Overexpression and cosuppression of xylem-related genes in an early xylem differentiation stage-specific manner by the AtTED4 promoter

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Summary

Tissue-specific overexpression of useful genes, which we can design according to their cause-and-effect relationships, often gives valuable gain-of-function phenotypes. To develop genetic tools in woody biomass engineering, we produced a collection of Arabidopsis lines that possess chimeric genes of a promoter of an early xylem differentiation stage-specific gene, Arabidopsis Tracheary Element Differentiation-related 4 (AtTED4) and late xylem development-associated genes, many of which are uncharacterized. The AtTED4 promoter directed the expected expression of transgenes in developing vascular tissues from young to mature stage. Of T2 lines examined, 42%, 49% and 9% were judged as lines with the nonrepeat type insertion, the simple repeat type insertion and the other repeat type insertion of transgenes. In 174 T3 lines, overexpression lines were confirmed for 37 genes, whereas only cosuppression lines were produced for eight genes. The AtTED4 promoter activity was high enough to overexpress a wide range of genes over wild-type expression levels, even though the wild-type expression is much higher than AtTED4 expression for several genes. As a typical example, we investigated phenotypes of pAtTED4::At5g60490 plants, in which both overexpression and cosuppression lines were included. Overexpression but not cosuppression lines showed accelerated xylem development, suggesting the positive role of At5g60490 in xylem development. Taken together, this study provides valuable results about behaviours of various genes expressed under an early xylem-specific promoter and about usefulness of their lines as genetic tools in woody biomass engineering.

Introduction

Gene overexpression has been widely used to examine gene functions or to improve useful phenotypes. Unlike gene knockout or knockdown technology, overexpression does not always require genome information of target organisms, allowing similar methods to be applied across species. However, we must pay careful attention to cause-and-effect relationships in gene overexpression studies. In higher plants, the cauliflower mosaic virus 35S promoter is one of the most frequently selected promoters to induce very high transgene expression in various cell types (Odell et al., 1985). Due to the ubiquitous expression pattern, however, it is more or less inevitable to cause pleiotropic effects in any cell types. Conversely, the 35S promoter-driven overexpression may be no longer stable in certain developmental stages (Labuz et al., 2010; Nilsson et al., 1996). In addition, undesired epigenetic regulations often affect transgene expression (e.g. RajeevKumar et al., 2015).

Many efforts have been made in developing technologies to utilize woody biomass as renewable materials or energy source for a sustainable future (Isikgor and Becer, 2015; Menon and Rao, 2012). A majority part of woody biomass consists of xylem cells with secondary cell walls (SCWs). We aimed to develop genetic tools to make SCWs valuable in engineering, as well as to understand molecular mechanisms involved in SCW formation. We first use Arabidopsis to build up our methodology. Arabidopsis plants develop woody xylem cells with SCWs in maturing inflorescence stems and hypocotyls, which offers a model for wood formation (Chaffey et al., 2002; Lehmann and Hardtke, 2015; Strabala and MacMillan, 2013; Zhang et al., 2011). In order to obtain many key factors in SCW formation, we started constructing a large collection of Arabidopsis overexpression lines to identify distinct phenotypes and causal genes. More uncharacterized genes that were expressed in accordance with the late SCW formation were possible candidates (Demura et al., 2002; Kondo et al., 2015; Kubo et al., 2005; Ohashi-Ito et al., 2010; Yamaguchi et al., 2011). In previous reports, some xylem overexpression approaches succeeded in efficiently inducing expected phenotypes by the use of the late SCW formation-specific promoters (e.g. Petersen et al., 2012; Ratke et al., 2015; Wilkerson et al., 2014). Alternatively, we looked for a promoter capable of inducing an early xylem differentiation stage-specific overexpression. Such promoter might replace SCW construction steps in new order if genes like the late SCW formation-associated ones were overexpressed.

In this report, we selected a promoter of Arabidopsis Tracheary Element Differentiation-related 4 (AtTED4) to modify gene expression for 48 different Arabidopsis genes at the very early...
stage of xylem differentiation. Each modified gene expression level in homozygous T3 lines was compared with the wild-type gene expression level. T-DNA repeat status was also investigated. Moreover, one novel overexpression phenotype is shown. The presented results describe how one plant tissue-specific promoter drove the expression of different genes and thereby affected wild-type gene expression in a comprehensive scale that has never been reported before.

Results and discussion

Selection of AtTED4 promoter to induce gene expression at an early stage of xylem differentiation

35S promoter can induce massive gene expression in not only xylem but also various tissues. However, we required closer cause-and-effect relationships in our gene overexpression study. In order to accurately modify gene expression in immature xylem, we looked for an alternative promoter to overexpress genes at an early stage of xylem differentiation. ZeTED4 was identified as a highly expressed gene in the Zinnia tracheary element differentiation system and ZeTED4 mRNA accumulated in procambium and immature xylem in Zinnia plants (Demura and Fukuda, 1994). We examined a promoter activity of its Arabidopsis homologue, At3g18280 (hereafter referred to as AtTED4), in Arabidopsis plants to assess whether it reproduced the expression pattern. The pAtTED4::GUS plants showed the fine procambium–immature xylem expression domain in developing vascular tissues from young to mature stage (Figures 1). The GUS expression was also detected in the developing inflorescence stem (Figure 1b). Quantitative PCR analysis of inflorescence stem samples revealed that AtTED4 mRNA level was 10- and 20-fold higher than that of At5g61480 (TDR/PXY) and At4g32880 (ATHB8), respectively, which were preferentially expressed in procambium and procambium–immature xylem. We selected 48 genes of our interest, many of which are uncharacterized genes specifically expressed in developing xylem cells, for overexpression under the control of the AtTED4 promoter (Table 1).

A survey of multiple T-DNA insertions at single loci

Transformation of Arabidopsis plants by the floral dip method frequently produces multiple T-DNA insertions at single loci (De Paepo et al., 2009). It has been well documented that such T-DNA repeats, especially inverted repeats, have often resulted in cosuppression of transgenes and wild-type genes (e.g. Jorgensen et al., 1996). First, we selected transgenic lines in which one or multiple T-DNA insertion(s) occurred at single loci, based on the 3 : 1 segregation ratio exhibited in the T2 generation. Next, to assess the T-DNA repeat status of the transgenic lines, we performed PCR-based observation of junctions of neighbouring T-DNAs. This simple test was designed to detect whether plants had no repeat or T-DNA repeats (Figure 2). As a result of total 246 T2 lines for 41 of the 48 genes, 42% and 58% were judged as lines with the nonrepeat type insertion and the repeat type insertion, suggesting that approximately more than a half of transgenic plants have repeats of the transgene (Table 2). Next, we examined the repeat type insertions. We expected that the 2-kb PCR product results from insertions of directly repeated T-DNA, the 1-kb PCR product was from insertions of invertedly repeated T-DNA, and the other sizes of PCR products may from various structures of insertions (Figure 2b). The 2-kb PCR products occupied most part of ones from the repeat type insertion (49% of 58%), suggesting that the direct-repeat type insertion occurs frequently in transgenic plants (Table 2). Therefore, we designed this insertion as the simple repeat. To confirm our expectation, we sequenced the junction of T-DNA repeats of the 2-kb PCR products as well as other size of PCR products (Figures 3 and S1). Of four 2-kb PCR products, two showed a junction (Figure 3, Lines 1 and 3) and two showed multiple junctions (Figure 3, Lines 5 and 7), suggesting that the former two result from a direct repeat of two T-DNAs and the latter two result from direct repeats of more than two T-DNAs. Because the 1.5-kb PCR product was amplified only with the forward primer, we judged that this product results from a structure with an insertion between two inverted T-DNAs (Figure 3, Line 6). Interestingly, the both PCR products of more than 3 kb showed

![Figure 1](image-url)
a direct repeat of two T-DNAs with a vector sequence insertion between the T-DNAs (Figure 3, Lines 2 and 4). These results suggested that the repeat type insertion in our transgenic plants contains not only the direct repeat of two T-DNAs but also of more than two T-DNAs.

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Quantitative PCR analysis revealed modified gene expression levels in total 174 T3 lines for 48 genes and the corresponding wild-type gene expression levels (Figure 4a; Table 1). Overexpression was observed for 37 genes. In addition, one or two cosuppression lines were also detected for eight of the 37 genes (At1g01360, At1g43790, At3g59690, At5g03170, At2g38080, At4g18780, At5g60490, At3g16920). For eight genes such as At5g01360, At4g09990, At1g10810, At5g11540, At1g29200, At1g69588, At2g20650, At5g49900, At1g70550, At2g38480, At5g54570, At1g48280, At1g501190, At1g29200, the promoter activity was high enough to overexpress all the genes and B of At5g49900 had the insertion of singly copy of T-DNA per genome (Figure 5a,b), and their AtTED4 mRNA level increased about two times more than that in the wild type (Figure 5c). In contrast, Line C of At5g49900 had multiple copies of T-DNA and its AtTED4 mRNA level was not more than that of the wild type (Figure 5a–c). Line C of At5g49900 had a 2-kb and a <2-kb PCR products (Figure 5a). The <2-kb product suggests an inverted repeat of T-DNA, which may result in the repression of AtTED4 mRNA level. Lines A to C of At1g43790 were simple repeat type lines (Figure 5d), and their copy numbers varied from 7 to more than 15 per genome (Figure 5e). Increases in T-DNA numbers appeared to associate with overexpression levels in Lines B and C of At1g43790, but Line A, which had the highest copy number, showed the cosuppression of At1g43790 gene expression (Figure 5f). Increases in T-DNA numbers appeared to associate with overexpression levels on one hand but to result in cosuppression on the other hand. Schubert et al. (2004) showed a close relationship between the gene number and the overexpression/cosuppression event using exogenous genes in Arabidopsis. Our study using various endogenous genes is consistent with their result.

The AtTED4 promoter could overexpress At1g75410, At4g28500, At4g18780, At5g60490 and At3g16920 genes in the nonrepeat type lines, even though their wild-type expression level was much lower than that of wild-type AtTED4 expression level (Figure 4b; Table 1). A comparison of the AtTED4 promoter only moderately overexpressed genes whose wild-type expression level was much lower than that of wild-type AtTED4 expression level (Figure 4). These results suggest that an intrinsic AtTED4 promoter activity was high enough to overexpress all the genes.
analysed in this study, although each transgene expression level in mRNA amount was somehow restricted down towards its wild-type gene expression level by unknown mechanisms.

Christie *et al.* (2011) reported that endogenous genes avoided gene silencing through intron splicing, and then, small RNA densities were high in exons of intronless genes. Therefore, we compared cosuppression frequency between intronless and intron-possessing genes. Seven of the nine intronless genes caused cosuppression by their overproduction, while nine of the 36 intron-possessing genes caused cosuppression (Tables 1 and S3). This result indicated that overexpression of intronless genes tend to cause cosuppression, which was consistent with the previous report (Christie *et al.*, 2011), even when a tissue-specific promoter was used. For cosuppression events, the threshold model has been proposed and carefully examined (Nagaya *et al.*, 2005; Schubert *et al.*, 2004). They showed that each model reporter gene had its expression threshold for silencing. The threshold probably depends on a quality control machinery of

Table 2  T-DNA repeat type

| PCR product | T-DNA repeat type | Numbers of T2 lines |
|-------------|-------------------|---------------------|
| No product  | Nonrepeat         | 103 (42%)           |
| 2 k only    | Simple repeat     | 121 (49%)           |
| Other than 2 k | Other repeat | 22 (9%)             |

analysed in this study, although each transgene expression level in mRNA amount was somehow restricted down towards its wild-type gene expression level by unknown mechanisms.

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overexpression phenotype, we used AtTED4 this time as a xylem differentiation marker. As expected, FLA12 overexpression in Lines A and B resulted in an increase in AtTED4 expression (Figure 6c,d). In Line C, the FLA12 cosuppression line, however, AtTED4 expression was not higher than wild-type level (Figure 6c, e). These results strongly suggest that FLA12 overexpression accelerated xylem and interfascicular fibre development. FLAs form a subfamily of arabinogalactan proteins, for which a variety of functions have been proposed (Geifert and Roberts, 2007). MacMillan et al. (2010) reported a reduction in cellulose content and inflorescence stem strength in fla11 fla12 double mutant. Recently, Eucalyptus FLA2, a closest homologue of Arabidopsis FLA12, was shown to be able to alter cellulose deposition in woody tissues in the induced somatic sector analysis when overexpressed by the cauliflower mosaic virus 35S promoter (MacMillan et al., 2015). Therefore, FLA12 is supposed to be a key regulator in SCW formation, although its precise function still remains unknown. In our experiments, immature xylem-specific overexpression of FLA12, which is originally expressed in SCW-forming cells (MacMillan et al., 2010; Ohashi-Ito et al., 2010), caused increased xylem and interfascicular fibre development. There have been few cell wall proteins that are proven to promote xylem differentiation. We reported that xylan, an arabinoarabogalactan protein with the glycosylphosphatidylinositol anchor, which promotes xylem differentiation through cell–cell interaction (Motose et al., 2004). FLAs are also arabinoarabogalactan proteins with Fasciclin domain that is related to cell adhesion domain. Therefore, FLA12 may function in promoting xylem and interfascicular fibre differentiation through cell–cell interaction.

In conclusion, we found that an early xylem differentiation stage-specific promoter sequence, pAtTED4, was able to overexpress a variety of genes with different wild-type expression levels in a range of 0.001–100 arbitrary units (Tables 1 and S4). The AtTED4 promoter-directed expression of late xylem development-associated genes was able to induce xylem-related phenotypes, as typically shown by overexpressing At5g60490. Thus, our comprehensive analysis can be a guide for producing tissue-specific overexpression lines in a large scale and also provides a collection of lines with AtTED4 promoter chimeric genes, which is useful as genetic tools in woody biomass engineering. Efforts are underway to analyse phenotypes of these lines.

**Experimental procedures**

**Plant growth conditions**

 Arabidopsis thaliana (L.) Heynh. accession Columbia (Col-0) was used as the background for all the lines analysed in this study. Selection of transgenic plants was performed on a 1/2 MS-based conventional medium containing 15 mg/L Hygromycin B under 10-h fluorescent white light at 22°C and 14-h dark at 20°C (100 μmol/m²/s). Seedlings were further grown on pots with a mixture of vermiculite (VS kakou) and PRO-MIX BX (Premier Horticulture) under 16-h LED light at 22 °C and 8-h dark at 20 °C (120 μmol/m²/s).

**Vector construction and Arabidopsis transformation**

The primers used in the following steps are shown in Table S1. The 2.8 kb of 5′ sequence from the translation start site of AtTED4 was integrated into the Hind III- and Xba I-treated pH35GS by In-Fusion HD Cloning kit (Clontech), resulting in introducing pAtTED4 in place of p35S in pH35GS (Kubo et al., 2005). Coding sequences were cloned into the modified vector.
Figure 5  Comparison of T-DNA structure, the number of T-DNAs and modified gene expression in At5g49900 and At1g43790 T3 lines. (a–c) Two ‘nonrepeat type’ and one ‘other repeat type’ lines of At5g49900. (d–f) Three ‘simple repeat type’ lines of At1g43790. (a, d) The PCR-based detection of the junctions as illustrated in Figure 2 for the indicated T3 lines. (b, e) Real-time PCR-based estimation of the number of AtTED4 promoter sequence in genomic DNA, when the number of AtTED4 promoter sequence in wild type is assumed to be 2. The data are the means ± SD (n = 3). (c, f) Expression level of target genes in wild type and the indicated T3 lines. The data are the means ± SD (n = 3).

Figure 6  Effect of At5g60490 overexpression on vascular development. (a, b) Developing inflorescence stems of wild-type (a) and of At5g60490 overexpression plants (b). A typical result observed in T1 lines is shown. The result was confirmed in another At5g60490 overexpression T4 line (Figure S2). The sections were stained with Alcian blue and Safranin. Bars = 100 μm. (c–e) Expression level of At5g60490 (c) and AtTED4 (d, e) in wild type and three independent At5g60490 T3 lines. Quantitative PCR analyses were performed using Lines A–C in (c), Lines A and B in (d) and Lines A and C in (e), independently. All the data are the means ± SD (n = 3). Asterisks indicate the differences between the wild type and each line (*1 P < 0.05, *2 P = 0.052, *3 P = 0.056 in Dunnett’s test).
destination vector by the Gateway system (Invitrogen). Using GV3101 (pMP90), Arabidopsis plants were transformed with the above-mentioned constructs by the floral dip method (Clough and Bent, 1998).

**Histology**

Fresh GUS-expressing samples were once treated with 90% acetone and then stained in a GUS detecting solution (2 mM X-Gluc, 0.5 mM potassium ferricyanide 0.5 mM potassium ferrocyanide, 0.1 mM sodium phosphate buffer, pH 7.4). The samples were transferred in 70% ethanol and sectioned at 80 μm thickness.

For Alcian blue/Safarin staining, samples more than 1.1 mm diameter were obtained from basal parts of inflorescence stems more than 20 cm in height, fixed with FAA solution (3.7% formaldehyde, 0.5% acetic acid, 50% ethanol), washed with 70% ethanol and sectioned at 80 μm thickness. The sections were stained with 0.05% Alcian blue for 1 min, followed by 0.005% Safarin for 30 sec, and then washed.

**PCR conditions**

Neighbouring T-DNA junctions were amplified with the following forward and reverse primers: 5'-AAT CCT GTT GCC GTT CTT GCC AT-3' and 5'-GTT GCA GAA TAA TCA AGC CTG AAT AT-3'. Template DNAs were simply prepared from immature leaves of young seedlings using an extraction buffer (0.5% SDS, 25 mM EDTA, 250 mM NaCl, 200 mM Tris-HCl buffer, pH 7.5). A standard PCR for 40 cycles was performed using Ex-Taq DNA polymerase (Takara).

Quantitative PCR was performed using TaqMan probe system with LightCycler 480 (Roche). Primers and probes used in the analysis are shown in Table S2. Total RNAs were prepared from 5- to 10-cm segments from the top of inflorescence stems of each four plants more than 20 cm in height, using RNeasy Plant Mini Kit (Qiagen). The RNAs were converted to cDNAs by SuperScript III (Invitrogen). Calculated by the threshold cycles and the amplification efficiencies of UBQ10 and each gene in the same PCR, expression levels were shown by arbitrary unit relative to UBQ10 level as 100. All the data were analysed in triplicates, except as indicated (Tables 1 and S3).

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Figure S1** Further investigation of the structure of T-DNA repeats.

**Figure S2** Effect of At5g60490 overexpression on vascular development.

**Table S1** Primers used for cloning.

**Table S2** Primers and probes used for real-time PCR.

**Table S3** Magnitude of modified gene expression level in individual T3 lines.

**Table S4** Gene annotation.