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Screening of Tissue-Specific Genes and Promoters in Tomato by Comparing Genome Wide Expression Profiles of Arabidopsis Orthologues

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Constitutive overexpression of transgenes occasionally interferes with normal growth and developmental processes in plants. Thus, the development of tissue-specific promoters that drive transgene expression has become agriculturally important. To identify tomato tissue-specific promoters, tissue-specific genes were screened using a series of *in silico*-based and experimental procedures, including genome-wide orthologue searches of tomato and Arabidopsis databases, isolation of tissue-specific candidates using an Arabidopsis microarray database, and validation of tissue specificity by reverse transcription-polymerase chain reaction (RT-PCR) analysis and promoter assay. Using these procedures, we found 311 tissue-specific candidate genes and validated 10 tissue-specific genes by RT-PCR. Among these identified genes, histochemical analysis of five isolated *promoter::GUS* transgenic tomato and Arabidopsis plants revealed that their promoters have different but distinct tissue-specific activities in anther, fruit, and root, respectively. Therefore, it appears these *in silico*-based screening approaches in addition to the identification of new tissue-specific genes and promoters will be helpful for the further development of tailored crop development.

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crops worldwide, and its fruit contains abundant minerals, vitamins, essential amino acids, and dietary fibers. For these economical important features, genetic engineering of tomatoes has been required to improve productivity, fruit quality, and resistance to biotic and abiotic stresses.

In genetic engineering, transformation is an important tool for crop improvement and gene function analysis. Biolistic and *Agrobacterium*-mediated transformation methods have been widely used to generate many transgenic plants with genes of industrial or agronomic interest. During the early phase of plant genetic engineering, certain constitutive promoters, such as the cauliflower mosaic virus 35S promoter and maize ubiquitin, have been used to express a wide range of traits in various plant species (Brisson et al., 1984; Cornejo et al., 1993). However, constitutive or overexpression strategies may lead to undesirable pleiotropic effects in transgenic plants (Hsieh et al., 2002; Kasuga et al., 1999). In some cases, the use of endogenous regulatory regions of promoters with particular developmental expression patterns has mitigated this problem (Kasuga et al., 2004; Lee et al., 2003). Therefore, it is important to achieve temporal and tissue-specific expression of foreign genes through promoter control.

The complete sequencing of the *Arabidopsis thaliana* genome represents a major step in plant genetic research (Kaul et al., 2000). This knowledge has enabled the monitoring of gene expression of this flowering plant on a genome-scale using microarrays such as the ATH1 full-genome array (Redman et al., 2004). The availability of a full-genome array and the accumulation of transcriptomic data have led to the development of online analysis tools. Genevestigator is an online analysis tool that provides simple visualized results through the analysis of large microarray datasets (Hruz et al., 2008; Zimmermann et al., 2004). This tool has been widely used to support experimental findings on gene expression as well as to determine the expression of a gene in a mutant background (McGrath et al., 2005). It has also been used to demonstrate where a gene is expressed or confirm gene expression in a particular tissue type (McCormack et al., 2005). Recently, Won et al. (2009) successfully applied Genevestigator (Gene Atlas) to a filtration procedure to screen root hair-specific genes in Arabidopsis.

Recently, there were several reports that orthologous genes from diverse angiosperm species displayed similar expression patterns (Kim et al., 2006; Wang et al., 2009). Therefore, to screen tissue-specific genes, we observed expression patterns of Arabidopsis genes (tomato orthologues) in Genevestigator and experimentally analyzed tissue specificity by reverse transcription-polymerase chain reaction (RT-PCR) analysis and histochemical beta-glucuronidase (GUS) assays in transgenic

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Table 1. Isolated tissue-specific genes in tomato

EST No.[a]	AGI No.[b]	Target tissues	Gene description[c]	Primer sequences for RT-PCR	RT-PCR product size (bp)	Primer sequences for promoter isolation	Size of promoter
E263304	At2g36190	Seed Fruit Flower	Cell wall invertase	5′-TACACCGGAGTAGTAGATTC-3′ 5′-GCATTCAGAGTCTCAATTGT-3′	1301	-	-
E234020	At1g17010	Seed Flower	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	5′-GATTGTGACAAATGG-3′ 5′-AAGTCCATCCAAATC-3′	328	-	-
E273715	At5g07330	Seed Fruit	Unknown protein	5′-CAAGGATATCACCAACAC-3′ 5′-GATCGATCACACAGTTAG-3′	731	5′-TCAAGCTTGCAATCAGTTAAAAGTTT-3′ 5′-ACTGGATCCAAATCTCCCTTCCGT-3′	1110
E723552	At2g23240	Fruit	Metallothionein-like protein	5′-ATGTAAGAGGAAGCAGTGG-3′ 5′-AGGTTCGCACTTTCAGGGTG-3′	316	-	-
E254367	At5g51030	Fruit Flower	NAD(P)-binding Rossmann-fold superfamily protein	5′-TCTGTGAACACATGAGGC-3′ 5′-GATCTCCCTTCTACGTAC-3′	740	5′-GGATCCACCAGGTCAAGGACATGAAC-3′ 5′-GAATTCTATTATTACTAGTATTATTTTC-3′	1498
E541096	At3g59850	Flower	Pectin lyase-like superfamily protein	5′-TAACCTTCTCATCTCATC-3′ 5′-GTTCTCTCCCCAAATCAG-3′	431	5′-GAATTCACATAATTCACATTATGACG-3′ 5′-GTCGACGATCGATCAGCTCCCCCGTC-3′	1505
E553370	At4g09960	Fruit Seed Flower	K-box region and MADS-box transcription factor family protein	5′-CAACTCACTCAGATCGATCG-3′ 5′-TTGTGATCAGGAGACAATGG-3′	694	-	-
E543254	At5g15130	Root	WRKY DNA-binding protein	5′-AATGTGAGATATATGAAGCC-3′ 5′-GTATAGATACTCTAGCCTTC-3′	468	5′-GGATCCTCATATAGATTTGGCTCG-3′ 5′-GGATTCTGCTCTATCTTTCCTTAG-3′	1485
E210745	At5g66390	Root	Peroxidase superfamily protein	5′-AGAGTAACAATGATAACC-3′ 5′-TCTGCATATTGCTTCACC-3′	808	-	-
E542814	At1g30870	Root	Peroxidase superfamily protein	5′-AAGGAAGTGAGAAGGATGCC-3′ 5′-CTCGCATTGATAGCA TAAGA-3′	778	5′-CTGCAGCACACTCTACAAGATACTC-3′ 5′-GTCGACTCTACGATATTTGTGGAG-3′	1534

[a]Sequences available in the Sol Genomic Network
[b]Arabidopsis orthologues of tomato ESTs were evaluated for tissue specificity in Genevestigator
[c]Gene description annotated with blastx homology search.

tomato and Arabidopsis plants. As a result, we identified various tissue-specific genes and promoters. Our results demonstrate that Arabidopsis microarrays can serve as a filter to screen certain tissue-specific genes in tomato.

MATERIALS AND METHODS

Plant materials and growth conditions

A. thaliana L. Heynh. ecotype Columbia (Col-0) plants and tomato (*S. lycopersicum* L.) cultivars Micro-Tom and Heinz were used. Plants were grown in soil (60% relative humidity) or *in vitro* on Murashige and Skoog medium (Murashige and Skoog, 1962), containing 3% sucrose and 0.25% phyta-gel (pH 5.8) with a 16-h light (100 μE/s/m^2)/8-h dark cycle at either 20°C or 26°C.

In silico analyses

Tomato expressed sequence tag (EST) genes were obtained from the 'Sol genomics network' (http://solgenomics.net). Tomato unigenes were assembled from tomato ESTs using the 'Stackpack' program (Christoffels et al., 2001). Tomato orthologue searches in Arabidopsis were performed using the TAIR blastx program (http://www.arabidopsis.org). Tissue specificities of each gene were validated using the Genevestigator Gene Atlas microarray database (https://www.genevestigator.com; Hruz et al., 2008; Zimmermann et al., 2004). To identify putative tissue-related *cis*-elements, the promoter regions were analyzed using the PLACE database (http://www.dna.affrc.go.jp/PLACE; Higo et al., 1999).

RT-PCR analysis

For RT-PCR analysis, cDNA was synthesized from 2 μg total

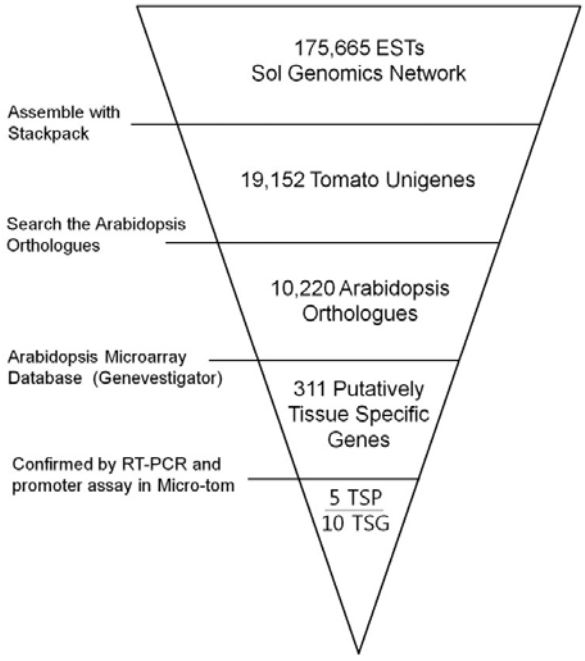

Fig. 1. Screening procedures for tissue-specific tomato genes. Four screening steps were used for gene isolation: 1) Assemble the tomato unigenes from the EST pool using the Sol Genomics Network Stackpack program; 2) Search Arabidopsis orthologues using blastx in TAIR; 3) Screen tissue-specific candidate genes in the Arabidopsis Microarray Database (Genevestigator); 4) Validate tissue specificities by RT-PCR and promoter assay. TSP, tissue-specific promoter; TSG, tissue-specific gene.

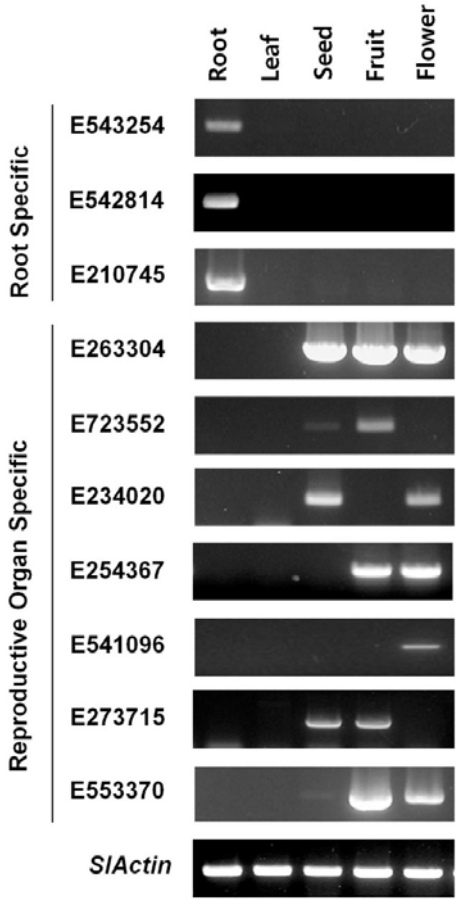

Fig. 2. Expression profiles of tissue-specific genes in tomato (Micro-tom). Total RNA was prepared from root, leaf, seed, fruit, and flower. RT-PCR was carried out using gene-specific primers, and the products were separated on a 1.0% agarose gel.

RNA. Each cDNA sample was diluted 1:10, and 1 μl diluted cDNA was used for PCR amplification in a volume of 50 μl with gene-specific primer sets (Table 1). The resulting PCR products were analyzed by electrophoresis and ethidium bromide staining. *SlActin* (U60480) was used as a control. The primers of *SlActin* are 5′-TGGCATCATACTTTCTAC AATG-3′ (forward) and 5′-CTAATATCCACGTCACATTTC AT-3′ (reverse).

Generation of transgenic tomato and Arabidopsis plants

To construct the *promoter::GUS* reporter systems, tissue-specific gene promoters were obtained from tomato (Heinz) genomic DNA by PCR using the two primers shown as a double set in the primer list (Table 1). The PCR products were cloned into *pMD18-T* (Takara, Japan) using TA overhangs, and the integrity of the constructs was verified by sequencing. The constructs were then inserted into the *pCAMBIA1381-GUS* plasmid (http://www.cambia.org.au). Recombinant plasmids were introduced into *Agrobacterium tumefaciens* EHA105 (for tomato) and GV3101 (for Arabidopsis), and transformation was performed for tomato (Micro-Tom) and Arabidopsis according to the procedure described by Park et al. (2003), and Clough and Bent (1998), respectively.

Histochemical GUS assays

Histochemical localization of GUS activity was performed as described by Jefferson et al. (1987). Briefly, transgenic tomato and Arabidopsis seedlings, organs, and tissues were vacuum-infiltrated in 50 mM sodium phosphate buffer (pH 7.0), 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, and 0.2% Triton X-100 containing 1 mM X-GlcA. The samples were incubated in the dark at 37°C for 12 h and subsequently transferred into 70% ethanol to remove chlorophyll.

RESULTS

Screening of organ-specific candidates in tomato by Arabidopsis orthologue search

To identify tomato tissue-specific genes, we applied several screening steps. First, we collected 175,665 tomato ESTs from the Solanaceae family database (SOL Genomic Network; http://sgn.cornell.edu), and these ESTs were assembled into 19,152 unigenes using the software program 'Stackpack.'

Genes that are similarly expressed in response to a given, unique stress might be coordinately regulated by common *cis*-elements (Rombauts et al., 2003). Orthologues are defined as genes from different species that have evolved from a common ancestral gene by speciation and generally retain a similar function in the course of evolution (Sonnhammer and Koonin, 2002). Moreover, many orthologues not only display similar expression patterns but also share common motifs in their promoter regions (Kim et al., 2006; Wang et al., 2009). Therefore, we hypothesized that if we could find tissue-specific genes in Arabi-

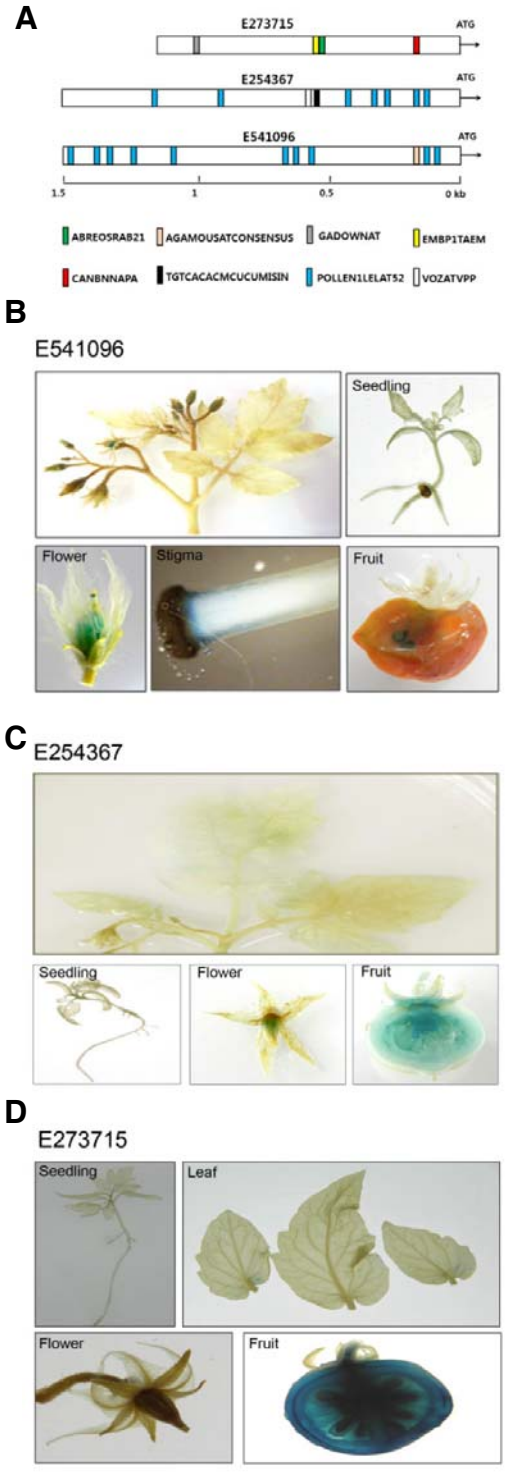

Fig. 3. Structural features of reproductive tissue-specific promoters and histochemical GUS staining of transgenic tomatoes (Micro-Tom) carrying the GUS-coding region fused to each tissue-specific promoter. (A) The location of tissue-specific *cis*-elements of interest was identified using the PLACE database. The symbols of each *cis*-element are annotated. Histochemical localization of *E541096P::GUS* (B) *E254367P::GUS* (C), and *E273715P::GUS* (D) expression in various tissues of tomato (Micro-Tom) transgenic plants.

dopsis, the tomato orthologues would be tissue-specific. The 10,220 Arabidopsis orthologues identified by blastx were compared against 19,152 tomato unigenes; the Arabidopsis genes were then analyzed by the Genevestigator Arabidopsis microarray database, which provided information on the tissue/organ specificity of gene expression (Hruz et al., 2008; Zimmermann et al., 2004). Through these screening steps, we found 311 tissue-specific candidate genes in tomato (Fig. 1 and Supplementary Table 1).

Semi-quantitative RT-PCR analysis for the screening of tissue-specific genes

To examine the specificity of 311 putative tissue-specific genes in tomato, we randomly chose 32 genes, and three independent total RNAs were isolated from root, leaf, seed, fruit, and flower in 9-week-old Micro-Tom. We then performed RT-PCR using specific primer sets (Table 1). Approximately 31.2% of the tested genes were tissue specific. Among them, three genes were specific for root, and seven genes were specific for reproductive organs, with differential expression in seed, fruit, and flower (Fig. 2). Moreover, as shown in Table 1, 10 genes were shown to be related to transcription, oxidation/reduction, carbohydrate metabolism, and flavonoid biosynthesis.

Isolation of the regulatory regions of five tissue-specific genes

We amplified the possible promoter regions of *E273715*, *E254367*, *E541096*, *E543254*, and *E542814* by PCR using tomato (cv. Heinz) genomic DNA as a template. Nucleotide sequences of the amplified fragments were compared with tomato sequences using the Solgenomic network (http://sgn.cornell.edu). No substitutions, insertions, or deletions within the isolated fragments were observed.

Generally, tissue-related *cis*-acting elements are found in the promoter regions of tissue-specific genes. All amplified promoter fragments were analyzed for tissue-specific *cis*-elements using PLACE software (Higo et al., 1999), suggesting that they may also function in tomato tissue-specific expression. We identified 12 potential regulatory elements associated with tissue expression-related transcription factor-binding sites on five isolated promoters of tissue-specific genes (Figs. 3A and 4A). Seed- and fruit-specific *E273715* contain four seed-related *cis*-elements (ABREOSRAB21, Marcotte et al., 1989; CANBNNAPA, Ellerström et al., 1996; EMBP1TAEM, Guiltinan et al., 1990; GADOWNAT, Ogawa et al., 2003) but do not contain fruit-related *cis*-elements. In the cases of *E254367* (flower- and fruit-specific) and *E541096* (flower specific) promoters, there were several flower- and pollen-related regulatory elements (VOZATVPP, Mitsuda et al., 2004; POLLEN1LELAT52, Bate and Twell, 1998; AGAMOUSATCONSENSUS, Shiraishi et al., 1993). In particular, we found that *E254367* contains fruit-specific *cis*-elements (TGTCACACUCUMSIN, Yamagata et al., 2002; Fig. 3A). On the other hand, four root-specific regulatory sequences (RHERPATEXPA7, Kim et al., 2006; SP8BFIBSP8BIB, Ishiguro et al., 1992; OSE1ROOTNODULE, Vieweg et al., 2004; WUSATAg, Kamiya et al., 2003) were differentially found in the *E543254* and *E542814* promoters (Fig. 4A). Since the promoter regions of these genes contain many *cis*-elements correlated with tissue-specific expression, the expression of these genes may be controlled by complex regulatory mechanisms that respond to different developmental cues for formation of specific tissues or organs.

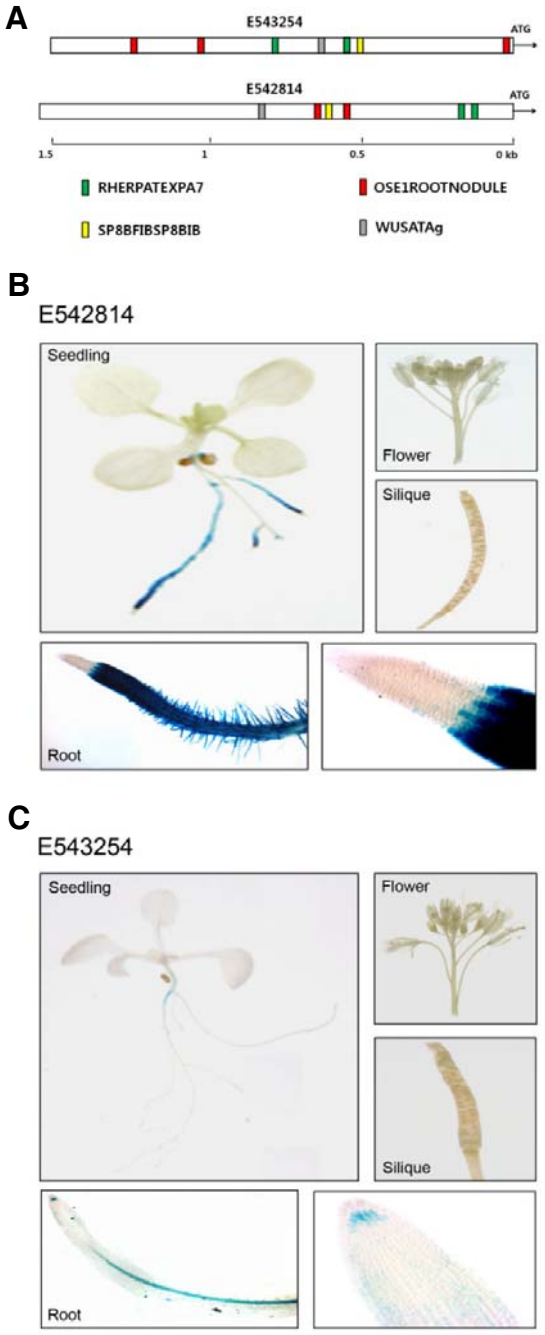

Fig. 4. Structural features of root-specific promoters and histochemical GUS staining of transgenic Arabidopsis carrying the GUS-coding region fused to each root-specific promoter. (A) The location of root-specific *cis*-elements of interest was identified using the PLACE database. The symbols of each *cis*-element are annotated. Histochemical localization of *E542814P::GUS* (B) and *E543254P::GUS* (C) expression in various tissues of Arabidopsis transgenic plants.

Tissue-specific promoter activities of five selected genes in Micro-Tom and Arabidopsis

Of the 10 tissue-specific genes identified by genome-wide screening, we wished to monitor their promoter activities in specific target tissues, because each promoter may contain different expression activities in each tissue. For this purpose, we choose three reproductive organ-specific (*E273715*, *E254367*, and *E541096*), and two root-specific (*E543254* and *E542814*) genes and isolated promoters from each. The promoters were fused to GUS in a binary vector, and *Agrobacterium*-mediated transformations were performed in Micro-Tom and Arabidopsis. We then monitored the activity of each promoter by histochemical GUS staining.

In the cases of *E273715* and *E254367*, which showed fruit expression by RT-PCR, GUS staining was performed in transgenic Micro-Tom (T1) plants. Alike with RT-PCR results, transgenic Micro-tom plants displayed no expression in root, leaf, node, and internode, *E254367P::GUS* expressed in anther and columella in fruit. While *E254367P::GUS* has a character to express in columella, *E273715P::GUS* showed overall expression in Micro-Tom fruit. *E541096* was shown to be flower-specific by RT-PCR, and Micro-Tom (T1) plants were examined for GUS staining. *E541096P::GUS*-containing plants showed specific activities in anthers but other organs and tissues had no activity (Fig. 3).

The root-specific activities of the *E543254* and *E542814* promoter regions were also evaluated by histochemical staining of GUS activity in *E543254P::GUS*- and *E542814P::GUS*-containing transgenic Arabidopsis plants (T2). Both transgenic plants revealed root-specific activity with no activity in other organs. However, *E543254P::GUS* activity was specific to the vascular bundle and root cap, and *E542814P::GUS* showed highest activities in whole root tissues except the root tip (Fig. 4).

After thoroughly examining the expression patterns of these five promoter::GUS transgenic lines, we evaluated the similarities in expressional specificities between tomato promoter activities and the expression of their Arabidopsis orthologue genes by using Genevestigator. As a result, they shared the expression patterns in seed (*E273715*-At5g07330, and *E541096*-At3g59850), stigma (*E541096*-At3g59850), anther (*E254367*-At5g51030), root hair (*E542814*-At1g30870), root cap (*E543254*-At5g15130), and root-vascular cells (*E543254*-At5g15130; Figs. 3, 4 and Supplementary Fig. S1). However, we also found slightly different expression patterns in *E254367* and *E273715*, which showed high expression in fruit, but their Arabidopsis orthologues (At5g51030 and At5g07330) did not express in silique (Figs. 3C, 3D, and Supplementary Fig. S1). Furthermore, *E541096* has high activity in anther, but there was no expression in Arabidopsis orthologue (At3g59850; Fig. 3B and Supplementary Fig. S1). Though *E541096* showed no expression in root, its orthologue (At3g59850) displayed low activity in Genevestigator (Fig. 4C and Supplementary Fig. S1). These unexpected minor expressional differences in tomato and Arabidopsis tissues may be a demerit for mining the tissue specific promoters by using orthologue search. Therefore, precocious consideration of these minor differences may be required to develop tissue specific promoters.

DISCUSSION

Constitutive overexpression of transgenes that interfere with normal plant processes emphasizes the need for refinement of transgene expression methodologies. The development of tissue-specific promoters to drive transgene expression has helped fill that need (Potenza et al., 2004). In this study, we devised a large-scale screening system for the isolation of tissue-specific genes in tomatoes using a genome-wide orthologue search and Genevestigator Arabidopsis microarray data-

base analysis between tomato and Arabidopsis and identified 311 candidate genes and 10 tissue-specific genes as a result.

In this study, RT-PCR results showed that only 31.2% (10 specific genes/32 tested candidates) of genes were tissue specific in our screening system. Moreover, even though the screened tissue specific promoters were used, we found unexpected minor expressional differences between tomato promoter containing transgenic plants and Arabidopsis orthologue genes in Genevestigator. These unexpectedly low yields and minor differences are most likely because we used Arabidopsis microarray database. DNA microarrays provide an unprecedented capacity for whole-genome profiling. However, the quality of gene expression data obtained from microarrays can vary greatly with the platform and procedures used (Morey et al., 2006). Therefore, this variability could be a reason for the low yield and expressional differences in our screening system. The second reason is likely due to the adoption of a heterologous comparative system. Taxonomically, though both plants are dicotyledonous, Arabidopsis (*A. thaliana*) is a genus in the family Brassicaceae (rosid clade), and tomato (*S. lycopersicum*) belongs to a genus in the family Solanaceae (asteroid clade). Moreover, mitochondrial DNA sequence comparisons show that the lineages of the Arabidopsis and tomato families separated about 112-156 million years ago (Ku et al., 2000; Yang et al., 1999). Therefore, it seems that these taxonomic and evolutionary distances of both plants caused expressional differences between orthologues, and these differences may be major factors in the low yield and minor expressional differences of our screening system. To develop a more advanced screening system, it might be necessary to adopt the databases of more highly related species, such as potato or pepper, in the same family. On the other hand, tissue/organ-related *cis*-acting elements are found in the promoter regions of tissue/organ-specific genes. Thus, Won et al. (2009) searched root hair element-containing promoters in their sequential filtering method to isolate root hair-specific genes. Actually, *in silico* analysis revealed that our isolated promoters also contained several tissue-specific *cis*-elements, suggesting that using databases of related species and tissue-specific *cis*-elements is very important for the genome-wide screening of tissue-specific genes.

Among the 10 tissue/organ-specific genes, we selected five genes and then generated transgenic plants expressing the GUS reporter under the control of each gene's promoter. The representative transgenic lines of each gene's promoter showed consensus results with RT-PCR. In many cases, the transformed plants were expressed exclusively in a specific tissue. However, in others, expression was not completely confined to a single tissue or plant part (Potenza et al., 2004). Therefore, it is necessary to select plant lines in which the genes of interest are expressed in the target tissues only (Wu et al., 2011). In this study, while one promoter (*E541096*)-containing transgenic line was expressed exclusively in a specific tissue, four promoter (*E273715*, *E254367*, *E543254*, and *E542814*)-containing plants were required to produce a set of transformed lines and select for plants showing the desired expression in a specific tissue only. These line-dependent specificities may due to the positional effects of the tomato genome on introduced *Promoter::GUS* gene constructs that might influence the expression levels and specificity of these promoters.

In this study, we attempted to develop a high-yielding screening system in tomato by *in silico* orthologue searches and comparisons using the microarray database of Arabidopsis. Moreover, many tissue-specific candidates were suggested and specificity among the candidates by RT-PCR and histochemical transgenic plant analysis was observed. Additionally, we found tissue-specific expression for each specific target tissue examined. Therefore, our experience with the screening of tissue-specific genes will provide useful information for the further development of agriculturally useful promoters.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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