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Induced somatic sector analysis of cellulose synthase (*CesA*) promoter regions in woody stem tissues

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Abstract

The increasing focus on plantation forestry as a renewable source of cellulosic biomass has emphasized the need for tools to study the unique biology of woody genera such as *Eucalyptus*, *Populus* and *Pinus*. The domestication of these woody crops is hampered by long generation times, and breeders are now looking to molecular approaches such as marker-assisted breeding and genetic modification to accelerate tree improvement. Much of what is known about genes involved in the growth and development of plants has come from studies of herbaceous models such as *Arabidopsis* and rice. However, transferring this information to woody plants often proves difficult, especially for genes expressed in woody stems. Here we report the use of Induced Somatic Sector Analysis (ISSA) for characterization of promoter expression patterns directly in the stems of *Populus* and *Eucalyptus* trees. As a case study, we used previously characterized primary and secondary cell wall related cellulose synthase (*CesA*) promoters cloned from *Eucalyptus grandis*. We show that ISSA can be used to elucidate the phloem and xylem expression patterns of the *CesA* genes in *Eucalyptus* and *Populus* stems, and also show that the staining patterns differ in *Eucalyptus* and *Populus* stems. These findings show that ISSA is an efficient approach to investigate promoter function in the developmental context of woody plant tissues and raise questions about the suitability of heterologous promoters for genetic manipulation in plant species.

Keywords: CAMV35S promoter; *Eucalyptus*; GUS reporter gene; *Populus*; Secondary cell wall; Wood formation

Abbreviations ATS, Average of transformed sectors per cm^2; ISSA, Induced Somatic Sector Analysis

Introduction

Plantation tree species such as those from the genera *Populus* and *Eucalyptus* are receiving attention world-wide for their capacity to produce cellulosic biomass which can be used for pulp and, potentially, biofuel production (Hinchee et al. 2011). Unlike first generation biofuel crops such as sugarcane and maize, forest trees are less likely to directly compete with food production and have a greater biomass production capacity (Rathmann et al. 2010), although the processing of lignin-rich woody biomass to liberate cell wall biopolymers remains a challenge (Mansfield 2009). Furthermore, the genetic improvement of forest trees is hindered by long generation times and late expression of mature traits. Tree breeders attempting to enhance properties such as wood quality and cellulose deposition will benefit from the application of molecular approaches such as marker-assisted breeding (MAB) and genetic modification (Grattapaglia et al. 2009; Seguin 2011). These molecular approaches are now also benefiting from the application of next-generation genomics technologies, which can be used to study the genetics of wood formation as a system and to rapidly identify candidate genes for further functional analysis (Mizrachi et al. 2012).

Cellulose is deposited in plant cell walls by large, membrane bound, protein complexes composed of several different cellulose synthase (CESA) proteins (Kimura et al. 1999), the identity of which depends on the type of cell wall being laid down (Song et al. 2010). In *Arabidopsis* and other plant species, three *CesA* genes (*CesA4*, *7* and *8*) have been associated with secondary cell wall deposition, while a different set of *CesA* genes were found to be involved in primary cell wall formation (Turner and Somerville 1997; Taylor et al. 2000, 2003; Hamann et al. 2004; Samuga and Joshi 2004; Ranik and Myburg 2006). During primary cell wall formation in *Arabidopsis* two *CesA* genes, *AtCesA1* and *3*, are essential for cell development with knock-out mutants being lethal (Arioli et al. 1998; Scheible et al. 2001). Five other *CesA* genes (*AtCesA2*, *4*, *5*, *6* and *9*) have been linked to

primary cell wall formation in *Arabidopsis*, but these are functionally redundant when mutated, and appear to be involved in tissue-specific primary cell wall formation (Beeckman et al. 2002; Desprez et al. 2002; Stork et al. 2010; Carroll and Specht 2011).

While there are many similarities in cellulose biosynthesis across plant genera (Popper et al. 2011), there are also a number of species-specific features. The *CesA* gene family has ten members in *Arabidopsis* (Richmond and Somerville 2000), while *Populus* has 18 expressed *CesA* genes (Djerbi et al. 2005; Suzuki et al. 2006; Kumar et al. 2009). A phylogenetic analysis of the *Populus CesA* gene family revealed that the 18 *CesA* genes grouped with the ten *Arabidopsis* orthologs in all of the primary and secondary cell wall related clades and that *Populus* has two or more paralogs of some *Arabidopsis* genes (Kumar et al. 2009). In particular, it was noted that *Populus* has duplicated genes for the secondary cell wall associated *AtCesA7* and *AtCesA8* genes. In each case, one of the two *Populus* paralogs (*PtiCesA7*-A or *PtiCesA8*-B) was more highly expressed in xylem, suggesting differential regulation of the paralogs and possible loss of regulation of the lower expressed paralog (Suzuki et al. 2006). Similarly, the primary cell wall associated *AtCesA3* gene has four close orthologs in *Populus*, and each of these have a different expression pattern (Suzuki et al. 2006). The differentiated expression patterns of the duplicated *CesA* genes in *Populus* suggest that the *Populus* paralogs may be undergoing subfunctionalization.

Inter-specific differentiation can affect regulatory sequences in promoters and produce discordant results when different orthologs are used in transgene constructs. Fei et al. (2006) found that a promoter construct which increased glutamine synthase expression in both *Lotus japonicus* and *Sesbania rostrata*, did not produce a corresponding increase in expression in *Pisum sativum*. In an extensive study on mammalian and *Drosophila* cell lines eight supposedly constitutive promoters were tested and most promoters showed variation in reporter gene expression between both cell line and species (Qin et al. 2010). Even the highly

utilized CAMV 35S promoter has been shown to have differential expression across different species (Benfey and Chua 1990; Zhang et al. 2003). This evidence suggests that for some promoters it may be desirable to perform functional analysis in the native genetic backgrounds, however, this may not be practical in species that are recalcitrant to genetic transformation.

Induced Somatic Sector Analysis (ISSA), first proposed by Spokevicius et al. (2005) and developed further by Van Beveren et al. (2006), uses a novel *in planta* transformation method, which has been successfully applied in the analysis of transgenes in woody stem tissues of *Pinus, Populus* and *Eucalyptus* (Hussey et al. 2011). In this method, *Agrobacterium* carrying the promoter and transgene of interest is applied to the exposed cambium on the stem of a living tree. The gene construct is transferred by *Agrobacterium* into actively dividing cambial, xylem, phloem and ray initial cells, creating a number of transformants in this small section (~1 cm^2) of the tree stem (Van Beveren et al. 2006). When the cambium is resealed and the stem is allowed to grow for a few months where the transformed cells divide and multiply within the stem, producing somatic sectors of transformed cells. This area of transformed cells can then be analyzed for transgene (e.g. β-glucuronidase) expression and changes in cell wall morphology by comparing transformed sectors to adjacent non-transformed stem cells. ISSA has great potential for functional genetic studies, as it allows for the analysis of transgenes and promoters directly in the stem tissues of the tree and, for wood-specific constructs, may give a more accurate picture of the native functions or expression patterns of transgenes in woody tissues (Spokevicius et al. 2007).

Here, we used ISSA to study the expression patterns of six previously characterized promoters of *Eucalyptus grandis CesA* genes (Creux et al. 2008) in woody stem tissues of *Eucalyptus* and *Populus* trees. The first objective of this study was to investigate the suitability of ISSA for the analysis of promoter function in various woody stem tissues.

Secondly, we assessed whether ISSA could be used to compare reporter gene expression patterns in *Populus* and *Eucalyptus* stems. To our knowledge, this is the first study to directly compare the expression patterns of promoter::reporter gene constructs in woody tissues of *Populus*, the model tree genus for molecular studies, and *Eucalyptus*, a globally important fibre crop.

Materials and methods

Plant material

Three-month-old ramets of five *Eucalyptus camaldulensis x globulus* and six *Eucalyptus camaldulensis x grandis* clones were purchased from a specialist forestry nursery (Narromine Transplants, Narromine, NSW, Australia), potted in premium potting mix and maintained in a greenhouse for another four months. A single *Populus alba* (L.) 'pyramidalis' clone, growing at the University of Melbourne Creswick Campus (Vic. Australia), was used to generate plant material through rooted cuttings. Dormant stems were sourced and established in cutting beds following treatment with a commercial rooting hormone powder (Yates Striking Powder, Homebush, NSW), transplanted into premium potting mix after six weeks and maintained in the greenhouse for three months until required. Greenhouse temperatures were maintained between 14°C and 17°C at night and between 21°C and 25°C during the day. A 16-hour photoperiod was kept through supplementary lighting. Supplementary lighting was supplied by six 1000W Metal Halide globes in a glasshouse chamber of approximately 16 m^2. All plants were watered regularly with tap water (as required, depending on season) and fertilised with a slow release formulation (Osmocote Exact Mini, Scotts-Sierra Horticultural Products, Marysville, OH, USA) every three months.

Promoter isolation, vector and *Agrobacterium* preparation

Kumar et al. (2009) proposed a revision of the *CesA* gene nomenclature for the *Populus CesA* genes, which allows for the direct comparison of the *Arabidopsis* and *Populus CesA* genes (See Table 1 for *CesA* gene orthology). The change in nomenclature has not yet been applied to the *Eucalyptus CesAs* and for this reason we have retained the naming convention first published by Ranik and Myburg (2006). The *Eucalyptus CesA* promoter regions (*EgCesA1-5* and *7*) and the *Arabidopsis CesA8* promoter region were cloned into the pCR8/GW/TOPO entry vector (Invitrogen). Orientation of the inserts was determined using restriction endonuclease digestion. Promoter DNA was transferred from the entry vectors to the binary vector pMDC162 (Curtis and Grossniklaus 2003) using LR Clonase (Invitrogen) according to the manufacturer's instructions. The expression cassette consisting of the promoter and GUS (β-glucuronidase) reporter gene was confirmed by sequencing prior to *Agrobacterium* transformation (Creux et al. 2008).

Two CAMV 35S promoter vectors were used as a positive control. The first, 35S-F, was the pCAMBIA1305.1 vector (http://www.cambia.org/ verified 5/5/10) and the second, 35S-G, was based on the same pCAMBIA1305.1 vector backbone, but with a Gateway recombinase cassette in the multi-cloning region. An empty (promoter-less) pMDC162 vector was also used as negative control.

All vectors were transformed into AGL-1, a disarmed strain of *Agrobacterium tumefaciens* containing a derivative of pTiBO542 (Lazo et al. 1991), using an *E. coli* pulsar (BIO-RAD Laboratories, Gladesville, NSW, Australia), 2 mm cuvette and 2.5 kV, and following protocol 26 'transformation of *E. coli* by electroporation' as described in Sambrook and Russell (2001). Bacteria were grown for 48 h at 28°C in LB medium containing 25 µg $mL^{\leq 1}$ rifampicin and 50 µg $mL^{\leq 1}$ kanamycin. The *Agrobacterium* suspension was then diluted

1:20 with fresh LB and grown to OD_{600} of 0.4 to 0.6 after which the cells were recovered by centrifugation (1150 *g* for 15 min) and resuspended in 1 mL of Murashige–Skoog (MS) media prior to inoculation (Table 2).

Inoculation and harvest of transformed tissues for Induced Somatic Sector Analysis

During the start of the growing season (early summer) 40 *P. alba 'pyramidalis'*, 20 *E. camaldulensis x globulus* (four ramets of each clone) and 24 *E. camaldulensis x grandis* (four ramets of each clone) potted plants were selected on the basis of good form and growth for experimentation (Table 2). Along the stem of each tree, eleven approximately 1 cm^2 cambial windows were opened using the *in vivo* stem ISSA method described in Van Beveren et al. (2006). Each cambial window was inoculated with 5 µL of *Agrobacterium* suspension containing one of the 11 promoter constructs under investigation (one promoter per window) and subsequently sealed using parafilm. Due to lower sector numbers for the *EgCesA1* and *EgCesA2* promoters (see Results) a further 30 windows were produced for each promoter in both species (*Eucalyptus* and *Populus*) during the following year using the same clonal material. Plant height and stem diameter (measured at a height of 10 cm from the trunk base) were recorded. Plants were fertilised after inoculation and maintained in the greenhouse until harvest.

At harvest, plant height and stem diameter (measured at a height of 10 cm from the trunk base) were again recorded and stem sections harbouring cambial windows were excised from the main stem and placed in 10 ml Falcon tubes for transport. Un-inoculated stem tissue (outside the window area) was removed and the remaining cambial tissue was cut transversely into 1 mm half-discs and placed back into the 10 mL tubes for GUS assays. Cambial discs were washed twice with 0.1 M $NaPO_4$ buffer (pH 7) prior to the addition of 5 mL (approx) of GUS solution (0.1 M $NaPO_4$ buffer pH 7, 0.5% v/v Triton X-100 (Sigma), 10

mM EDTA (Sigma), 0.5 mM potassium ferricyanide (III) (Sigma), 0.5 mM potassium hexacyanoferrate (II) trihydrate (Sigma), 0.5 mM X-Gluc). Cambial discs were incubated in a water bath in GUS solution at 55°C for 10 min prior to being placed upright in the dark on a rotary shaker (150 rpm) at 37°C overnight. The GUS solution was then replaced with 70% ethanol and samples were stored at 4°C until assessment (Spokevicius et al 2005; Alwen et al 1992).

Assessment of GUS staining patterns

For the purpose of promoter expression analysis, it is important to note that the observed "sector types" described in this study in all cases represent the net staining pattern produced by the combined effect of the initial cell transformed (determining the total sector of transformed cells) and the cell/tissue specificity of the promoter construct tested (specifying the subpopulation of cells within the sector that express GUS). Cambial windows were initially assessed for GUS staining using protocols described in previous ISSA studies where whole sectors were investigated (Spokevicius et al. 2006; Van Beveren et al. 2006). In most cases, the sector was transversely cut into two and each half was examined under a microscope. Generally, correct identification of sector type and staining pattern could be made without any further examination due to a combination of the intensity of the GUS staining, the transparency of the wood, sector size and the experience of investigators. Where identification was not certain upon initial investigation, serial sectioning was undertaken on the two halves until correct identification could be made. The sector categories described in the previous studies (Spokevicius et al. 2006; Van Beveren et al. 2006) were also used in this study in addition to new sector categories. The same ‘tylose’ and ‘wound parenchyma’ sector categories were used as before, but ‘cambial’ and ‘phloem’ sectors were redefined to include the addition of two new sector pattern types, indicative of the cell type that was initially

transformed and the subsequent expression pattern of the promoter tested. In the case of ‘cambial’ sectors, these were reclassified into two subcategories. The first subcategory, ‘xylem mother sectors’ (Fig. 1a), was characterized by GUS staining in the newly derived xylem cells extending for a short distance from the wound parenchyma. ‘Xylem mother sectors’ are indicative of the initial transformation of a xylem mother cell and the formation of a transgenic xylem sector which terminates after a number of cell divisions when the xylem mother cell is replaced by an adjacent, non-transgenic mother cell, or when it terminally differentiates and therefore ceases to be a mother cell. A second subcategory was defined as ‘cambial sector proper’ (Fig. 1a), where GUS staining was observed in and around the cambial region, which indicates that an undifferentiated cambial initial was transformed which continued to produce xylem and phloem mother cells and sectors of xylem and phloem cells derived from these mother cells. The definition of a ‘cambial sector proper’ had to be expanded for the promoter analysis because the tissue specificity of the promoters investigated could produce GUS staining patterns that differ from the original sector descriptions which were based on constitutive GUS expression (Fig. 1a - original descriptions and Fig. 1c – new cambial staining patterns). While a "cambial sector proper" may produce transgenic xylem and phloem sectors, GUS staining would only be seen in the xylem for example if a xylem-specific promoter was tested. During this stage of assessment, the amount of new growth (wound parenchyma and xylem tissue) was measured in millimetres using a dissecting microscope to give an indication of the extent of growth that occurred post inoculation.

Cambial sectors were further analyzed to gain insight into the temporal and spatial activity of the promoters under investigation, referred to here as ‘cambial sector ratio’. For this analysis, cambial sectors were assessed for presence or absence of GUS staining in three distinct regions defined as X1, X2, and P (Fig. 1b). The X2 region extended outward from the

initial wound parenchyma cells up to the end of the mature xylem. Staining observed in this region was indicative of promoter activity in ray cells which extend radially through the stem. Most xylem fibres and vessels in the X2 region have already undergone programmed cell death (PCD) and one would therefore only expect X2 GUS expression and staining in ray cells which have not undergone PCD. The X1 region was characterized by staining in developing xylem cells close to and including the cambial zone (but no staining on the phloem side). Staining observed in the X1 region was indicative of promoter activity in differentiating xylem cells (before the onset of PCD). The P region comprised all phloem tissues and GUS staining in this region was indicative of promoter activity in phloem tissue in general. It is important to again note here that the final staining pattern observed (combination of P, X1 and X2) was determined by the cell type initially transformed and the specificity of the promoter tested.

Statistical analysis of ISSA results

Details of the statistical analysis are outlined in the Results section. Confidence intervals (95%) were calculated for growth data using Minitab (Minitab Inc., State College, PA, USA) to compare growth rates. Chi-squared tests were performed using Minitab to compare the frequency of GUS expressing sectors observed in the X1, X2 and P regions (at $\alpha = 0.05$). Promoter constructs for which fewer than 10 sectors were observed were excluded from the statistical analysis, as was the case for the *EgCesA2* and *EgCesA4* in *Populus* stems. However, the majority of sectors observed for these promoters were cambial sectors and data for these promoters were included in the graphs, but should be validated in future experiments.

Results

The number of transformed sectors varied dependent on species and promoter

In total, inoculated stem tissue with a surface area of 559 cm^2 (*Eucalyptus:* 258 cm^2 and *Populus:* 240 cm^2) was harvested and stained for GUS activity, with 2558 transformed tissue sectors identified for the 10 promoter constructs investigated (Table 2). Overall, more GUS expressing sectors were identified in *Eucalyptus* stem tissues with a total of 1661 and an average of 6.4 transformed sectors per cm^2 of inoculated tissue (ATS^{-2}). In *Populus* stems only 897 sectors were counted, with an ATS^{-2} of 3.7 (Table 2). In the *Populus* and *Eucalyptus* stems, the *35S*::GUS (F and G) constructs resulted in the highest ATS^{-2} values ranging from 12.0 to 27.1 (Table 3), which is a measure of overall transformation efficiency as the 35S promoters are expressed ubiquitously in most plants. No sectors were observed in windows inoculated with the promoter-less pMDC162 vector (negative control). The highest ATS^{-2} value for the *CesA* promoters was observed for the *AtCesA8* promoter construct in *Eucalyptus* (ATS^{-2} = 9.4) and *Populus* stems (ATS^{-2} = 5.2), while the lowest values were observed for the *EgCesA2* promoter with an ATS^{-2} value of 0.6 for *Eucalyptus* and ATS^{-2} of 0.3 in *Populus* stems (Table 3). It is important to note that the ATS^{-2} values for the *CesA* promoters are more likely to reflect the spatio-temporal regulation of these promoters and the lower values are likely due to the smaller subset of tissues in which the promoters are active.

Sector type and frequency differed between promoter constructs

Overall, the most abundant sector types observed were phloem, xylem mother and cambial sectors, while periderm sectors only occurred at very low frequencies in *Populus* and *Eucalyptus* stems (Fig. 2a and b). As expected, the constitutive 35S promoter constructs (F and G) produced a wider range of sector types than the *CesA* promoter constructs (Fig. 2a and

b). All of the major sector types were represented in *Populus* stems inoculated with the two 35S constructs, including periderm and tylose sectors, which are induced upon wounding (Van Beveren et al. 2006). In *Eucalyptus* stems, no periderm or tylose sectors were recorded for the 35S constructs, and the ratio of sector types observed for the 35S constructs was distinctly different to that of the *CesA* promoter constructs (Fig. 2b). In both species, the *CesA* promoter constructs produced a high frequency of cambial and xylem mother sectors (Fig. 2a and b). In *Populus* stems, the *CesA* promoter constructs also produced phloem sector types at high frequencies (15% to 40%) in five (*EgCesA3, 4, 5, 7* and *AtCesA8*) of the seven *CesA* promoter constructs investigated (Fig. 2a). This included the secondary cell wall related *CesA* promoters (*EgCesA3* and *AtCesA8*) for which low numbers of phloem sectors were observed in *Eucalyptus* stems (Fig. 2b).

Cambial sectors were the most abundant and varied among species and promoter constructs

Cambial sectors were highly abundant in *Populus* and *Eucalyptus* stems (Fig. 2a and b) and these sectors were further classified into expression patterns (Fig. 2c and d). Five different cambial expression patterns were observed in the two species which included X2+X1+P, X2+X1, X1+P , X1-only and P-only (Fig. 1b). The X2+X1+P sector type was most likely produced by the transformation of a cambial initial, which subsequently gave rise to a ray sector extending into the P, X1 and X2 region followed by promoter activity in all three regions (Fig. 1c). The X2+X1 sector type could be the result of transformation of a ray initial on the xylem side, or xylem-specific promoter activity in a cambial sector giving rise to ray cells. The X1+P sector type was most likely the result of transformation of a cambial initial differentiating into phloem (P) and xylem (X1), but terminating at the zone of PCD (X1/X2 border), and subsequent promoter activity in phloem and xylem cells. X1-only and P-only sectors could be produced by the transformation of a cambial initial followed by xylem or

phloem promoter activity, or the transformation of a xylem or phloem mother cell, respectively, followed by promoter activity in the resulting xylem or phloem sector. No X2-only or X2+P staining patterns were detected in either species.

In the case of the two *35S* promoter constructs (F and G) all cambial sector types (Fig. 1b) were found in *Eucalyptus* stems (Fig. 2d), but three (X2+X1+P, X1+P and P-only) accounted for the majority of cambial staining patterns observed in *Populus* stems (Fig. 2c). In both species, the X2+X1+P sector type was the highest frequency cambial staining pattern observed for the 35S promoter constructs (approx 40% in *Eucalyptus* and 65% in *Populus* stems). For the *CesA* promoter constructs, all cambial staining patterns (Fig. 1b) were identified, but with distinct differences in the frequencies of staining patterns between *Populus* and *Eucalyptus* stems.

In *Eucalyptus* stems, cambial sector X1-only was the most frequent type observed for the promoters of secondary cell wall related genes *EgCesA1, 2, 3* and *AtCesA8,* whereas for the promoters of the primary cell wall related genes *EgCesA 4, 5* and *7* much higher frequencies of the X1+P cambial sector types in addition to X1-only were observed (Fig. 2d). In *Populus* stems, the secondary cell wall related *CesA* promoters also showed a high frequency of X1-only staining patterns, however, in *Populus* these promoters also displayed a higher frequency of the X1+P type sectors. The primary cell wall related *CesA* promoters produced a high proportion of X1+P sectors in the *Populus* stems, similar to the pattern observed in *Eucalyptus,* but there was also a number of X2+X1+P and P only sectors present (Fig. 2c).

Some *CesA* promoters showed similar activity to the *35S* promoter

We next investigated whether any of the *CesA* promoter constructs exhibited similar or different cambial sector (staining pattern) ratios when compared to the *35S* promoter

constructs (Chi-squared tests, Table 4). Cambial sector ratios were derived from the spatial temporal data sourced from cambial sectors (ratio of X2, X1, P). In *Eucalyptus* stems, all of the *CesA* promoter constructs exhibited significantly different ($\alpha = 0.05$) cambial sector ratios from that of the *35S* promoter constructs (Fig. 3b and Table 4). In *Populus* stems, the cambial sector ratios of some *CesA* promoters, such as *EgCesA4* and *5*, were not significantly different from that of the *35S* promoter constructs (Table 4) and in general the expression pattern seemed to be more variable than in *Eucalyptus* (Fig. 2c). This result suggests that there is a difference in *Populus* and *Eucalyptus* stems, either on a developmental or anatomical level as a result of different cell/tissue patterning, and/or on a genetic level with different transcriptional regulation of the *CesA* promoters.

Patterns of individual *CesA* promoter activity between tree species

We directly compared the cambial staining patterns observed for each promoter construct between the two species. We found that the *35S* promoter staining patterns were not significantly different in *Populus* and *Eucalyptus* tissues irrespective of the vector backbone (Table 5). This confirmed that the *Populus* and *Eucalyptus* stem tissues did not have significantly different relative transformation efficiencies in cells giving rise to X1, X2 and P staining patterns, although the *Populus* stems exhibited lower overall transformation efficiency (Table 3). The similarity of the staining patterns observed for the 35S promoter in *Eucalyptus* and *Populus* stems also suggests that differences in the amount of diffusion of GUS observed in the *Populus* and *Eucalyptus* stems did not greatly influence the resulting sector frequencies. In contrast, all the *CesA* promoter constructs (except the *EgCesA5* promoter), exhibited statistically significant differences ($\alpha = 0.05$) in cambial sector ratios between *Eucalyptus* and *Populus* stems (Table 5). Differences in *EgCesA1*, *2* and *3* and

AtCesA8 promoter activity could be attributed to activity being confined mostly to the X1 (developing xylem) region of *Eucalyptu*s (Fig. 3b and Fig. 4a,b), whereas in *Populus* stems, activity was observed at similar frequencies in both the X1 and P regions (Fig. 3c and Fig. 4e,f). In the case of the *EgCesA4* and *7* promoters, the majority of activity was observed in the X1 and P regions in *Eucalyptus* (Fig. 3b), while in *Populus* stems a higher proportion of observations were in the P and X2 (mature xylem) regions (Fig. 3a).

Discussion

ISSA provides a rapid and efficient approach to evaluate promoter expression in woody stems

Gene and promoter testing in tree genera such as *Eucalyptus*, *Populus* or *Pinus* require time-consuming and laborious manipulation through tissue culture and greenhouse studies. In this study we investigated the use of Induced Somatic Sector Analysis (ISSA, Spokevicius et al. 2005; Van Beveren et al. 2006) as an approach for rapid functional genetic analysis of promoter expression patterns in developing woody tissues based on large numbers of independent transgenic events. We demonstrate the suitability of ISSA for promoter expression analysis of six *Eucalyptus* cellulose synthase (*CesA*) genes in the stems of *Eucalyptus* and *Populus* trees. We show that in the *Eucalyptus* genetic background the *EgCesA* promoters produced distinct staining patterns, which were consistent with the primary and secondary cell wall associated expression patterns previously demonstrated for these genes (Samuga and Joshi 2004; Ranik and Myburg 2006), whereas in the heterologous *Populus* genetic background the staining patterns of the two groups of *Eucalyptus CesA* genes were less distinct.

There are a number of key advantages to using ISSA for functional genetic analysis of wood formation genes and promoters. First, it allows the testing of transgenic constructs directly in native woody tissues (e.g. *Pinus*, *Populus* and *Eucalyptus*) in a relatively short period of time yielding measurable results within a few months (Spokevicius et al. 2005). Second, this technique requires little greenhouse space because multiple constructs or replicate transformations (up to 10 windows) can be performed on a single tree stem. Finally, a major advantage of ISSA is that each transgenic sector represents an independent transformation event, and with ten inoculation windows per tree a large number of independent events are produced, which can then be statistically analysed. For these reasons ISSA can be a useful technique to quickly screen promoter constructs for expression in woody stem tissue, in order to select candidate promoters for more detailed whole-plant analyses in model species such as *Arabidopsis* or *Populus*.

An important aspect to consider when analysing ISSA data is that stem tissue is comprised of different cell types at different developmental stages (Plomion et al. 2001) each of which may respond differently to transformation by *Agrobacterium*. Similarly, genetic background and species-specific developmental patterns may affect transformation efficiency. For example the periderm sectors, which are a result of transformed cells near the cut surface of the cambial window and have undergone rounds of division during the wounding response, were observed for most of the promoter constructs transformed into *Populus* stems. No such sectors were observed in *Eucalyptus* stems (Fig. 2) suggesting that this tissue is recalcitrant to transformation or responds differently to wounding in *Eucalyptus*. Another important consideration for analysing promoter regions using ISSA is the cell fate of the initially transformed cell and the cell- or tissue-specificity of the promoter construct. These two factors determine the final staining pattern observed and have to be jointly considered in the analysis of tissue- or cell type- specific promoters. We found it useful to

compare the sector type frequencies obtained for the *CesA* promoters to those obtained for the CAMV35S promoter constructs (Fig. 3), which for the purpose of this study we assumed to be constitutively expressed in all cells derived from transformed initials. The latter is supported by the observation of a more diverse set of sector types for the CAMV35S promoter constructs including a higher frequency of wound parenchyma and tylose sectors (Fig. 2a and b), suggesting that these tissues are indeed susceptible to transformation, but that tissue-specific regulation resulted in low sector counts for these tissues when transformed with the *CesA* promoter constructs.

The CAMV35S control promoter was expressed in all stem tissues and exhibited similar cambial expression patterns in *Eucalyptus* and *Populus* stems

One of the aims of this study was to investigate the suitability of ISSA for assessing and comparing promoter activity in woody stems across plant species and genera. Towards this end, we first asked whether the observed sector types and staining patterns were indeed comparable among species, because it has been noted before that even constitutive promoters such as CAMV35S can show variable expression across species (Qin et al. 2010). Similar to previous results (Van Beveren et al. 2006) we found that the CAMV35S promoter was active in most sampled stem tissue types (Fig. 2a,b) and this was to be expected as the CAMV35S promoter is constitutive and will express GUS in most plant tissues (Odell et al. 1985; Jefferson et al. 1987; Benfey and Chua 1990). The comparison of CAMV35S driven GUS expression patterns in cambial derived sectors observed in *Eucalyptus* and *Populus* stems did not show any significant differences (Table 5), however, other sector types such as the tylose sectors exhibited very different frequencies presumably due to differences in the susceptibility of cell types to transformation (Fig. 2a and b). Together these results suggest

that ISSA can be used to compare promoter expression patterns across species using either sector type (Fig. 2) or cambial staining patterns (Fig. 3), provided that the inherent differences in transformation efficiency are accounted for by using a standard constitutive promoter construct such as CAMV35S.

Staining patterns for *CesA* promoters in cambial derived tissues showed clear grouping of primary and secondary cell wall related promoters

In a previous study, the expression patterns of the *Arabidopsis* (*AtCesA8*) and *Eucalyptus* (*EgCesA1*) promoters were analyzed using promoter::GUS assays in *Arabidopsis* plants (Creux et al. 2008). This confirmed the secondary cell wall related expression patterns of these two functional orthologs (Ranik and Myburg 2006). We included the same two promoter constructs in this study to allow comparison of the ISSA results to that obtained by whole-plant transformation in *Arabidopsis*. We found that the cambial staining patterns obtained in *Eucalyptus* stems for these two promoter constructs, as well as for the other *CesA* genes (Fig. 3) were consistent with the expression patterns previously observed for these genes (Taylor et al. 2003; Taylor 2008; Ko et al. 2012). The *EgCesA1*, *2*, *3* and *AtCesA8* promoters produced GUS staining mostly in developing xylem cells (X1) which actively deposit secondary cell walls before the onset of PCD (Plomion et al. 2001) and are thus expected to show high *EgCesA1*, *2* and *3* expression levels. Their distinct expression patterns may explain the lower net ATS^{-2} values (ATS^{-2} = 0.7 for *EgCesA1* to ATS^{-2} = 2.0 for *EgCesA3*) observed for the secondary cell wall associated *Eucalyptus CesA* promoters (Table 3). In contrast, higher ATS^{-2} values were observed for the primary cell wall related promoters (*EgCesA4*, *5* and *7*), which reflected their expression in a wider range of cell types such as phloem (P), developing xylem (X1) and ray cells in mature xylem (X2) tissues. These results

demonstrate that the ISSA approach was able to discriminate the distinct expression patterns of the *Eucalyptus CesA* genes in woody stem tissues.

The staining patterns of the *CesA* promoter constructs were not as distinctive in *Populus* stems as was observed for the primary and secondary cell wall associated *CesA* genes in *Eucalyptus* stems (Fig. 2c,d and Fig. 3a). In particular, the three secondary cell wall related *Eucalyptus CesA* promoters (*EgCesA1*, *2* and *3*) did not predominantly produce developing xylem (X1) expression in *Populus* stems, but were expressed at equal frequency in phloem (P) and developing xylem (X1) tissues. This could be the result of differences between the regulatory networks of the two genera and has been reported in a number of other plant promoter studies (Zhang et al. 2003; Fei et al. 2006; Qin et al. 2010). While the transcriptional network regulating secondary cell wall deposition is thought to be largely conserved across plant species and genera (Zhong et al. 2010), there may be important differences in promoter sequence and transcription factor binding sites of these species. In well-studied models such as humans, fruit flies and yeast, cis-regulatory variation has been shown to be relatively common (Ho et al. 2009; Dowell 2010; Mu et al. 2011) and could underlie differences in reporter gene expression observed for the same promoter construct in different species, as was found in this study. Cis-element evolution within promoter sequences can give rise to subfunctionalization of duplicated gene loci in organisms such as *Populus*, which have undergone genome-wide or segmental duplications (Tuskan et al. 2006). Furthermore, the NAC domain transcription factor family harboring many of the key transcription factors involved in secondary cell wall formation is highly expanded in some plant genomes and the duplicated genes may be under different evolutionary pressures (Hu et al. 2010). These differences may explain the variation observed in reporter gene expression from different genetic backgrounds. Other possible sources of variation in the reporter gene

expression observed for these two species could be on an anatomical or development level, but would require further investigation to elucidate this complex issue.

Conclusion

In this study we show that ISSA is an efficient approach to investigate promoter expression in the stems of woody plants such as *Populus* and *Eucalyptus*. ISSA requires less time and space to test promoters in woody stems than whole-plant transformation and regeneration, and provides ample independent transformation events for statistical analysis. However, it is important to include appropriate controls to interpret the ISSA staining patterns produced by transforming multiple cell types and using promoters with cell type- or developmental stage-specific expression. We found that the *CesA* promoter constructs produced distinct staining patterns in woody stem tissues consistent with the predicted roles of the corresponding *CesA* genes in primary and secondary cell wall formation. Our results suggest that, while many aspects of the secondary cell wall transcriptional network are conserved (Zhong et al. 2010), there are regulatory differences which should be considered when testing promoters in heterologous systems. ISSA should be applicable to a wider range of woody plants and various secondary cell wall related promoters could be analyzed in this manner, which will be important for elucidating the transcriptional control of woody biomass production.

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Tables

Table 1 *Arabidopsis thaliana* and *Populus trichocarpa CesA* orthologs and the corresponding *Eucalyptus grandis CesA* genes included in this study

	Arabidopsis[a]	***Eucalyptus***[b]	***Populus***[c]
Primary cell wall	*AtCesA1*	*EgCesA5*	*PtiCesA1*-A
associated *CesA* genes	*AtCesA2*	*EgCesA7*	*PtiCesA6*-A
	AtCesA3	*EgCesA4*	*PtiCesA3*-D
Secondary cell wall	*AtCesA4*	*EgCesA2*	*PtiCesA4*
associated *CesA* genes	*AtCesA7*	*EgCesA3*	*PtiCesA7*-A
	AtCesA8	*EgCesA1*	*PtiCesA8*-A

[a] All *Arabidopsis* ortholog information was obtained from TAIR (www.arabidopsis.org)

[b] All *Eucalyptus* ortholog information was obtained from Phytozome (www.phytozome.org) and nomenclature for the *EgCesA* genes taken from previous publications (Ranik and Myburg 2006; Yin et al. 2009)

[c] All *Populus* ortholog information was obtained from Kumar et al. (2009) in which the *Populus* and *Arabidopsis* naming conventions were unified

Table 2 Growth parameters and overall transformation efficiency for *Eucalyptus* and *Populus* plants

Growth Parameters	***Populus***	***Eucalyptus***
Average height at inoculation	91.7 cm (SE = 5.0 cm)	168.9 cm (SE = 6.7 cm)
Average height at harvest	205.8 cm (SE = 6.9 cm)	247.7 cm (SE = 9.186 cm)
Average diameter at inoculation (at stem height = 10 cm)	6.2 mm (SE = 0.09 mm)	7.5 mm (SE = 0.17 mm)
Average diameter at harvest (at stem height = 10 cm)	11.4 mm (SE = 0.22 mm)	11.2 mm (SE = 0.33 mm)
Average total radial growth of cambial window xylogenic tissue (from wound site)	2.55 mm (SE = 0.03 mm)	1.67 mm (SE = 0.069 mm)
Average total radial expansion rate	0.022 mm/day (SE = 0.001 mm/day)	0.013 mm/day (SE = 0.001 mm/day)
Total number of sectors counted	897	1661
[a]ATS^{-2}	3.7	6.4

[a] ATS^{-2} is the average number of transformed sectors per cm^2 of inoculated stem tissue

Table 3 Average number of transformation events per cm^{-2} of tissue (ATS^{-2}) observed for the different promoter constructs

Promoter	*Populus*[a]	*Eucalyptus*[a]
EgCESA1	0.5 (*0.07*)	0.7 (*0.13*)
EgCESA2	0.3 (*0.17*)	0.6 (*0.12*)
EgCESA3	1.6 (*0.28*)	2.0 (*0.45*)
EgCESA4	0.6 (*0.17*)	8.1 (*1.26*)
EgCESA5	1.3 (*0.28*)	2.7 (*0.82*)
EgCESA7	3.0 (*0.45*)	5.3 (*1.01*)
AtCESA8	5.2 (*0.75*)	9.4 (*1.58*)
35SF	19.1 (*0.85*)	27.1 (*2.20*)
35SG	12.0 (*0.98*)	17.8 (*2.10*)

[a] ATS^{-2} values for each promoter in *Eucalyptus* and *Populus* with the standard error in brackets

Table 4 Comparison of β-glucuronidase (GUS) expression frequencies observed for the *CesA* promoter constructs and for the CAMV35S promoter constructs in *Eucalyptus* and *Populus* stem tissues. Values below the diagonal are pair-wise comparisons within *Eucalyptus* and above the diagonal are within *Populus*

Chi-square values	***Populus***							
	EgCesA1	***EgCesA2***	***EgCesA3***	***EgCesA4***	***EgCesA5***	***EgCesA7***	***AtCesA8***	**35SA**[d]
Eucalyptus								
EgCesA1		0.295[c]	1.373[c]	6.169[b]	6.46[b]	6.986[b]	3.863[a]	13.754[b]
EgCesA2	0.708[c]		1.112[c]	4.79[a]	4.518[a]	5.317[a]	2.855[c]	9.186[b]
EgCesA3	2.839[c]	1.11[c]		2.223[c]	2.449[c]	2.941[c]	1.003[c]	7.555[b]
EgCesA4	8.856[b]	11.05[b]	15.151[b]		0.676[c]	0.661[c]	1.463[c]	1.869[c]
EgCesA5	9.976[b]	12.008[b]	16.123[b]	4.852[c]		0.015[c]	1.047[c]	1.591[c]
EgCesA7	9.18[b]	11.363[b]	15.488[b]	0.108[c]	3.275[c]		1.871[c]	3.952[a]
AtCesA8	0.024[c]	1.122[c]	3.258[c]	27.224[b]	27.855[b]	27.32[b]		14.897[b]
35SA	29.165[b]	30.234[b]	37.791[b]	46.858[b]	11.278[b]	36.082[b]	103.367[b]	

[a] Significance determination: >3.84 shows significant difference with one degree of freedom (dark grey shading)

[b] Significance determination: >5.99 shows significant difference with two degrees of freedom (light grey shading)

[c] White cells indicate promoter comparisons where there was no significant difference in expression patterns

[d] Only one of the two 35S promoter data sets were used as there was no significant difference between the two datasets

[e] Chi-squared values determined by pair-wise comparison of the frequency of GUS expression for different promoter constructs across the different stem tissues (P: phloem, X1: immature xylem and X2: mature xylem) with null hypothesis of equal expected frequency ratios for each comparison

Table 5 The inter-specific comparison of β-glucuronidase (GUS) expression patterns observed in *Populus* and *Eucalyptus* cambial tissues for the *EgCesA* and CAMV35S promoters

Chi-squared value[e]	***Populus***							
	EgCesA1	***EgCesA2***	***EgCesA3***	***EgCesA4***	***EgCesA5***	***EgCesA7***	***AtCesA8***	**35SA[d]**
Eucalyptus								
EgCesA1	8.584[b]	4.613[a]	9.488[b]	14.438[b]	15.776[b]	20.837[b]	16.503[b]	32.828[b]
EgCesA2	-	7.125[b]	11.841[b]	17.072[b]	17.69[b]	22.588[b]	18.394[b]	33.858[b]
EgCesA3	-	-	16.049[b]	22.917[b]	22.418[b]	27.903[b]	23.5[b]	41.617[b]
EgCesA4	-	-	-	5.462[a]	11.024[b]	16.697[b]	9.004[b]	49.714[b]
EgCesA5	-	-	-	-	2.437[c]	4.369[a]	1.476[c]	13.97[b]
EgCesA7	-	-	-	-	-	12.978[b]	6.421[b]	39.142[b]
AtCesA8	-	-	-	-	-	-	47.554[b]	101.493[b]
CAMV35SA	-	-	-	-	-	-	-	1.568[c]

[a] Significance determination: >3.84 shows significant difference with one degree of freedom (dark grey shading)

[b] Significance determination: >5.99 shows significant difference with two degrees of freedom (light grey shading)

[c] White cells indicate promoter comparisons where there was no significant difference in expression patterns

[d] Only one of the two 35S promoter data sets were used as there was no significant difference observed between the two datasets

[e] Chi-squared values determined by comparison of the frequency of GUS expression for different promoter constructs across the different stem tissues (P: phloem, X1: immature xylem and X2: mature xylem) of *Populus* and *Eucalyptus* plants with null hypothesis of equal expected frequency ratios for *Populus* and *Eucalyptus* tissues

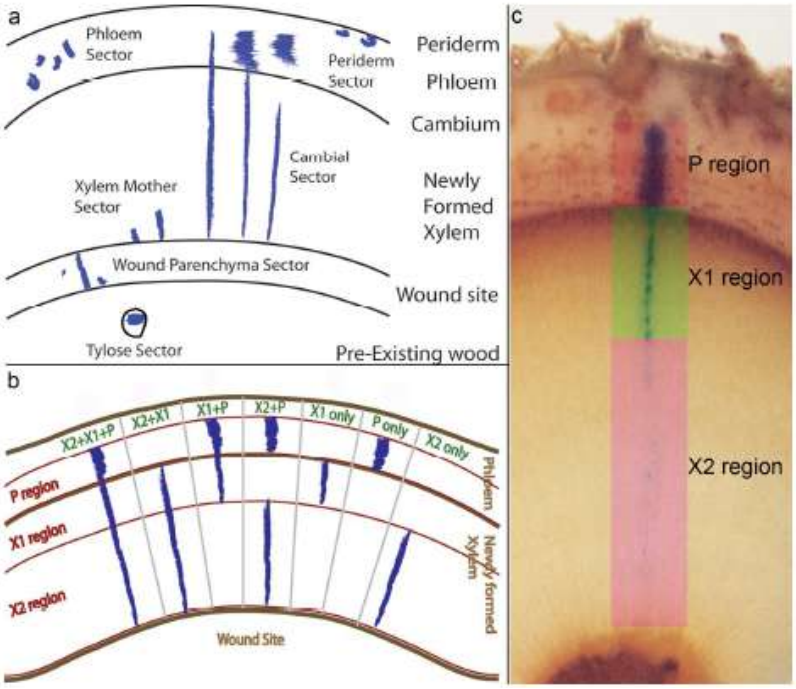
Figure legends

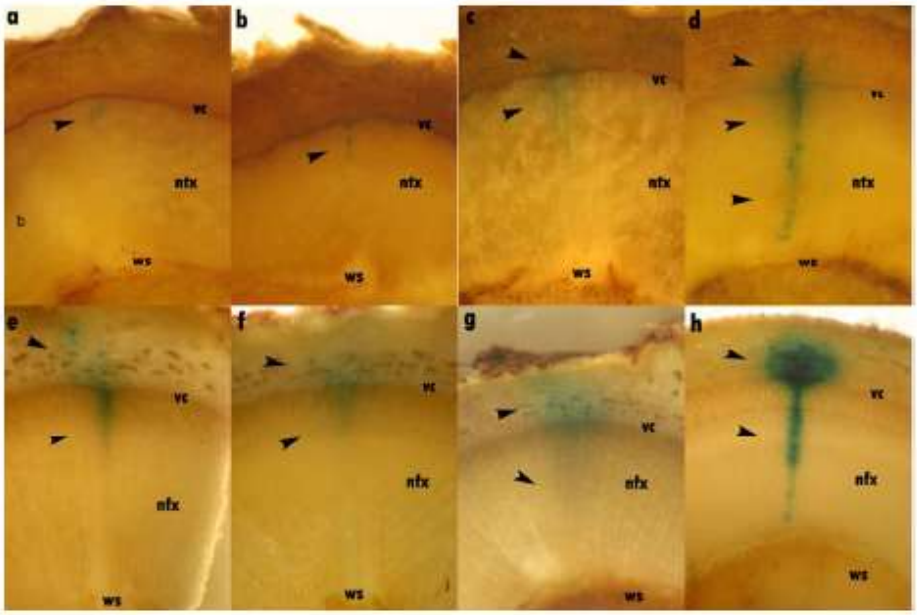
Fig.1 Representative somatic sectors (expected and observed) for different promoter types in woody stem tissues. **a** Schematic representation of the different somatic sectors that are typically observed in cross sections of transformed woody stem tissue during induced somatic sector analysis (ISSA) with constitutive CAMV35S driven GUS expression. Only tylose sectors are observed in the pre-existing xylem at the centre of the stem. All other sector types are observed in the wound site and across the newly formed cambial zone. **b** Reclassification of the different sector types (staining patterns) that can be formed during ISSA in woody stem tissues depending on the type of cell transformed (cambial initial, phloem mother cell or xylem mother cell) and the cell or tissue specificity of the promoter used to drive GUS expression. Sector types were classified depending on the presence of GUS in the phloem (P), immature xylem (X1) mature xylem (X2) or a combination of these. The only sector types observed during this study were X2+X1+P, X1+X2, X1+P, X1 only and P only. **c** Cross section of a *Populus* stem showing a transformed cambial sector with GUS expression in the phloem (P), immature xylem (X1) and the mature xylem (X2) driven by the CAMV35S promoter, indicating that a cambial initial was transformed which continued to divide and produce xylem and phloem cells

Fig.2 The overall observed frequency of somatic sector types and cambial sector types observed in *Populus* and *Eucalyptus* stem tissues. The frequency of the different sector types for each promoter in *Populus* (**a** and **c**) and *Eucalyptus* (**b** and **d**) plants is indicated on the y-axis, while the promoters used for each transformation are indicated on the x-axis. The number next to each promoter name (*n*) indicates the total number of sectors observed for that promoter. Of all sector types (**a** and **b**) counted, cambial sectors were found to be most highly abundant in *Populus* and *Eucalyptus* stems. The cambial sectors (**c** and **d**) were further classified into different subtypes (Fig. 1c) depending on GUS staining patterns in phloem (P), immature xylem (X1) and mature xylem (X2) regions: P+X1+X2, X1+X2, P+X1, X1 only and P only

Fig.3 Spatiotemporal frequencies of cambial staining patterns observed for the different promoter constructs in the woody stem tissues of *Populus* (**a**) and *Eucalyptus* (**b**) plants. The sector frequency of GUS expression in the cambially derived stem tissues (phloem P-green, immature xylem X1-red and mature xylem X2-blue) is indicated on the y-axis and the promoter constructs are listed on the x-axis. The *n* indicates the number of sectors counted for each promoter

Fig.4 Examples of cambial staining patterns observed in *Eucalyptus* and *Populus* stems. *EgCesA3* (**a**) and *AtCesA8* (**b**) promoter constructs showing activity in the X1 region only (black arrows) in *Eucalyptus* stems, whereas the same promoters (**e** and **f**, respectively) showed activity in the X1 and P regions in *Populus* stems. The *EgCesA5* promoter construct showing activity in the X1 and P regions in *Eucalyptus* (**c**) and Populus (**g**) stems. *35S* promoter activity was often seen in all three regions (X2, X1 and P) in *Eucalyptus* (**d**) and *Populus* (**h**) stems. nfx = newly formed xylem, vc = vascular cambium and ws = wound site


a
Phloem Sector
Periderm Sector
Periderm
Phloem
Cambium
Cambial Sector
Xylem Mother Sector
Newly Formed Xylem
Wound Parenchyma Sector
Wound site
Tylose Sector
Pre-Existing wood
b
X2+X1+P
X2+X1
X1+P
X2+P
X1 only
P only
X2 only
Phloem
Newly formed Xylem
P region
X1 region
X2 region
Wound Site
c
P region
X1 region
X2 region


a
b
c
d
e
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g
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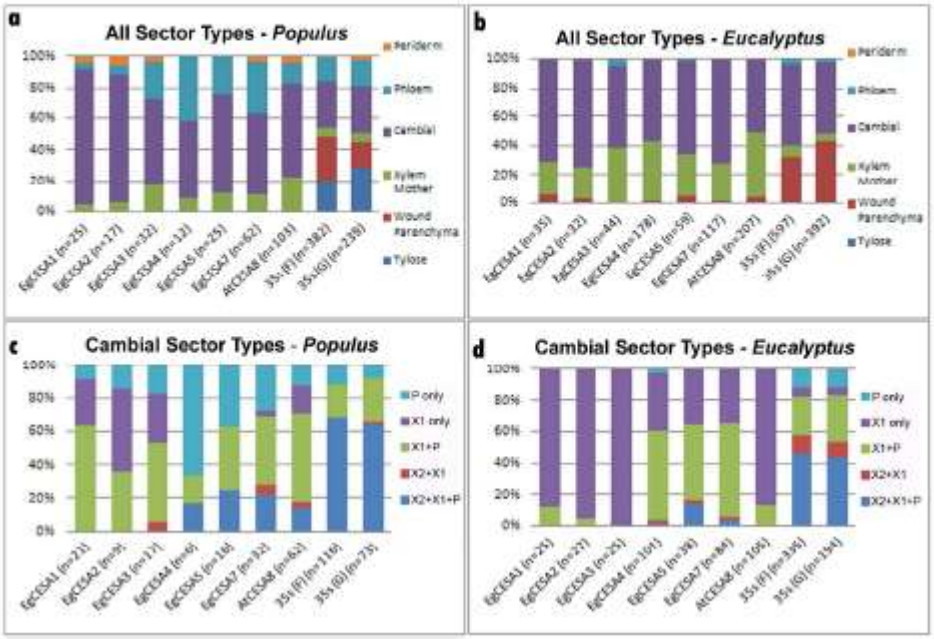
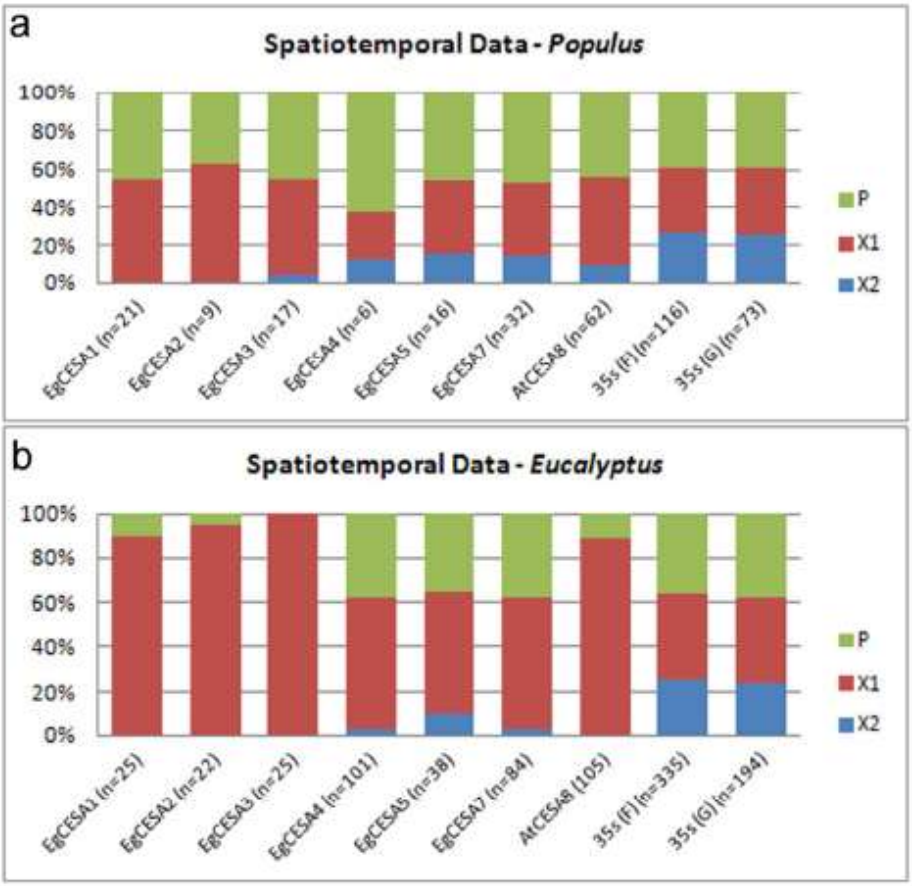
vc
ntx
ws


a
All Sector Types - Populus
b
All Sector Types - Eucalyptus
c
Cambial Sector Types - Populus
d
Cambial Sector Types - Eucalyptus


a
Spatiotemporal Data - Populus
P
X1
X2
EgCESA1 (n=21)
EgCESA2 (n=9)
EgCESA3 (n=17)
EgCESA4 (n=6)
EgCESA5 (n=16)
EgCESA7 (n=32)
AtCESA8 (n=62)
35s (F) (n=116)
35s (G) (n=73)
b
Spatiotemporal Data - Eucalyptus
EgCESA1 (n=25)
EgCESA2 (n=22)
EgCESA3 (n=25)
EgCESA4 (n=101)
EgCESA5 (n=38)
EgCESA7 (n=84)
AtCESA8 (105)
35s (F) (n=335)
35s (G) (n=194)

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