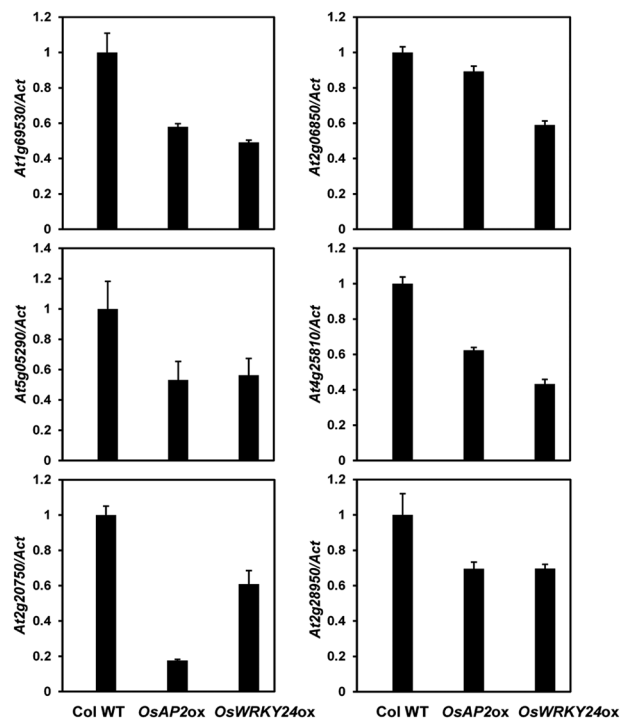

Figure 3. Expression of genes encoding cell-wall loosening proteins in *Arabidopsis*. Expression level of *AtEXPA1* (At1g69530), *AtEXPA2* (At5g05290), *AtEXPA6* (At2g28950), *AtEXPB1* (At2g20750), *AtXTH4* (At2g06850) and *AtXTH23* (At4g25810) was investigated in 2-week-old seedlings of wild-type Columbia, p*35S:OsAP2* and p*35S:OsWRKY24* plants. qPCR was performed on a CFX96TM real-time system (Bio-Rad) using Maxima SYBR Green qPCR Master Mix (Thermo). The primers used for quantification are listed in Supplementary Table S1. *Actin* (*Act*) was used as a standard for quantification of gene expression. Data are represented as mean±SD of three biological replicates.

chemicals inducing abiotic stresses such as drought and salt stresses are also similar. Results of lamina inclination assay with brassinolide (Sigma-E1641) treatments demonstrated that induced expression of the two genes in the lamina joint under the control of the *OsBUL1* promoter confers increased sensitivity to brassinolide resulting in increased lamina angles confirming the expression hierarchy among *OsBUL1*, *OsBDG1* and *OsAP2* and *OsWRKY24* with brassinosteroid response (Figure 1C; Jang and Li 2017; Jang et al. 2017a).

To examine whether the functional activities of *OsAP2* and *OsWRKY24* are conserved in *Arabidopsis*, the two genes were ectopically expressed in *Arabidopsis* using CaMV*35S* promoter. Compared with the WT, transgenic *Arabidopsis* showed much smaller plant body size starting from the seedling stages (Figure 2A–C, 2G–I, 2K). Moreover, the flower size of the transgenic plants was reduced (Figure 2J). Particularly, transgenic *Arabidopsis* overexpressing *OsWRKY24* exhibited an extremely tiny body and produced a small number of short seeds (Supplementary Figure S1). To test whether cell size is also affected in those plants, epidermal cells of the first true leaf from each transgenic plant were examined using a scanning electron microscope (SEM) and indeed, smaller size epidermal cells were observed in the transgenic plants (Figure 2D–F). In order to examine the phenotype by molecular analyses, expression of several representative genes involved in cell elongation was investigated by quantitative RT-PCR (Figure 3). The expression level of *Expansin* genes including *Expansin-A1* (At1g69530), *Expansin-A2* (At5g05290), *Expansin-A6* (At2g28950) and *xyloglucan endo-transglycosylase/hydrolase* (*XTH*) genes such as *XTH4* (At2g06850) and *XTH23* (At4g25810) was lower in transgenic plants showing smaller epidermal cells than the WT control. Subcellular localization of OsAP2 and OsWRKY24 was also examined using *Arabidopsis*

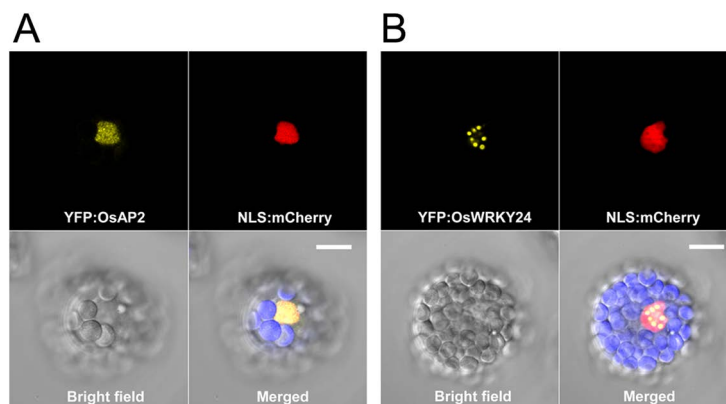Figure 4. Subcellular localization of OsAP2 and OsWRKY24 in *Arabidopsis* mesophyll cells. (A) YFP:OsAP2 and nuclear localization signal (NLS):mCherry were co-transformed into *Arabidopsis* protoplasts. YFP signals from YFP:OsAP2 are exclusively localized in the nucleus. (B) YFP:OsWRKY24 and NLS:mCherry were co-transformed into *Arabidopsis* protoplasts. YFP signals from YFP:OsWRKY24 form nuclear speckles. Red florescence is from a nuclear marker, NLS:mCherry (Lee et al. 2012). Isolation and transfection of *Arabidopsis* protoplasts followed, as described by Wu et al. (2009). Bar=10 μm.

mesophyll protoplasts. Yellow florescence signals are exclusively detected in the nucleus from both Yellow Florescence Protein (YFP):OsAP2 and YFP:OsWRKY24 fusion proteins (Figure 4), which is similar to the results in rice mesophyll protoplasts (Jang and Li 2017). Interestingly, however, the YFP:OsWRKY24 protein forms nuclear-speckles only in *Arabidopsis* mesophyll cells (Figure 4B) implying the distinct characteristics between mesophyll cells of the two different plant species, rice and *Arabidopsis*. Nuclear speckles are believed to serve as an active hub coordinating transcription of genes with the processing of their pre-mRNAs (Hall et al. 2006). Thus, OsWRKY24 is likely to participate in active transcription of downstream genes in *Arabidopsis*. Furthermore, in order to test whether OsWRKY24 acts as a transcriptional activator in plants, a dominant repressor version of OsWRKY24 [OsWRKY24-*SUPERMAN* repressive domain X (SRDX)] was generated by the fusion of SRDX (LDLDLELRLGFA) repressor domain to the carboxyl terminal of OsWRKY24. It was found that fusion of this motif to transcriptional activators converts them into dominant repressors (Hiratsu et al. 2003). Transgenic plants overexpressing OsWRKY24-SRDX exhibited no significant phenotypic alterations compared to the WT (Figure 5) indicating OsWRKY24 may act as a transcriptional activator in *Arabidopsis*.

Since TFs are master controllers of gene expression through the formation of homo/hetero-multimeric complexes, overexpression of TFs can be anticipated to produce distinct phenotypes in different plant species. For instance, although overexpression of a key flowering gene of *Arabidopsis*, *FD* encoding a basic leucine zipper (bZIP) TF, causes early flowering in *Arabidopsis*, transgenic rice overexpressing *FD* exhibits no significant alteration in heading date but has smaller grain size with semi-dwarfism (Abe et al. 2005; Jang et al. 2017b). Previously, we identified two rice genes, *OsAP2* and *OsWRKY24* as putative downstream genes of *OsBDG1*, a positive regulator controlling rice leaf angle through cell elongation of the lamina joint (Jang and Li 2017). The spatiotemporal expression of the two genes using various organs at different developmental stages displayed similar patterns and the induced expression of *OsAP2* and *OsWRKY24* was observed by exogenous treatment of phytohormones such as gibberellin and/or brassinosteroid (Jang and Li 2017). Furthermore, ectopic expression of *OsAP2* and *OsWRKY24* under the control of *OsBUL1* promoter, which is preferentially active in lamina joints and spikelets caused increased leaf angle and grain size with elongated epidermal cells in lamina joint and spikelet lemma (Jang and Li 2017).

In this study, we surveyed the co-expression network of rice genes through the RiceFREND database (Sato et al. 2013) with *OsAP2* and *OsWRKY24* and found
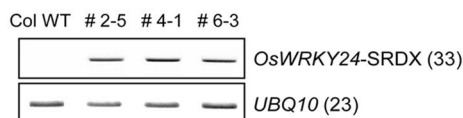

Figure 5. Transgenic *Arabidopsis* plants overexpressing a chimeric repressor of *OsWRKY24* (*OsWRKY24-SRDX*). The *OsWRKY24-SRDX* DNA fragment for an entry clone was amplified by PCR using the following primers, 5′GGG GAC AAG TTT GTA CAA AAA AGC AGG CTT CAT GAC AAC CTC GTC GTC3′ and 5′GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG CTA TGC GAA TCC TAG TTC CAG TTC GAG ATC CAG GTC TAG GTA GAG CGA GTT CTG3′. Transgenic plants containing p*35S:OsWRKY24-SRDX* construct with high expression level of the transgene exhibit an indistinguishable phenotype to WT.

their expression patterns to be closely linked. Since some genes of the AP2 family and WRKY family are known to play pivotal roles in the adaptation of plants to abiotic stresses including drought stress (Bakshi and Oelmüller 2014; Phukan et al. 2017), we also investigated expression patterns of the two genes under several abiotic stress-inducing conditions using abscisic acid (60 µM ABA; Sigma-A4906), polyethylene glycol (15% PEG6000; Alfa Aesar-A17541) and NaCl (150 mM; Amresco-0241). Of note, the two genes showed similar expression patterns under different stress conditions. Functional study using transgenic rice plants expressing *OsAP2* and *OsWRKY24* under the control of *OsBUL1* promoter demonstrated that the two genes confer increased sensitivity to brassinolide supporting the notion that genes with similar expression patterns tend to be functionally related across several experimental conditions. Transgenic *Arabidopsis* overexpressing *OsAP2* and *OsWRKY24* also showed similar phenotypes such as small plant body with decreased cell size, and expression of *expansin* and *XTH* genes involved in cell expansion/elongation through cell-wall loosening was also reduced in the transgenic plants (Cosgrove 2000; Eklöf and Brumer 2010). Notably, the phenotype of smaller cells in *Arabidopsis* caused by *OsAP2* and *OsWRKY24* is opposite to that of transgenic rice with increased expression of those genes (Jang and Li 2017) suggesting that the two TFs may control the

expression of distinct target genes in *Arabidopsis* and rice. Nuclear speckled localization of YFP signals from YFP:OsWRKY24 in *Arabidopsis* mesophyll protoplasts, which is different from that in rice mesophyll protoplasts (Jang and Li 2017) may support this notion. Previously, overexpression of *BOLITA*, an *Arabidopsis AP2* gene caused small and compact flowers in *Arabidopsis* while it produced bigger flowers with broader petals in tobacco (Marsch-Martinez et al. 2006) supporting the notion that ectopic expression of TFs in heterologous systems may cause novel phenotypes, which may be due to divergent *cis*-elements in the regulatory region of gene expression in genomes of distinct species and/or modification of transcription machinery for downstream genes through interaction with other TFs. Thus, we believe that the two genes, *OsAP2* and *OsWRKY24* have potential to control cell size in plants, and it would be also interesting to identify their target genes in *Arabidopsis* and rice.

Acknowledgements

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