Functional Differentiation of *Lotus japonicus* TT2s, R2R3-MYB Transcription Factors Comprising a Multigene Family

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Leguminous plants have many paralogous genes encoding enzymes involved in the flavonoid biosynthetic pathway. Duplicate genes are predicted to contribute to the production of various flavonoid compounds and to have resulted in a diversity of legume species. We identified gene duplication in the transcription factors regulating flavonoid biosynthesis in the model legume *Lotus japonicus*. Three copies of a homolog of *Arabidopsis thaliana* TRANSPARENT TESTA2 (TT2), which is a MYB transcription factor that regulates proanthocyanidin biosynthesis, were present in the *L. japonicus* genome. The organ specificity and stress responsiveness differed among the three LjTT2s, and correlations between proanthocyanidin accumulation and the expression levels of the three LjTT2s, and correlations between proanthocyanidin accumulation and stress responsiveness differed among the three LjTT2s. These results suggest that LjTT2 factors have diverse functions in the tissues in which they are expressed; in particular, LjTT2a is predicted to have evolved flexibility in interaction with other transcription regulators to resist environmental stresses.

**Keywords:** *Lotus japonicus* — Multigene family — MYB — Proanthocyanidin — Transcription factor.

Abbreviations: AD, activation domain; ANR, anthocyanidin reductase; BD, binding domain; bHLH, basic helix–loop–helix; DMACA, 4-(dimethylamino) cinnamaldehyde; EST, expressed sequence tag; GFP, green fluorescent protein; LAR, leucoanthocyanidin reductase; LUC, firefly luciferase; ORF, open reading frame; RT–PCR, reverse transcription–PCR; RUC, *Renilla* luciferase; 35S, cauliflower mosaic virus 35S promoter; TAC, transformation-competent artificial chromosome; TT2, TRANSPARENT TESTA2; TT8, TRANSPARENT TESTA8; TTG1, TRANSPARENT TESTA GLABRA1.

The nucleotide sequences reported in this paper have been submitted to DDBJ under accession numbers AP009374-5 (TAC clone LjT02E24a-b), AB300033 (LjTT2a), AB300034 (LjTT2b) and AB300035 (LjTT2c).

**Introduction**

Gene duplications contribute to the establishment of new gene functions and underlie the origins of evolutionary novelty (Ohno 1970, Moore and Purugganan 2005). Annotation of the first four complete plant genomes has revealed that plants have many paralogous genes that make up multigene families (*Arabidopsis* Genome Initiative 2000, International Rice Genome Sequencing Project 2005, Tuskan et al. 2006, French–Italian Public Consortium for Grapevine Genome 2007). The origin of multigene families is attributed to gene duplication arising from region-specific duplication or genome-wide polyploidization, which is a prominent feature in plant genome evolution. Gene redundancy contributes to many aspects of plant cell functions, including secondary metabolism and vegetative and reproductive development.

In leguminous plants, genes involved in flavonoid biosynthesis make up multigene families and form gene clusters. For example, eight copies of chalcone synthase are encoded at locus I of the soybean genome (Akada and Dube 1995, Clough et al. 2004). Six isoforms of chalcone synthase have also been reported in *Phaseolus* (Ryder et al. 1987). *Lotus japonicus* is a model plant for the study of the molecular genetics of leguminous plants; an expressed sequenced tag (EST) analysis (Asamizu et al. 2000, Endo et al. 2000, Asamizu et al. 2004) and a genome sequencing project (Sato et al. 2001, Nakamura et al. 2002, Asamizu et al. 2003, Kaneko et al. 2003, Kato et al. 2003, Sato et al. 2006) are in progress for this species. In *L. japonicus*, genes encoding chalcone synthase, chalcone isomerase and dihydroflavonol reductase form respective clusters (Shimada et al. 2003, Shimada et al. 2005). The paralogous genes were suggested to have acquired new functions different from those of their ancestral types as a result of accumulated...
nucleotide substitutions during their molecular evolution. Some duplicated genes show different expression patterns, suggesting that some specialization may allow subtle shifts to optimize the pathway throughput in different tissues and at different points in the plant’s life cycle. Various expression patterns of structural genes in the pathway are achieved via transcription factors with distinct specificities.

Transcriptional regulators for flavonoid biosynthesis, including the anthocyanin, flavonol and proanthocyanidin pathways, are controlled by members of protein families containing R2R3-MYB domains, basic helix-loop-helix (bHLH) domains and conserved WD40 repeats (WDRs). The combination and interaction of the R2R3-MYB, bHLH and WDR factors determine the set of genes to be expressed (Quattrocchio et al. 1998, Baudry et al. 2004, Broun 2005, Koes et al. 2005, Ramsay and Glover 2005). In Arabidopsis thaliana, the biosynthetic pathway leading to proanthocyanidin accumulation has been characterized using transparent testa (tt) mutants, which fail to accumulate proanthocyanidins in the seed coat (Shirley et al. 1995, Abrahams et al. 2002). Among the identified TT loci, TRANSPARENT TESTA2 (TT2), TRANSPARENT TESTA8 (TT8) and TRANSPARENT TESTA GLABRA1 (TTG1) encoding MYB, bHLH and WDR proteins, respectively, regulate the expression of several flavonoid structural genes, including anthocyanidin reductase (ANR; Nesi et al. 2000, Nesi et al. 2001, Baudry et al. 2004, Fig. 1). AN2, a MYB transcriptional factor of Petunia hybrida, activates genes of the late anthocyanin pathway, beginning with dihydroflavonol 4-reductase in petal limbs (Quattrocchio et al. 1999). In a transient assay, AN2 can interact with either of two distinct bHLH factors, JAF13 and AN1. In Zea mays, transcriptional regulation of the entire anthocyanin biosynthetic pathway, starting from c2 (encoding chalcone synthase), is controlled by the combinatorial action of C1/Pl and R/B families, which encode MYB and bHLH proteins, respectively (Paz-Ares et al. 1987, Ludwig et al. 1989, Ludwig et al. 1990, Lloyd et al. 1992, Cone et al. 1993), whereas the R2R3-MYB transcription factor Z. mays P activates a subset of genes for 3-deoxyflavonoids and phlobaphene biosynthesis without an additional bHLH factor in floral organs of Z. mays (Grotewold et al. 1994; Fig. 1). Thus, combinatorial interactions between transcription factors are of central importance in the regulation of gene expression and seem to be common in several plant species.

Of the transcription factors that make up the transcriptional complexes, R2R3-MYB proteins form a large superfamily. R2R3-MYB proteins are involved in many physiological and biochemical processes.

Fig. 1 The flavonoid biosynthetic pathway and its regulators. Enzymes of the flavonoid biosynthetic pathway are indicated in uppercase letters. Parentheses on each side indicate the regulatory factors involved in the biosynthesis of the compounds shown in black boxes. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone-3-hydrogenase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase; UFGT, UDP-glucose:flavonoid 3-O-glucosyltransferase; PAP1, PRODUCTION OF ANTHOCYANIN PIGMENT1; TT, TRANSPARENT TESTA; TTG1, TRANSPARENT TESTA GLABRA1; C1, COLOURLESS1; Pl, PURPLE LEAF; R, RED; B, BOOSTER.
Approximately 125 R2R3-MYB genes occur in the *A. thaliana* genome (Riechmann and Ratcliffe 2000, Stracke et al. 2001) in contrast to the small number of genes that encode MYB homologs in the animal and fungal kingdoms (Lipsick 1996).

We isolated and characterized three genes of *L. japonicus*, i.e. *LjTT2a*, *b* and *c*, which encode MYB transcription factors closely related to *A. thaliana* TT2. The sequence homology and phylogenetic analysis of these three MYB proteins imply that they duplicated recently. *LjTT2a*, *b* and *c* can activate the promoter of *A. thaliana* and *L. japonicus* ANR, indicating that *LjTT2a*, *b* and *c* have orthologous functions to *A. thaliana* TT2, which is involved in proanthocyanidin biosynthesis. *LjTT2a*, *b* and *c* displayed not only diverse patterns of expression in various organs and in response to environmental stresses, but also different transcriptional activities mediated by interactions with bHLH and WDR proteins. Therefore, these closely related MYB proteins may differentially regulate proanthocyanidin biosynthesis by producing various patterns of ANR gene expression.

### Results

**TT2 homologs comprise a multigene family in the *L. japonicus* genome**

A similarity search of *A. thaliana* TT2 was carried out against the 333 Mbp region of the *L. japonicus* genome obtained from the genome sequencing program, which covers 90.2% of *L. japonicus* ESTs. Among the MYB factors identified in the search, three paralogous genes show significant similarity to TT2. The isolated open reading frames (ORFs), named *L. japonicus* TT2*α*, *b* and *c* (*LjTT2a*, *b* and *c*; GenBank accession Nos. AB300033, AB300034 and AB300035), share high similarities of 74–82% (Table 1) in the full-length sequences, indicating that they comprise a multigene family formed by recent duplication. An analysis of the deduced amino acid sequences revealed that *LjTT2a*, *b* and *c* contain R2R3 repeat DNA-binding conserved domains located tandemly at the N-terminal regions (Fig. 2A) that share high amino acid sequence similarities of 93–95% (Table 1). The R2R3 repeat DNA-binding domains of *LjTT2s* contain the motif [D/E]Lx3[R/K]x3Lx2Lx3R for interaction with bHLH protein (Stracke et al. 2001, Fig. 2A). Nesi et al. (2000) determined a sequence conserved between TT2 and OsMYB3 in the highly variable C-terminal region. However, this region was not found in the amino acid sequence of the *LjTT2s*.

On the phylogenetic tree created using the neighbor-joining method in the MEGA program (Kumar et al. 2004) and amino acid sequences of R2R3-MYB domains of plant MYB factors that are involved in pigmentation or epidermal cell shape, *LjTT2b* was more related to *LjTT2c* than to *LjTT2a*, and these three proteins formed a subgroup with TT2 (Fig. 2B), which regulates proanthocyanidin biosynthesis in the seed coat of *A. thaliana* (Nesi et al. 2001). This group further clustered with ZmCl and ZmPl, which are involved in anthocyanin regulation in *Z. mays* (Piazza et al. 2002).

Comparison of the gene structure among the three *LjTT2s* revealed that these genes have a similarly structured 4 kb region containing three exons. Although the lengths of each coding sequence differ, the sizes of the first and second exons are very similar among these three genes (Fig. 2C).

**Table 1 Sequence homology of TT2 and LjTT2s**

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<th>Homology (%)</th>
<th>Nucleotide sequence of the ORF</th>
<th>Amino acid sequence of the R2R3 domain</th>
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<td>LjTT2a</td>
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<td>56.9</td>
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<td>LjTT2b</td>
<td>74.5</td>
<td>59.2</td>
</tr>
<tr>
<td>LjTT2c</td>
<td>76.6</td>
<td>58.5</td>
</tr>
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</table>

*LjTT2a* interacts with *TT8* and *TTG1*

Yeast two-hybrid experiments of *A. thaliana* transcription factors have shown that TT2, TT8 and TTG1 interact to form a complex (Baudry et al. 2004). To examine protein–protein interactions of *LjTT2s* with *TT8* and *TTG1*, yeast two-hybrid experiments using *LjTT2s* as well as TT2 were performed. TT2 and *LjTT2s* were fused to the GAL4 DNA-binding domain (BD) and assayed for their ability to bind TT8 and TTG1 proteins fused to the GAL4 activation domain (AD) (Fig. 3). Among the clones grown on the dropout media lacking tryptophan, leucine, histidine and adenine, only clones containing *LjTT2a* showed a similar growth pattern to TT2, whereas AD-fused *LjTT2b* and *LjTT2c* showed weak interacting activity with TTG1 (Fig. 3).

The three *LjTT2s* activate the *ANR* promoter in plant cells

TT2, TT8 and TTG1 directly activate *ANR* expression in *A. thaliana* (Baudry et al. 2004). To determine whether the three *LjTT2s* can complement the function of TT2 in vitro, transient expression experiments were conducted in *A. thaliana* leaf cells (Fig. 4). Both TT2 and TT8 are necessary to activate *ANR* in plant cells, and TTG1 overexpression strongly increases the *ANR* activation initially conferred by TT2 and TT8 (Baudry et al. 2004). Therefore, we co-bombarded the effector constructs containing the genes of TT8 and TTG1 from *A. thaliana* driven by the cauliflower mosaic virus 35S (35S) promoter with a tested effector containing *A. thaliana* and *L. japonicus*
TT2 genes. For comparative analysis of the three LjTT2s, the effector constructs with and without the TT2 gene were used as positive and negative controls, respectively (Fig. 4A). About 1.6 kb of the promoter regions of A. thaliana ANR (Baudry et al. 2004) and L. japonicus ANR and leucoanthocyanidin reductase (LAR, data not shown) fused in-frame to the green fluorescent protein (GFP) and firefly luciferase (LUC) genes were used as
reporter constructs (Fig. 4A). The effects of the ectopic expression of various combinations of MYB, bHLH and WDR factors on ANR activation were determined by detecting GFP fluorescence and quantifying LUC activity. For internal controls in quantitative analysis, constructs containing the Renilla luciferase (RUC) gene driven by the 35S promoter were co-bombarded with the effector and reporter constructs in A. thaliana leaf cells. The reporter constructs did not result in significant GFP expression (Fig. 4B) or LUC activity (Fig. 4C) without MYB factors. However, the co-expression of TT2 or the three LjTT2s with TT8 and TTG1 resulted in significant expression of GFP (Fig. 4B) and LUC activity (Fig. 4C). In agreement with the result obtained by Baudry et al. (2004), the combination of TT2 and TT8 induced the activation of the AtANR promoter, and TTG1 expression strongly increased the AtANR activation conferred by TT2/T8 (Fig. 4C).

Whereas LjTT2b and c induced the activation of the AtANR promoter similarly to TT2, LjTT2a showed a different mode of induction. First, the transient transformation of A. thaliana leaf cells with LjTT2a alone induced significant activation of the reporter gene. Secondly, the co-expression of TT8 had a distinct effect on LjTT2a activity, leading to increased LUC activity. Thirdly, the effect of TTG1 on the LjTT2a/TT8 induction of the AtANR promoter was lower than those of the other tested MYB factors (TT2, LjTT2b and LjTT2c; Fig. 4C).

To confirm that LjTT2s activate the structural genes of L. japonicus involved in proanthocyanidin biosynthesis, the promoter regions of L. japonicus ANR and LAR were used in transient expression experiments (Fig. 4D). The co-expression of TT2, as well as the LjTT2s, with TT8 and TTG1 and the LjANR promoter increased the LUC activity in A. thaliana leaf cells. The levels of activation of the reporter gene differed among the four MYB factors, with the highest activity induced by LjTT2a (Fig. 4D). In contrast to ANR, the LjLAR promoter was not activated by any combination of TT2 or the LjTT2s with TT8 and TTG1 (data not shown).

Transcription levels of the three LjTT2s differ with developmental and environmental stimuli

To gain an overview of the expression profiles of the three LjTT2s in L. japonicus, we performed semi-quantitative reverse transcriptase–PCR (RT–PCR) with total RNA prepared from various tissues and/or organs, including seedlings at different stages of development (Fig. 5A). Transcripts of the three LjTT2s were more or less detected in all seedling stages examined (Fig. 5A). The accumulation of transcripts increased during seedling development for all three LjTT2s. The organ specificity of expression in mature plants differed among the three LjTT2s (Fig. 5A). Transcripts of LjTT2a were detected only slightly in all organs examined, in contrast to LjTT2c, the transcripts of which accumulated at high levels compared with those observed in seedlings. LjTT2b was mainly expressed in roots, and a few transcripts were detected in the aerial plant parts.

To address the relationships of the three LjTT2s with proanthocyanidin accumulation in L. japonicus, we examined proanthocyanidin accumulation, as well as the transcripts of putative ANR and LAR of L. japonicus, which may be involved in proanthocyanidin biosynthesis (Fig. 5A). To detect proanthocyanidin accumulation, L. japonicus seedlings and organs at the same developmental stages and growth conditions used in the expression analysis of the LjTT2s were stained with DMACA [4-(dimethylamino) cinnamaldehyde], which specifically reacts with proanthocyanidin and its precursors and visualizes the compounds as blue staining (McMurrough
Fig. 4  Activation of the AtANR and LjANR promoters in A. thaliana leaf cells. (A) The reporter and effector plasmid constructs are shown. Putative cis-elements, which are recognized by MYB and bHLH factors, were detected by analyzing the LjANR promoter region using the SignalScan server (PLACE database, http://www.dna.affrc.go.jp/PLACE/signalscan.html) and are indicated by arrowheads on the LjANR promoter region. The PA enhancer that is the regulatory region of the AtANR promoter responsible for expression in pigmented cells (Debeanjon et al. 2003) is indicated by the double-headed arrow. In the LUC assay, 35S::RUC plasmid was used as the internal control. (B) Transient expression of the reporter construct, which contains the GFP gene as a reporter gene, with the expression of TT2 (TT2, LjTT2a, b or c), TT8 and TTG1 in particle-bombarded A. thaliana leaf cells. A control assay was conducted without an effector plasmid containing the TT2 factor. The scale bar represents 0.5 μm. Transient expression of the reporter constructs of AtANR (C) and LjANR (D) promoters, which contain the LUC gene as a reporter gene, with different combinations of TT2, TT8 and TTG1 in particle-bombarded A. thaliana leaf cells. The columns and error bars denote the mean and standard deviation of the activity of each reporter plasmid after bombardment. The reporter gene activity, measured as firefly luciferase (LUC) enzyme activity, is expressed in arbitrary units and was normalized to Renilla luciferase (RUC) activity expressed by the co-bombarded internal control plasmid 35S::RUC.
Increasing expression of the three \textit{LjTT2s} and \textit{ANR} during seedling development was correlated with proanthocyanidin accumulation in \textit{L. japonicus} (Fig. 5A). Although \textit{ANR} and \textit{LAR} were expressed in all organs tested, proanthocyanidin accumulation was limited to the stems and roots (Fig. 5A). \textit{LAR} expression remained stable in all tissues examined.

Some genes involved in the flavonoid biosynthetic pathway are induced by environmental stresses (Shimizu et al. 1999, Shimada et al. 2000, Winkel-Shirley 2001). To assess the response of the three \textit{LjTT2s} to stress, total RNA of \textit{LjTT2a} and \textit{ANR} was
transiently induced at 1 h after wounding and began increasing from 6 h after wounding (Fig. 5B). Although elicitor treatment with reduced glutathione (Shimada et al. 2000) had no effect on ANR expression, the three LjTT2s showed different patterns of expression after elicitation (Fig. 5B), suggesting the diversified functions of the LjTT2s in response to environmental stresses.

Discussion

Leguminous plants have many paralogous genes that encode enzymes involved in the flavonoid biosynthetic pathway. These duplicate genes are predicted to contribute to the production of various flavonoid compounds and to have resulted in a diversity of legume species. Because gene duplications occur at a higher frequency in plants than in animals and fungi, the transcription factor families also have a much higher expansion rate in plants than in animals (Shiu et al. 2005). We determined that gene duplication occurs in the transcription factors that regulate flavonoid biosynthesis in the model legume L. japonicus. Three copies of homologs of the A. thaliana MYB transcription factor TT2 which appears to be encoded by a single copy in the A. thaliana genome were present in the L. japonicus genome (Fig. 2A). TT2 homologs have been reported thus far only in Z. mays (C1; Ludwig and Wessler 1990), Brassica napus (BnTT2; Wei et al. 2006) and Vitis vinifera (VvMYBPA1; Bogs et al. 2007). However, an orthologous function was identified only in VvMYBPA1, which activates the VvANR promoter. C1 activates the AtANR promoter, but whether C1 can activate Z. mays ANR to regulate proanthocyanidin biosynthesis is not clear. Thus, this is the first report of putative proanthocyanidin regulators that possess the characteristics of multiplex genes in a legume.

Three LjTT2s form a tandem cluster in regions containing a high number of repetitive sequences derived from transposable elements including retrotransposons and miniature inverted repeat transposable elements (MITEs). Insertions of transposable elements are associated with gene clusters and generate further variation in these complexes and in gene expression (Richter and Ronald 2000, Feschotte et al. 2002, Casacuberta and Santiago 2003). Therefore, these elements are predicted to have contributed to the inversion of LjTT2b, as well as the diversification of the three genes (Fig. 2C).

According to the nucleotide and amino acid homology and the phylogenetic tree (Table 1, Fig. 2C), LjTT2b seems to have arisen from the duplication of LjTT2a. LjTT2b was then duplicated, giving rise to LjTT2c. The differences in the reporter activity affected by the three LjTT2s and physical interaction with other proteins were consistent with the branching pattern of the phylogenetic tree (Figs. 2B, C, 4C). On the other hand, LjTT2a and LjTT2c activated the AtANR promoter to some degree without the co-expression of TT8 and TTG1, in contrast to LjTT2b and TT2, which require TT8 and TTG1 to transactivate AtANR (Fig. 4C; Baudry et al. 2004). Because transcription factors of the bHLH family are involved in protein–protein interaction, including specific DNA binding and the activation of target genes (Goff et al. 1992), LjTT2a and c should also interact with another bHLH factor that is constitutively expressed in A. thaliana leaf cells to activate the AtANR promoter.

The effect of TT8 on the transactivation of LjTT2a appears to be much stronger than on that of the other TT2 factors TT2, LjTT2b, and LjTT2c. Moreover, the effect of TTG1 on the transactivation of LjTT2a was much lower than that on TT2, LjTT2b and LjTT2c under co-expression with TT8. Six positions that are highly conserved within the group of MYB proteins interact with bHLH factors; the amino acid motif [DE]Lx2[RK]x3Lx6Lx3R at positions 12–33 in the R3-MYB consensus contributes to the interaction strength (Zimmermann et al. 2004). However, these amino acid residues were highly conserved among the three LjTT2s and TT2 (Table 2). In the highly conserved amino acid sequences of the R3 repeat domain in the three LjTT2s, only two positions (3 and 11) were specific for LjTT2a. Site-directed mutagenesis of each residue will allow the determination of whether the strong interaction of LjTT2a with TT8 and non-specific interaction with other bHLH proteins depend on the R3-MYB domain. A weak effect of TTG1 on LjTT2a activity could be explained in this context because one of the proposed functions of WDR proteins is to stabilize bHLH proteins (Payne et al. 2000, Baudry et al. 2004). Strong interaction of MYB-bHLH would not require the stabilization effect of WDR proteins. Although no significant interactions of LjTT2b and LjTT2c with TT8 were observed in the yeast two-hybrid analysis, LjTT2b and LjTT2c significantly activated the AtANR promoter when co-expressed with both TT8 and TTG1. Baudry et al. (2004) reported that interaction of a ternary complex comprising TT2, TT8 and TTG1 resulted in increased stability compared with their dimers. Therefore, we concluded that LjTT2b and c required WDR proteins to form a stable complex with bHLH proteins.

Lotus japonicus accumulates proanthocyanidin in several organs and tissues, including the seed coat, roots and stems, whereas proanthocyanidin accumulation in A. thaliana is limited to the seed coat, in which TT2 is specifically expressed to induce the biosynthesis of proanthocyanidin and its precursors in collaboration with TT8 and TTG1 (Nesi et al. 2001). The expression patterns of the three LjTT2s were not limited to certain organs or tissues (Fig. 5). Correlations between proanthocyanidin accumulation and the expression levels of all LjTT2s were
Proanthocyanidins act in defense against plant disease. LjTT2a mature organs were much lower than those in seedlings, although the transcription levels of LjTT2a pathways could also be a possible explanation for this. Thus, the dual pathways via ANR and LAR (Fig. 1) appear to be regulated by different transcriptional mechanisms. Therefore, the paralogs of LjTT2 may be an evolutionary model of a transcription factor that acquired novel functional roles within the L. japonicus genome for survival in severe environmental conditions, resulting in a diversity of leguminous plants.

### Materials and Methods

**Plant materials**

Seeds of L. japonicus accession Gifu B-129 were offered by the Department of Biological Production and Environmental Science, Miyazaki University. Seeds were germinated on a 0.8% agarose gel. One week after germination, the seedlings were grown on soil under a 16 h photoperiod at 24°C. Various organs were harvested under a 16 h photoperiod at 24°C. They were then extracted and analyzed for the expression of LjTT2a, LjTT2b, and LjTT2c. The expression patterns as well as the physical interactions and transactivating properties imply the diversified functions of the three LjTT2s for proanthocyanidin biosynthesis (Fig. 6). LjTT2b expression appeared to be limited to the roots, and its activation of the AtANR promoter showed the most similarity with that of TT2 interacting with TT8 and TTG1, suggesting that LjTT2b is most related to TT2. LjTT2c was expressed in all organs examined and showed weak transactivity without TT8 and TTG1. However, the effect of TT8 and TTG1 on LjTT2c did not differ from that on TT2. Therefore, we predict that the nucleotide sequence substitutions occur in the LjTT2c promoter region, allowing its broad expression in various organs. LjTT2a was expressed in response to environmental stresses and had the most diversified activation of the three LjTT2s for proanthocyanidin accumulation in L. japonicus, whereas LAR has supplementary functions, at least in the organs examined. In L. corniculatus, the ectopic expression of ZmSn transactivates both ANR and LAR (Paolocci et al. 2007), implying that another bHLH factor is required to activate LjLAR. Thus, the dual pathways via ANR and LAR (Fig. 1) appear to be regulated by different transcriptional mechanisms.

The three LjTT2s showed distinct transcription patterns in leaves, stems and roots (Fig. 5A), in contrast to their expression in seedlings, suggesting functional differences among the three LjTT2s in mature organs. Transcripts of LjTT2b and LjTT2c accumulated specifically in roots and in all three organs examined, respectively (Fig. 5A), and may contribute to the expression of ANR leading to the organ-specific production of proanthocyanidin. However, both LjTT2c and ANR transcripts were observed in leaves without proanthocyanidin accumulation. It is predicted that other steps needed for proanthocyanidin accumulation such as transport of flavan-3-ol to the vacuole and/or following polymerization are lacking. The shift of flows of substrate or the product of LjANR to other metabolic pathways could also be a possible explanation for this. Although the transcription levels of LjTT2a in mature organs were much lower than those in seedlings, LjTT2a showed stress-inducible expression (Fig. 5B). Proanthocyanidins act in defense against plant disease (Debeaujon et al. 2000, Peters and Constabel 2002), and their antioxidant properties imply that they have roles in other stress responses. The increase in the expression of LjTT2a that was induced by mechanical wounding was correlated with the expression of ANR (Fig. 5B), implying a role for proanthocyanidin in resistance against predators and insects. Therefore, the divergence of expression patterns of paralogous genes within a species may reflect different functions in various cell types and in response to environmental stimuli.

### Table 2  Amino acid motif in the R3 repeat of TT2 and three LjTT2s

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<th>Position at R3</th>
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about 4 weeks after transfer to soil. Elicitation of *L. japonicus* with reduced glutathione was performed as described by Shimada et al. (2000).

**Genome analysis**

Generation of a genome library using the transformation-competent artificial chromosome (TAC) vector (Liu et al. 1999) from the genomic DNA of *L. japonicus* accession Miyakojima MG-20, the sequencing strategy and gene assignment were carried out as described previously (Sato and Tabata 2006).

**Gene isolation and semi-quantitative RT–PCR**

Total RNA was extracted from 100 mg of frozen cells with extraction buffer [4.2 M guanidine thiocyanate, 0.5% (w/v) N-lauroylsarcosine, 25 mM sodium citrate, 14 mM 2-mercaptoethanol] and phenol/chloroform, and precipitated with 2 M lithium chloride. Total RNA (500 ng) from *L. japonicus* was used for first-strand cDNA synthesis at a concentration of 125 ng μl⁻¹ with an oligo(dT) primer and Superscript III (Invitrogen, Carlsbad, CA, USA). *LjTT2a*, *b* and *c* were isolated by PCR using the primer pairs LjTT2 FP/LjTT2-1 RP, LjTT2 FP/LjTT2-2 RP and LjTT2 FP/LjTT2-3 RP (see Supplementary Table S1), which were designed based on the sequence information of LjT02E24. PCR was performed with ExTaq DNA polymerase (TAKARA SHUZO CO. LTD, Kyoto, Japan). Amplified cDNAs were ligated into pT7Blue T-Vector only (Novagen, Madison, WI, USA) to construct pT7LjTT2a, pT7LjTT2b and pT7LjTT2c.

The level of mRNA of *LjTT2* s and putative *LjANR* and *LAR* was estimated by semi-quantitative RT–PCR. The PCR primer pairs LjTT2-1 FP/LjTT2-1 RP, LjTT2-2 FP/LjTT2-2 RP and LjTT2-3 FP/LjTT2-3 RP were used to amplify *LjTT2* s. The primers LjANR FP/LjANR RP, LjLAR FP/LjLAR RP and ACTIN FP/ACTIN RP were used to amplify *LjANR*, *LjLAR* and *LjACTIN*, respectively (Supplementary Table S1).

**DNA sequencing**

Nucleotide sequences were determined using a BigDye Terminator v. 3.1 Cycle Sequencing Kit and an ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems).

**Construction of plasmids**

The plasmids for yeast two-hybrid assay were constructed by fusing the ORFs of TT2 and LjTT2a, b, c to the activation domain of the pGADT7 vector and those of TT8 and TTG1 to the binding domain of the pGBK7 vector (BD Bioscience). The ORFs were amplified with and without a stop codon by PCR using 35S::TT2 and 35S::TT8 vector (Shimada et al. 2006) for TT2 and TT8, *A. thaliana* cDNA for TTG1, and p71LjTT2-1, p71LjTT2-2 and p71LjTT2-3 for LjTT2a, b and c as the templates with KOD plus DNA polymerase (Toyobo, Osaka, Japan), with the primer sets: BD-AtTT2 FP/BD-AtTT2 RP, AD-AtTT8 FP/AD-AtTT8 RP, AD-AtTTG1 FP/AD-AtTTG1 RP, BD-LjTT2 FP/BD-LjTT2-1 RP and BD-LjTT2 FP/BD-LjTT2-2 RP and BD-LjTT2 FP/BD-LjTT2-3 RP, respectively (Supplementary Table S1).

The reporter plasmids for transient assay were constructed by inserting the ~1.6 kb fragment of the promoter region of *AtANR* (Baudry et al. 2004) and putative promoter region of *L. japonicus* *ANR* and *LAR* into the multicloning sites of the 35S::sGFP(S65T) vector, which was a kind gift of Dr. Niwa (University of Shizuoka, Japan). The fragments were generated by PCR from *A. thaliana* and *L. japonicus* genomic DNA using the set of primers: AtANRpro FP/AtANRpro RP, LjANRpro FP/LjANRpro RP and LjLARpro FP/LjLARpro RP, with restriction sites added (Supplementary Table S1). The PCR products of the *AtANR*, *LjANR* and *LjLAR* promoter were ligated in-frame to the GFP gene in the 35S::sGFP(S65T) vector, replacing the 35S promoter. The resulting plasmids were named AtANRpro::GFP, LjANRpro::GFP and LjLARpro::GFP, respectively.

For the luciferase assay, the fragments were generated by PCR from *A. thaliana* and *L. japonicus* genomic DNA using the set of primers: AtANRpro FP/AtANRpro RP, LjANRpro

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**Fig. 6** Hypothesis of the transcription regulation mediated by the three LjTT2s. In roots and/or stems, LjTT2b and c are predicted to contribute to the organ-specific expression of the *ANR* gene in cooperation with both bHLH and WDR factors homologous to TT8 and TTG1, respectively. LjTT2a may be involved in stress responses and may interact with other bHLHs and/or WDRs.
FP/LjANRpro FP and LjLARpro FP/LjLARpro FP, with restriction sites added (Supplementary Table S1), using KOD plus DNA polymerase. The PCR products of the AtANR, LjANR and LjLAR promoters were ligated in-frame to the 35S::LUC vector (Shimada et al. 2006), replacing the 35S promoter. The resulting plasmids were named ATANRpro::LUC, LjANRpro::LUC and LjLARpro::LUC, respectively.

The effector plasmids for transient assay were constructed by fusing the ORFs of LjTT2a, LjTT2b, LjTT2c and TTG1 to the 35S::cGFP(S65T) vector, replacing the GFP gene. The ORFs were amplified with a stop codon by PCR using pLjTT2a, pLjTT2b, pLjTT2c and A. thaliana cDNA as templates with the set of primers: LjTT2 FP/LjTT2-1 RP, LjTT2 FP/LjTT2-2 RP, LjTT2 FP/LjTT2-3 RP and AtTTG1 FP/AtTTG1 RP with restriction sites added (Supplementary Table S1), with KOD plus DNA polymerase. The PCR products were ligated in-frame to the 35S::cGFP(S65T) vector, constructing 35S::LjTT2a, 35S::LjTT2b, 35S::LjTT2c and 35S::TTG1. The other effector plasmids (35S::T2 and 35S::T8) and internal control plasmid 35S::RUC have been constructed previously (Shimada et al. 2006).

**Yeast strain, transformation and two-hybrid assays**

All yeast two-hybrid analysis was performed in the yeast strain AH109 (James et al. 1996). The yeast transformation was performed with the lithium acetate/single-stranded DNA/polycyethylene glycol method (Gietz and Woods 2002). Co-transformed cells were spotted onto synthetic dropout medium lacking leucine, tryptophan, adenine and histidine to investigate interaction of the hybrid proteins according to a protocol provided with the Matchmaker system (Clontech Laboratories, Inc., CA, USA).

**Transient expression assay in plants**

Transient expression assays were carried out as previously described (Shimada et al. 2006). The rosetta leaves of A. thaliana were incubated for 20 min on a 50% MS plate (Murashige and Skoog 1962) containing 1.5% (w/v) sucrose and 0.25 M mannitol. The cells were subjected to bombardment using a pressure of 450 p.s.i. and a 26 mmHg vacuum. After transformation, the tissues were incubated in the dark for 20 h at 25°C. Observations were made with a Leica DM IRBE microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) equipped with a mercury lamp and a GFP filter set (480/40 nm excitation and 525/50 nm emission). The grapevine transcription factor VvMYBPA1 regulates proanthocyanin biosynthesis in Arabidopsis thaliana. Plant J. 39: 366–380.


Functional differentiation of the LjTT2 family


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