Transgenic Plant Journal ©2007 Global Science Books

Whole Plant and Plant Part Transgenic Approaches in the Study of Wood Formation – Benefits and Limitations

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ABSTRACT

Studying the molecular basis of wood formation (secondary xylogenesis) in trees is complicated by a suite of biological factors that place trees on top of the list of 'most cumbersome study organisms'. The identification of genes suspected to be involved in wood formation has advanced rapidly through the application of modern genomics tools such as large-scale gene expression studies and, to a lesser extent, through studies which aim to associate DNA sequence polymorphism with phenotypic variation. Transgenesis provides a powerful complementary approach enabling specific functional gene assessment at specific stages of wood formation. Genetic transformation as a tool to understand plant development has been widely used in annual plant species and, with the development of new molecular and culturing techniques, is becoming increasingly available for the study of woody perennials. The generation of full transgenic trees allows the study of individual gene effects on a whole tree basis, albeit only in few individuals, while new plant part methods for *in vitro* culturing of specific cells or tissue types enables analysis during defined developmental stages in isolation. Also, new plant part *in vivo* transformation methods that directly target cambial tissue in tree stems and developing vascular tissue in dormant lateral buds allow the analysis of independent transformed somatic wood sectors against surrounding control tissue within a single plant, and within short time frames. Here we review the more traditional whole plant transgenic systems and the recently developed plant part transgenic systems with the aim of identifying benefits and limitations of each approach in the study of wood formation.

Keywords: herbaceous annuals, *in vitro* culture, induced somatic sector analysis, model systems, reverse genetics, transgenesis, transgenic wood, woody perennials, xylogenesis

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INTRODUCTION

Transgenic science and technology is increasingly being applied to basic questions of plant molecular biology, especially the functional characterisation of genes, and to the development of genetically modified (GM) crops (Vain 2006). In order to apply transgenic approaches in a particular species efficient transfer and regeneration systems from single cells have been seen as essential prerequisites (Wenzel 2006). In model annual plant species and numerous annual crop species methods are well developed with the strategy being based on three steps; transformation, whole plant regeneration and characterisation (Sharma *et al*. 2005). In general, once an efficient system encompassing these three steps is developed within a species it is applied to both questions of basic molecular biology and to the development of GM crops.

The main drivers of transgenic system development in trees have been efforts directed at crop improvement through GM deployment. For many species this has resulted in the development of *Agrobacterium* or bombardment based methods for transformation with subsequent whole plant regeneration through embryogenesis or organogenesis. Recent reviews have covered most aspects of the development of these whole plant systems, their current state of development and application in trees (Confalonieri *et al*. 2003; Tang and Newton 2003; Merkle and Nairn 2005; Nehra *et al*. 2005; Poupin and Arce-Johnson 2005). For many tree species the efficient transformation and regeneration of plantlets from single cells remains problematic. Progress using transgenics in trees is therefore still in its infancy compared to many crop and other model annual species and certainly lags behind the advances being made through the application of other molecular tools such as transcript profi-

Received: 27 February, 2007. Accepted: 16 April, 2007.

Invited Review

Table 1 Comparative assessment of reverse genetics approaches in the study of wood formation.

Features	Whole Plant Systems		Plant Part Systems			
				Induced Somatic Sector Analysis		
	Woody perennials	**Herbaceous annuals**	***Pinus radiata* callus cultures**	***In vitro* transformation of apical stem segments**	**Transformation of exposed cambial cells *in vivo***	***In vivo* transformation of wounded dormant lateral buds**
Amount of tissue	Not limiting	Limiting	Limiting	Limiting	Limiting	Limiting
Wood cell types produced	All	Some	Some	All	All	All
Wood formation stages	All	Some	Some	All	All	All
Current ability to confirm transgene expression	Yes	Yes	Yes	No	No	No
Proof of Concept	Yes	Yes	Yes	No	Yes	No
Transgene carried in germline	Yes	Yes	No	No	No	No
Time frames for secondary wood or tracheary element formation	Long	Short	Short	Short	Short	Short
Loss due to contamination	Medium/High	Medium/High	Medium/High	High	Low	Low
Regulation of growth conditions	Confounded	Confounded	Tight	Tight	Confounded	Confounded
Technical and labour demands	High	High	High	High	Low	Low
Application in wide host range	No	No	No	No	Yes	No
Detail of phenotypic characterisation	High	Medium	Medium	Medium	Medium	Medium
Potential throughput	Low	Low	Medium	Low	Medium	Medium

ling and proteome profiling (for recent review see Boerjan 2005). While the ultimate goal of transgenic studies may be tree crop improvement, this improvement may not necessarily be achieved through the release of transgenic (GM) trees, even if the understanding is gained (wholly or partially) through the use of transgenic methods. In this respect it may be that transgenic systems will be increasingly used to improve our basic understanding of tree development and its molecular control. Systems developed around the strategy of whole plant recovery following transformation may therefore not be the most suitable.

Many basic biological and molecular questions in trees cannot be adequately studied using model annual systems (Boerjan 2005). One example where model annual systems do not enable complete biological understanding is wood formation (secondary xylogenesis) where cell type, seasonal and age dependant effects and stress response effects, such as the development of tension or compression wood, can only be studied in true wood producing whole plant or plant part systems. The application of tree based whole plant transgenics to such problems lags well behind the now rapid identification of genes putatively involved in important aspects of wood formation (see Nehra *et al*. 2005 for review). Recently a number of reports have described the development of plant part transgenic systems (Moller *et al*. 2003; Spokevicius *et al*. 2005; Spokevicius 2006; Spokevicius *et al*. 2006; van Beveren *et al*. 2006) aiming to facilitate simpler and faster elucidation of gene function, especially in the study of wood formation. A number of these systems have now moved through the early development stages and for a few, proof of concept has been demonstrated. With system development now moving into application it is timely to review these newer systems against the more traditional whole plant systems with the aim of clearly identifying current benefits and limitations of each approach.

Results from this review indicate that, in trees, the application of transgenic science will be inefficient if investigations of gene function during wood formation are solely base on classical whole plant regeneration systems. New plant part approaches offer significant advantages in particular for preliminary investigations of gene function and have the potential to bridge the current gap between the expansion of genomic resources and our ability to assess individual wood formation gene function at a higher throughput and within acceptable timeframes. It is also clear that all available systems have specific benefits and limitations with no system offering, as yet, a complete solution to the inherent difficulties of studying wood formation. A summary of the utility of each of these systems is presented in **Table 1**.

Wood formation

In order to orient the reader we first give a brief outline of the process of wood formation. Readers requiring more detail are referred to a recent review by Plomion *et al*. (2001). Wood formation is a secondary growth process that comprises the division of cambial initials, followed by secondary cell wall formation and programmed cell death (PCD). In mature tree stems the cambium forms a complete cylinder around a core of conducting xylogenic tissue and is surrounded by phloem and bark tissue. Centripetally, fusiform cambial initials give rise to fibres, tracheids and vessel cells (only tracheids are formed in conifers) while ray cell initials give rise to ray parenchyma cells which, combined, form the ray system. In many tree species a final developmental process of heartwood formation occurs during which conducting sapwood, characterised by living ray parenchyma cells, enters into a transition to heartwood. In some angiosperms vessel-associated ray cells block neighbouring vessels through the formation of tylose plugs. During heartwood formation ray cells undergo PCD and translocate polyphenolic substances into the surrounding cell walls. Heartwood formation leads to large decreases in wood permeability and provides protection from pathogens (Stewart 1966; Wilson 2001).

Following division, but prior to the commencement of secondary cell wall deposition, the immediate xylogenic derivatives of the fusiform cambial initials (i.e. xylem mother cells) undergo cellular expansion to reach their final form. The extent of this turgor driven expansion is regulated by a range of enzymes that assist in the association and disassociation of primary cell wall components including pectin, randomly aligned cellulose microfibrils and other non-cellulosic polysaccharides (Carpita and Gibeaut 1993). The deposition of lignified secondary cell walls marks the end of cellular expansion and is central to the process of xylogenesis and ultimately determines the functional properties of wood. Secondary cell walls consist of cellulose, lignin and other non-cellulosic polysaccharides which typically represent 90% of the wood dry weight and provide support for the cell. Other minor yet important contributions to cell wall structure and function are made by cell wall proteins and other compounds (Savidge 2000). Quantitative and qualitative changes in the composition of these elements can lead to changes in cell wall properties and are responsible for the large variation in wood properties observed within and between tree species.

Secondary cell wall thickening is characterised by the deposition of cellulose microfibrils in three layers; S1, S2, S3, which differ in their thickness and microfibril order and orientation. At the same time cell wall pits are formed which

allow for continued xylem sap transport and communication between cells (Timell 1986; Savidge 1996). The S2 layer is the thickest of these layers, and accounts for approximately 75-85% of the total cell wall cellulose content. As far as wood properties are concerned the S2 layer also has the largest influence (Plomion *et al*. 2001). For example 'Microfibril Angle (MFA)', one of the most important complex wood quality traits, refers specifically to the microfibril angle in this layer of the fibre (or tracheid) cell wall. MFA is known to influence the stiffness or elasticity of individual cells and wood and wood products. Understanding the development of such traits at the molecular level, in part through the use of trangenesis, is fundamental to the manipulation of wood properties to desired end uses.

WHOLE PLANT SYSTEMS

The creation and analysis of whole transgenic plants has been the most popular approach for reverse genetic investigations into gene function during wood formation. These systems follow the strategy of transform, regenerate and characterise. Whole transgenic plants have been produced through the initial *in vitro* transformation of a variety of tissue and cell types using, in most cases, either *Agrobacterium tumefaciens* mediated transformation or particle bombardment. Where the gene constructs used for transformation contain an antibiotic resistance gene (e.g. *npt*II conferring resistance to kanamycin) subsequent selection is often made using antibiotics. Regeneration of stably transformed individuals is achieved through root and shoot induction or through somatic embryogenesis (Nehra *et al*. 2005). The decision regarding which transformation method to use or which tissue or cell type to target is almost solely dependent on the species investigated (Birch 1997).

Both woody perennials and herbaceous annual whole plant systems have been used in the functional analysis of wood formation genes. Altered expression of candidate genes and their resultant phenotype have been successfully assessed in the woody perennial plant genera *Populus*, *Eucalyptus*, *Picea* and *Luecaena*, and in annual herbaceous plant genera including *Arabidopsis* and *Nicotiana*. The majority of genes studied in these systems are involved in lignin biosynthesis (phenylpropanoid and monolignol biosynthesis) with the selection driven mainly by commercial interest. Current understanding of lignin biosynthesis during cell wall formation is to a large degree based on these studies (Anterola and Lewis 2002).

The first and probably most obvious benefit of using whole transgenic plants is that the transgene is present in all cells of the organism. This leads to advantages in mass propagation of individual transformed lines enabling more detailed and precise measurement and characterisation of phenotypic changes associated with the transgene. In this regard, many researchers have taken up the opportunity to undertake further studies on previously created transgenic plants lines (Vailhe *et al*. 1996; Kajita *et al*. 1997; Ralph *et al*. 1998; Marita *et al*. 1999; Chabannes *et al*. 2001; Ralph *et al*. 2001; Ruel *et al*. 2001; Tamura *et al*. 2001; Schlimme *et al*. 2002; Huntley *et al*. 2003; Blaschke *et al*. 2004 and others). For example, the ability to mass propagate plant lines from work undertaken by Elkind (1990) has allowed for the utilisation of these plant lines in later studies with more specific biochemical (Howles *et al*. 1996; Korth *et al*. 2001) and molecular (Israelsson *et al*. 2003) investigations undertaken. Detailed assessments of whole plant performance under greenhouse and field conditions is also possible (e.g. Pilate *et al*. 2002; Hawkins *et al*. 2003). Opportunities also exist for relatively simple quantification of trans/target gene expression (e.g. Halpin *et al*. 1994; Baucher *et al*. 1996) and for the crossing between individuals to create double transformants (e.g. Blount *et al*. 2000; Coleman *et al*. 2006) and to create homozygous lines (e.g. Lagrimini *et al*. 1997).

Aside from the well recognised problem of regenerating many woody species following transgenesis a significant problem of using whole transgenic plants for the study of wood formation is the low efficiency by which suitable up/down regulation occurs leading to measurable phenotypic change. Due to the low efficiency of expression alteration, results in most whole plant studies are based on the analysis of few transgenic lines. For example, where poplar was used to study the phenotypic effects of changes in expression of a number of lignin associated genes, 10-25 individual transgenic lines were created with only 2-4 of these lines showing sufficiently altered gene expression to see significant changes in phenotype (van Doorsselaere *et al*. 1995; Jouanin *et al*. 2000; Meyermans *et al*. 2000; Ranocha *et al*. 2002). Similarly, this is also the case when using herbaceous annuals (Lee *et al*. 1997; Kawaoka and Ebinuma 2001; Shani *et al*. 2004) and can be attributed to the low efficiency of transgenic plant production, lack of well characterised and highly efficient wood specific promoters as well as the specific candidate gene. Although attempts have been made to identify and characterise wood-specific promoters (e.g. Feuillet *et al*. 1995; Lauvergeat *et al*. 2002), constitutive promoters such as the Cauliflower Mosiac Virus 35S (CaMV35S) promoter have been found to produce larger phenotypic changes when compared with wood-specific promoters (Yahiaoui *et al*. 1998). Accordingly the CaMV35S promoter continues to be frequently utilised.

The use of constitutive promoters in the study of wood formation can however, lead to undesirable changes in other plant parts. For example, Piquemal *et al*. (1998) down regulated cinnamoyl-CoA reductase in tobacco which led to altered plant development including reduced plant size, altered leaf morphology and collapsed vessels. Goicoechea *et al*. (2005) up-regulated a MYB transcription factor in tobacco and found this led to reduced plant size and loss of apical dominance. In contrast, Huntley *et al*. (2003) described how increases in the syringl lignin content as a result of increased ferulate-5-hydroylase expression under the control of cinnamate-4-hydroxylase promoter, a lignification gene, did not lead to any undesirable phenotypes in transgenic poplar plants. In addition, ectopic expression of some wood formation genes during various stages of development could also prove lethal, e.g. over-expression of α- or β-tubulin in maize callus cells lead to a loss of cell viability (Anthony and Hussey 1998). Suggested alternatives to wood-specific promoters include chemical induction of gene expression with specific inducible promoters (Groover 2005).

In whole plant systems there is limited control over environmental conditions at a tissue level. This is of particular relevance, for example, in the assessment of interactions between transgene expression and plant growth regulator concentrations. In this regard *in vitro* plant part systems have the advantage as the cambial environment can be more tightly controlled in culture (Leitch and Bossinger 2004).

Herbaceous annuals

Candidate genes for wood formation have been studied successfully in *Arabidopsis thaliana* and in *Nicotiana* spp. Investigations using arabidopsis are supported by the often cited benefits of vast genomic and mutant resources paired with a short life cycle and well developed transformation protocols. *Nicotiana* species on the other hand have often been utilised specifically for trangenesis work as wood formation more similar to that in trees has been observed in tobacco. Although possible in tree species, classic genetic studies using controlled pollination have been limited to herbaceous annual species. Such studies have been conducted in both tobacco (e.g. Tamagnone *et al*. 1998; Chabannes *et al*. 2001; Kawaoka and Ebinuma 2001; Ruel *et al*. 2001; Coleman *et al*. 2006) and arabidopsis (Sibout *et al*. 2002) providing a major advantage over the use of woody perennial species in transgenic studies. To date, however, few crosses have been performed for the study of wood formation genes. Examples come from tobacco where two plants, one with a down regulated cinnamic acid 4-hydrolase gene and the

other with an up regulated L-phenyalanine ammonia lyase gene were crossed in order to assess for metabolic flux in the lignin biosynthetic pathway (Blount *et al*. 2000).

Another advantage of using these annual plants is their relatively fast growth which considerably reduces the time taken for the production of the desired transgenic tissue. Time intervals between placing plantlets in a glasshouse and harvesting for phenotypic assessment of wood formation processes have been as little as 3-5 weeks in arabidopsis (Meyer *et al*. 1998; Baima *et al*. 2001) and around 2-4 months in tobacco (Halpin *et al*. 1994; Yahiaoui *et al*. 1998; Franke *et al*. 2000; Blee *et al*. 2003). This compares to reports of 2 months in whole plant systems using poplar (Ranocha *et al*. 2002) although, 7-10 months are more commonly reported in this system (Hu *et al*. 1999; Zhong *et al*. 2000; Li *et al*. 2003; Lapierre *et al*. 2004). The speed at which phenotypic traits can be assessed in annual plant systems gives a distinct advantage for early, basic investigations of gene function over proceeding straight to a woody plant system.

Both arabidopsis and tobacco produce a comparatively small amount of secondary xylem limiting phenotyping options. Problems associated with dissecting the target tissue from surrounding tissues also often leads to the analysis of tissue composites rather than just the tissue of interest. Apart from morphological studies involving microscopic techniques, methods used successfully for assessing the effect of wood formation genes in tobacco have included those that assess the chemistry of the cell wall; e.g. Nuclear Magnetic Resonance imaging (e.g. Ralph *et al*. 1998) and Fourier-transform infrared (FT-IR) microspectroscopy (e.g. Stewart *et al*. 1997). Methods, such as silviscan (Washusen and Evans 2001), that require larger amounts of tissue and which determine gross wood fibre traits important for wood quality, cannot be currently used for assessment of wood properties in annual plant systems.

While the use of herbaceous annuals has substantially furthered our understanding of wood formation the transfer of results to woody perennial species may be problematic and individual gene effects will require confirmation in tree systems prior to commercial application. Chemically, the cell walls of both arabidopsis and tobacco are more representative of hardwood tree species, with for example both containing guaiacyl (G) and syringyl (S) residues in their lignin (Kajita *et al*. 1996; Meyer *et al*. 1998). They are not representative for conifer wood where, for example S units are not present. The functional characterisation of transgenes in herbaceous annuals is therefore best seen as a preselection tool for the identification of potential wood formation candidate genes prior to commencement of more difficult studies in woody perennials. A more detailed discussion of the individual attributes of the arabidopsis and tobacco systems follows.

Arabidopsis thaliana

Arabidopsis is one of the world's most studied plants and is used as a model for dissecting many plant developmental and functional processes (Ye 2002; Oh *et al*. 2003). The plant itself is attractive for reverse genetic studies in general, due mainly to its high transformation and regeneration efficiency but also because it has a short generation time, small physical size, high seed production rate and a small genome size (Goodman *et al*. 1995; Birch 1997; Roberts and McCann 2000). Induction of secondary growth-like processes in arabidopsis have also been described but distinct differences to wood formed in perennial tree species do exist (Nieminen *et al*. 2004). Being a small plant, arabidopsis does not normally undergo extensive secondary growth but can be manipulated to do so under certain conditions for example, where flowering is delayed via decapitation of inflorescences and/or by manipulation of day length. Secondary growth is initiated in either the root hypocotyl or the inflorescence stem leading to the creation of secondary xylem tissue (Zhao *et al*. 2000; Ko *et al*. 2004). Secondary growth processes have been initiated in seedlings as little as six days old (Busse and Evert 1999). More recently, an arabidopsis *in vitro* cell culture system for the study of treachery element differentiation has been developed and successfully used for stable transformation (Oda *et al*. 2005).

Detailed anatomical examinations of wood formation in arabidopsis have been undertaken (Busse and Evert 1999; Altamura *et al*. 2001; Chaffey *et al*. 2002). Like in most trees, the arabidopsis cambium is non-storied but is comprised of fusiform initials only which produce xylary elements including fibres, vessels and axial parenchyma as well as phloem elements including sieve-tube elements, companion cells and phloem axial parenchyma. Morphological assessment of fibres and vessels showed that they shared similar morphological characteristics with these elements in poplar but these differed in that they were considerably smaller in size (Chaffey *et al*. 2002).

Reverse genetics studies of wood formation in arabidopsis have discovered genes important in influencing a number of different processes including cell division, elongation and lignification during secondary cell wall deposition. Increased expression of a Homeodomain Leucine Zipper transcription factor for example has been linked with changes in cell division and elongation rates, whereas down regulation showed no distinguishable phenotype (Baima *et al*. 2001). Changes in the expression of phenylpropanoid pathway genes including down regulation of 4-coumarate coenzyme A ligase (Lee *et al*. 1997) and cinnamoyl coenzyme A reductase (Goujon *et al*. 2003) and up regulation of ferulate-5-hydroxylase (Meyer *et al*. 1998; Sibout *et al*. 2002) all led to changes in lignin composition, particularly to changes in the S/G ratio. Decreases in the expression of a putative β-xylosidase gene, shown to be expressed in tissue undergoing secondary cell wall formation, did not lead to any significant change in cell wall sugar composition but did affect plant morphology (Goujon *et al*. 2003).

Many of the putative candidates identified in gene discovery studies in arabidopsis share high homology with expressed sequences found in tree species (Allona *et al*. 1998; Sterky *et al*. 1998; Kirst *et al*. 2003; Tuskan *et al*. 2006). In particular, genes involved in lignocellulosic cell wall formation in arabidopsis show high sequence identity with members of some gene families in poplar but in most cases the size of the gene family is larger in poplar (Tuskan *et al*. 2006). For example, some CesA genes involved in cellulose synthesis in the cell wall of arabidopsis share up to 98% amino acid sequence identity with their poplar counterpart (Joshi *et al*. 2004) although only ten CesA genes are present in arabidopsis while eighteen have been putatively identified in poplar (Tuskan *et al*. 2006). This suggests, as far as the cell wall is concerned, that biochemical function is likely to be conserved, but the exact functional characterisation of each gene will still need to be determined in true wood forming tissues.

Much has been gained from studying wood formation in arabidopsis, but its role as a general model for this process has been questioned by some (Chaffey 1999; Taylor 2002). The main arguments are that xylogenic tissue in arabidopsis lacks: a) anatomical wood features such as ray initials; although ray parenchyma cells have been observed in the early stages of pericycle derived secondary growth in the hypocotyl (Busse and Evert 1999), b) the ability to undergo seasonal change such as juvenile/mature wood development, earlywood/latewood formation, cambial dormancy-activity, and, c) processes associated with plant longevity such as heartwood formation (Chaffey *et al*. 2002; Taylor 2002). This suggests that not all genes involved in these processes could be present or active in arabidopsis thus potentially limiting its application to only some aspects of secondary xylogenesis. In addition, even though arabidopsis cell walls contain both G and S lignin residues, they do not occur in a consistent ratio along the stem and changes are often cell specific (Meyer *et al*. 1998) making accurate phenotyping difficult.

Nicotiana

Wood formation more similar to that in trees has been observed in tobacco (i.e. presence of ray parenchyma tissue) and consequently *Nicotiana* species have been extensively used for the study of lignin biosynthesis and its molecular control in particular (Anterola and Lewis 2002). Tobacco plants have also been used for the study of genes involved directly in secondary cell wall biosynthesis but only one case has been reported (Coleman *et al*. 2006). Tobacco plants have the ability to undergo some secondary growth near the base of the stem and compared to arabidopsis, these plants are larger and have longer generation times, providing the opportunity to produce more secondary xylogenic tissue for phenotypic assessments. Apart from this studies in tobacco have similar benefits as arabidopsis.

Investigations to date have focused on the commercial goal of increasing lignin extractability. These studies have also provided many fundamental insights into the lignin biosynthetic pathway. Genes studied include the LIM domain (Kawaoka *et al*. 2000; Kawaoka and Ebinuma 2001) and MYB (Tamagnone *et al*. 1998; Patzlaff *et al*. 2003; Goicoechea *et al*. 2005) transcription factors, phenylalanine ammonia-lyase (Elkind *et al*. 1990), 4-coumarate coenzyme A ligase (Kajita *et al*. 1996; Lu *et al*. 2003, 2004), cinnamic acid 4-hydroxylase (Blount *et al*. 2000), caffeoyl coenzyme A 3-*O*-methyltransferase (Zhao *et al*. 2005), caffeic acid *O*-methyltranferase (Ni *et al*. 1994), *O*-methyltransferase (Dwivedi *et al*. 1994; Atanassova *et al*. 1995), ferulate-5-hydroxylase (Franke *et al*. 2000), cinnamoyl-coenzyme a reductase (Piquemal *et al*. 1998; O'Connell *et al*. 2002), cinnamyl alcohol dehydrogenase (Halpin *et al*. 1994; Hibino *et al*. 1995; Stewart *et al*. 1997; Yahiaoui *et al*. 1998) and peroxidase (Lagrimini *et al*. 1997; Talas-Ogras *et al*. 2001; Blee *et al*. 2003). In addition, antisense strategies have been used for the down-regulation of both caffeoyl coenzyme A 3-*O*-methyltransferase and caffeic acid *O*-methyltranferase individually and in combination (Zhong *et al*. 1998; Pincon *et al*. 2001; Zhao *et al*. 2002). Non-lignification genes have also been investigated in tobacco including a secondary cell wall UDP-glucose pyrophosphate and sucrose synthase and up-regulation led to consistently shorter fibres and an increase in soluble carbohydrates in the secondary cell wall (Coleman *et al*. 2006). The vast amount of functional information already gained using this genus and the relatively efficient way by which it was obtained will ensure its continued use in the study of wood formation.

Woody perennials

Reverse transgenic studies of wood formation have been undertaken in a small number of tree species. Well defined protocols for transformation and regeneration have been developed in only a small number of woody perennial species and regeneration efficiency remains a limiting factor to the broad application of these techniques to a wide variety of species (for recent reviews see Confalonieri *et al*. 2003; Tang and Newton 2003; Merkle and Nairn 2005; Poupin and Arce-Johnson 2005). In particular, gymnosperms are often recalcitrant and only a small number of species have been identified that regenerate efficiently following transformation (Tang and Newton 2003).

By far the most extensively used genus for reverse transgenic studies of wood formation is *Populus* (Confalonieri *et al*. 2003). Poplar is considered a 'model' woody perennial species for the study of biochemical, physiological and molecular processes in trees (Mellerowicz *et al*. 2001; Taylor 2002). The poplar genome sequence has been completed (Tuskan *et al*. 2006) and many genes expressed during various stages of wood formation have been identified (e.g. Sterky *et al*. 1998, 2004). Consequently the majority of wood formation candidate genes, and in particular those involved in lignin biosynthesis, have been tested in poplars. Genes thought to influence other aspects of wood formation including cell division, cell elongation and secondary cell wall formation have also been studied but to a lesser extent. Other genera, including *Eucalyptus*, *Picea* and *Luecaena* have been used to study genes involved in lignification but not to the same extent as in poplars, presumingly due to lower plant regeneration efficiencies. Interestingly the three other genera investigated are phylogenetically diverse with the important inclusion of *Picea*, a gymnosperm with major differences in wood anatomy and genetic structure as compared to angiosperm species.

In poplar, transgenic lines with altered expression of lignin biosynthetic genes including the MYB transcription factor (Karpinska *et al*. 2004), 4-coumarate coenzyme A ligase (Hu *et al*. 1999; Jia *et al*. 2004), caffeic acid/5-hydroxyferulic acid *O*-methyltransferase (van Doorsselaere *et al*. 1995), caffeoyl coenzyme 3-*O*-methyltransferase (Meyermans *et al*. 2000; Zhong *et al*. 2000; Wei *et al*. 2001), caffeic acid *O*-methyltransferase (Tsai *et al*. 1998; Jouanin *et al*. 2000), cinnamyl alcohol dehydrogenase (Baucher *et al*. 1996; Lapierre *et al*. 2004), peroxidase (Morohoshi and Kajita 2001; Li *et al*. 2003) and laccase (Ranocha *et al*. 2002) have been produced. One case of a double transformant has been reported where the expression of both 4-coumarate coenzyme A and coniferaldehyde 5-hydroxylase were down and up regulated respectively (Li *et al*. 2003). Similarly, gene expression of cinnamate 4-hydrolase and cinnamyl alcohol dehydrogenase has been altered in *Eucalyptus* (Chen *et al*. 2001; Valerio *et al*. 2003), *O*-methyltransferase in *Leucaena* (Rastogi and Dwivedi 2006), and peroxidase in *Picea* (Elfstrand *et al*. 2001). Down regulation of superoxide dismutase, a gene known to be involved in the oxidative stress response, led to changes in xylem cell morphology and the up regulation of many lignification genes (Srivastava *et al*. 2007). Genes involved in cell division, cell elongation and secondary cell wall formation, including gibberellin biosynthesis (Eriksson *et al*. 2000), an endo-1,4-β-glucanase (Shani *et al*. 2004), a xyloglucanase (Park *et al*. 2004) and a sucrose synthase (Konishi *et al*. 2004) have also been investigated using whole plant transgenics approaches in poplars.

The most obvious benefit in using whole woody perennials for the study of wood formation is that all major anatomical features and cell types of wood are present including fibres, vessels, ray parenchyma cells, tracheids, and that processes associated with seasonal growth (e.g. early wood/latewood) and longevity (e.g. heartwood formation, reaction wood formation) occur. Another major benefit is that the amount of tissue that can be produced for analysis is only limited by the length of time that a plant is left to grow. Although at the same time there is significant within tree heterogeneity in many wood properties making comparison between and within trees problematic. This enables accurate determination of phenotype at both a cellular level, e.g. individual wood fibres, and at the tissue and even whole tree level. This is especially relevant where the aim is to incorporate transgenic lines into commercial tree improvement programs.

In contrast the long timeframes involved in the production, growth and assessment of wood in whole tree systems, up to two years in some species (Moller *et al*. 2005), provides a major limitation to high throughput gene studies. Technical requirements for transformation and regeneration are also considerable and continue to be specialised when compared to annual herbaceous species. Downstream genetic analysis is also limited due to long generation and flowering times making any breeding and backcrossing attempts difficult (Taylor 2002). The use of early flowering *Populus* plants as described by Meilan *et al.* (2004) might prove useful in overcoming this limitation.

PLANT PART SYSTEMS

Plant part systems provide an opportunity to undertake functional gene assessment in distinct tissues or cells and in isolation from the rest of the plant. Transgenesis in plant part systems differs from whole plant approaches in that transformation is followed directly by characterisation of the somatic transgenic cells and tissues. This alleviation on the need to regenerate plants between transformation and characterisation removes the most problematic step hindering the application of transgenic methods in many tree species and also considerably shortens the time between transformation and characterisation, potentially to a few weeks or months.

Plant part systems include a range of *in vitro* methods, i.e. xylogenic tissue is produced in culture, and *in vivo* methods that target for transformation individual cells within developing or mature plant stems. Following transformation, multiplication and differentiation of initially transformed cells leads to the production of distinct somatic tissue sectors. In some systems these sectors can be phenotypically assessed in comparison to the surrounding untransformed tissues and cells that have otherwise been subjected to the same treatment and have developed in the same microenvironment. Methods have been developed for the production of *in vitro Pinus radiata* callus cultures (Moller *et al*. 2003) and for Induced Somatic Sector Analysis (ISSA) which include methods for the transformation and analysis of *in vitro* apical stem segments (Spokevicius *et al*. 2005), *in vivo* transformation of exposed cambial cells within mature tree stems (Spokevicius 2006; van Beveren *et al*. 2006) and *in vivo* transformation of developing tree stems after wounding of dormant lateral buds (Spokevicius 2006; Spokevicius *et al*. 2006). A *Zinnia* cell culture system has been widely used for gene discovery and expression studies during tracheary element differentiation (Demura *et al*. 2002) but to our knowledge successful stable transformation using this system has not been reported.

One of the main benefits of undertaking functional studies in plant parts is that only target tissues and cell types will harbor the transgene. This overcomes problems associated with negative candidate gene effects where expression during other developmental stages or ectopic expression in a whole plant can hinder regeneration (lethal effects) or lead to significant deformation of the plant. This tissue and/or cell type specificity therefore removes the need for the discovery or development of strong developmental stage or tissue specific promoters.

Like for the annual whole plant systems, all plant part systems produce only small amounts of tissue for phenotypic assessment. In ISSA this limitation is compensated to some degree by the small variance expected between the control and transformed tissue arising from positional or other environmental effects. Also in ISSA, where transgenes' effects result in phenotypes requiring visual assessment e.g. the determination of cell fate, polarity, cell morphology, microfibril angle, etc. the availability of small amounts of transformed tissue is less problematic as suitable cell numbers for statistical comparisons are generally available. Phenotypic assessment of biochemical cell wall features on the other hand, e.g. cellulose, non-cellulosic polysaccharides and lignin composition, present a substantial technical challenge to both ISSA methods and to the *in vitro P. radiata* callus culture approach. A number of phenotyping techniques have however, been successfully used including analytical pyrolysis in the cell culture approach (Moller *et al*. 2005) and analytical pyrolysis and FT-IR microspectroscopy on sectors created using the *in vivo* transformation of exposed cambial cells within mature tree stems (our unpublished results). As all these systems are still newly developed there is no doubt that further phenotyping capability will be developed on a needs basis and the small tissue quantities available are unlikely to present a significant limitation to their application in future functional assessment of gene effects.

In *P. radiata* callus cultures, cells containing the transgene can be selected by antibiotic screening due to the presence of an antibiotic resistance gene (*npt*II) in the gene construct (Moller *et al*. 2003). This enables quantitative measures of both the antibiotic resistance gene expression and the target gene to be assessed using standard molecular techniques for each independent transformant line. ISSA on the other hand requires visual identification of tissue sectors in which potential effects of the target candidate gene can be expected. The GUS reporter gene has been successfully used for this purpose but, due to the need for destructive sampling, confirmation of changes in transgene expression are currently difficult to perform (Spokevicius 2006). Real time reporters such as Green Fluorescent Protein (GFP), Cyan Fluorescent Protein (CFP) and Yellow Fluorescent Protein (YFP) have recently been modified to contain peroxisome targeting signal peptides leading to successful application in woody perennials (Nowak *et al*. 2004). Use of these non-destructive reporter systems is currently being investigated for use in ISSA and may enable transgene expression to be measured using standard molecular techniques in each independent transformed sector.

In vitro cultures

In vitro approaches for the study of xylogenesis allow individual cells or tissues to be treated as distinct entities. Tight regulation of growth conditions can be efficiently achieved and cell development can be controlled with high synchronicity (Fukuda 1996). One limitation identified for whole plant systems is the inherent variability in the wood formation environment between different plants and even between different regions within the same plant. Combining *in vitro* methods with transgenesis potentially offers a powerful means for detailed investigation of gene function under tightly controlled conditions. *In vitro* systems in general require the establishment and maintenance of tissues in aseptic conditions. This is technically demanding and time consuming and often low efficiency is achieved (Spokevicius *et al*. 2005).

Systems using eucalypt *in vitro* stem segments have been developed providing a means to study *de novo* wood formation from cambial cells in culture (Leitch and Bossinger 2004). In these systems apical stem segments (ASS) or main stem explants (MSE) from *Eucalyptus globulus* have been used and, following an initial wound response, cambial activity resumed leading to the formation of secondary tissue in timeframes of only a few months (Leitch 1999). The extent of secondary growth in explants was found to be affected by cambial age, position in the tree and seasonal conditions at the time of harvest. In this ASS system most cell types in explants, including cambial initials were shown to be susceptible to *Agrobacterium*-mediated transformation (**Fig. 1A, 1B**) and stably transformed *de novo* wood sectors were produced (Spokevicius *et al*. 2005). These systems potentially allow for the direct comparison of transformed and non-transformed neighbouring tissue within the same plant segment and under nearly identical treatment conditions reducing the inherent variation between samples. Efficient use of these systems with *Agrobacterium*-mediated transformation has proven difficult as continued contamination following bacterial inoculation has been difficult to control. This results in a low recovery of stably transformed sectors and limited sector size due to the reduced growth periods (Spokevicius *et al*. 2005). To date phenotypic characterisation of candidate gene function in these systems has not been attempted.

The use of *Pinus* species for the study of wood formation using transgenics has been difficult due to low transformation and regeneration efficiencies (Whetten *et al*. 2001; Tang and Newton 2003). The recent development of an *in vitro* wood-forming callus culture system has now made studies in pines more accessible (Moller *et al*. 2003). Xylem strips are induced to form callus in culture and, after a period, these are transferred to an induction medium in

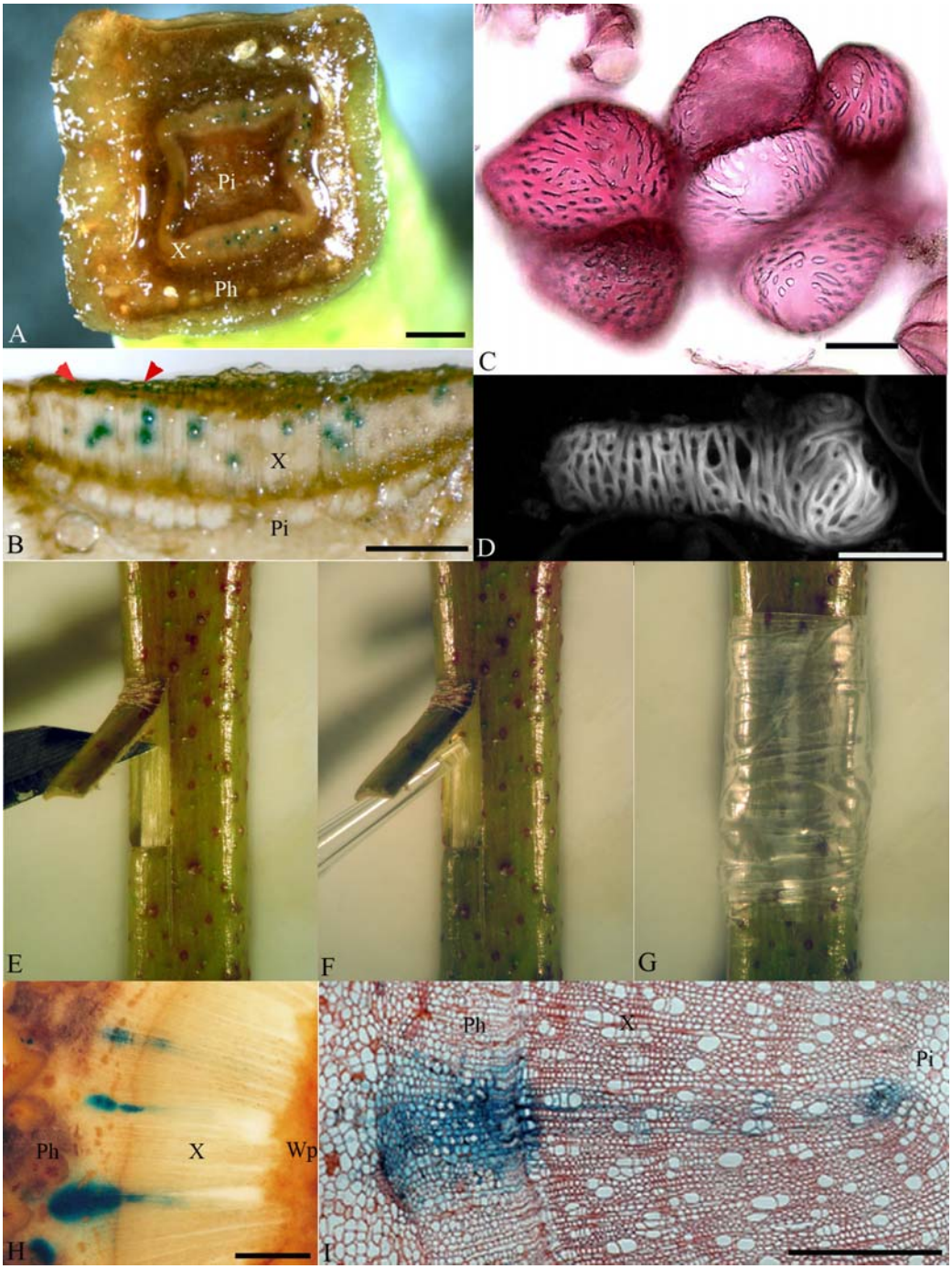

Fig. 1 Plant part systems. Susceptibility (as visualised by GUS staining) of several tissue types including cambial initials (red arrows) to *Agrobacterium*-mediated transformation in the *in vitro* apical stem segment system (**A, B**). Tracheary elements produced in the *P. radiata* callus culture system after induction (**C, D**) (Images kindly provided by C Walter Scion Research, Rotorua, New Zealand). Method of introducing *Agrobacterium* to cambial initials in the *in vivo* transformation of cambial initials including the creation of the cambial window (**E**), introduction of *Agrobacterium* suspension (**F**) and binding of cambial window to decrease wound responses and to improve cambium re-establishment (**G**). Transgenic sectors (as visualised by GUS) in the phloem and xylem created after transformation of cambial initials *in vivo* (**H**). Large transgenic sector (as visualised by GUS) created by *in vivo* transformation of dormant lateral buds (**I**). Abbreviations: Pi, pith; Ph, phloem; Wp, wound parenchyma; X, xylem. Scale bars: 10 µm (**C**), 30 µm (**D**), 400 µm (**I**), 1 mm (**A, B, H**).

which the undifferentiated callus mass is induced to form tracheary elements. This is reported to occur in up to 50% of newly formed cells (Moller *et al*. 2003). Newly differentiated tracheary elements also feature secondary cell wall thickenings and partial lignification with similar morphological characteristic of tracheary elements formed in the *Zinnia* tracheary element cell cultures (**Fig. 1C, 1D**). Cell walls show characteristic chemical fingerprints of *in arbor*-produced tracheary elements and importantly efficient stable transformation *via* particle bombardment has been demonstrated. Cinnamyl alcohol dehydrogenase was successfully down regulated in this system with marked effects on lignin content and composition (Moller *et al*. 2005). These results, and the short time frames involved (a few months) make this a suitable model system for medium throughput characterisation of candidate genes involved in secondary cell wall formation and lignification. An added benefit is that cell lines can be perpetuated and used for a variety of different studies e.g. feeding experiments for metabolite flux assessments (Moller *et al*. 2005). The suitability of this system for investigations into other aspects of wood formation on the other hand are limited by the lack of an active and/or functioning cambium and the current inability to induce synchronised differentiation.

In vivo transformation

In vivo transformation methods target, for transformation, cambial cells at various developmental stages within the growing tree stem (Spokevicius 2006; Spokevicius *et al*. 2006; van Beveren *et al*. 2006). Conceptually these approaches are similar to the *in vitro* stem segment method described above. In all systems transgenic wood sectors derive from individual transformed initials with each *in vivo* transformation protocol differing in the developmental stage at which initials are targeted for transformation. *In vivo* transformation of cambial cells in tree stems involves exposing cambial cells in the growing stem through the creation of a cambial window via partial bark peeling (**Fig. 1E**). The severed phloem strip is lifted upward and an *Agrobacterium* suspension is applied onto the exposed tissue before the strip is moved back into place and bound tightly to minimise wound responses and to encourage

rapid cambium reestablishment (**Fig. 1F, 1G**). Following inoculation, growth of stably transformed cambial initials leads to the formation of transformed wood and phloem sectors (**Fig. 1H**). Candidate gene effects are then determined in relation to neighbouring untransformed tissue. The efficiency of this system has been recently demonstrated with experiments in eucalypts describing the effects of a tubulin gene on microfibril angle orientation in transgenic wood sectors (Spokevicius 2006).

The *in vivo* transformation of dormant lateral buds in poplar targets, for transformation, stem tissue at an earlier stage of development and leads to the stable transformation of pro-cambial initials (Spokevicius *et al*. 2006). This produces sectors that occupy a larger proportion of the poplar stem (fewer initials are available to contribute to stem formation) over a similar timeframe to the above mature stem transformation approach and with high efficiency (**Fig. 1I**). To date this approach has only been successful in poplar where well developed and robust lateral buds, that can be induced to break dormancy, are available (Spokevicius 2006). A range of putative wood development genes are currently being tested using this system.

In vivo transformation methods remove the need for specialised and labour-intensive *in vitro* culturing procedures and high transformation efficiencies are achieved. Also, depending on the tree species, transgenic tissue production is limited only by the time for which treated stems are allowed to grow (usually a few months). Using *in vivo* methods transgenic tissue sectors are produced directly in the growing stem allowing for the functional analysis of genes involved during all stages of wood formation. These features combine to produce a system with the potential for medium throughput gene analysis. Little effort, beyond keeping plants alive, is required to maintain transgenic lines following inoculation. This stands in contrast to both whole plant and *in vitro* methods where extensive culturing is required, restricting the number of lines that can be created or carried.

In vivo transformation of cambial cells in tree stems to date has been successful in all woody perennial species investigated, including a conifer (*Pinus radiata*) and angiosperm trees (*Populus alba* and *Eucalyptus globulus*; van Beveren *et al*. 2006). Transformed sectors have also been successfully created in acacia (our unpublished results). These methods therefore potentially allow functional analysis of wood formation genes in tree species that have so far proven difficult to regenerate or culture.

Several limitations do exist for the application of *in vivo* transformation methods for transgenic studies of wood formation. As already mentioned, the amount of tissue available for phenotypic assessment, per sector, are small when plants are grown only for a few months limiting assessment to methods capable of utilising small tissue samples. Another technical limitation for these systems is the difficulty of performing quantitative, sector by sector, RNA-based gene expression and protein studies. While reporter transgene insertion and expression was demonstrated using RT-PCR (van Beveren *et al*. 2006) detailed quantitative expression analysis will depend on the successful integration of reporter systems requiring non-destructive visualisation, such as fluorescent proteins.

CONCLUDING REMARKS

Over recent years new options have become available for assessing gene function during wood formation. Whole plant regeneration systems have been improved or developed and innovative plant part systems have become available which offer the potential for medium throughput gene analysis. New plant part approaches offer significant advantages in particular for preliminary investigations of gene function and have the potential to bridge the current gap between the expansion of genomic resources and our ability to assess individual wood formation candidate gene function at a higher throughput and within acceptable timeframes. It is clear that all available systems have specific benefits and limitations with no system offering, as yet, a complete solution to the inherent difficulties of studying wood formation. The combination of currently available systems though holds much promise for rapid progress.

ACKNOWLEDGEMENTS

We thank C Walter of Scion Research, Rotorua, New Zealand for providing images for Figures **1C** and **1D**.

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