

Transformation as a Tool for Genetic Analysis in *Populus*

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Abstract We summarize the outlook for using transformation as a genetic tool in *Populus*. Transformation approaches avoid the major obstacle to performing genetics experiments in trees – namely long generation cycles and the difficulty of inbreeding to reveal loss of function alleles. Dominant transgenic alleles allow modifications in gene function to be readily observed in primary transformants. Although transformation has been mainly used for reverse genetics (where the gene sequence of interest is known), transgenic mutagenesis approaches such as activation tagging and gene/enhancer traps have also been shown to enable forward genetics (where the phenotype, not the gene, is known). We outline challenges and needs for more efficient use of transformation tools. These include expansion of the transformation toolbox (e.g., promoters, vectors, targeting), and improved ability to conduct field trials to study gene function in native and plantation environments (in spite of regulatory obstacles). Because of the power of transformation, it will remain a major genetic research tool for dissection of gene function in *Populus* for many years to come. It is the key biological attribute that makes poplar the most powerful model organism for genetic analysis of woody plant growth, adaptation, and development.

1 Introduction

Following the development of gene transfer technology for plants approximately two decades ago (De Block et al., 1984), genetic transformation has become an indispensable tool for dissection of gene function. It is used extensively in *Arabidopsis*, rice, tomato, and many other model herbaceous plant species. Its main uses are for insertional mutagenesis, complementation, ectopic gene expression and

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gene silencing. Here we review the role of transformation as a tool for functional dissection and gene discovery in *Populus* – the model taxon for woody perennial development.

Trees dominate many kinds of terrestrial ecosystems, and have a number of exaggerated developmental features that are poorly expressed in herbaceous species. These include extensive secondary meristem development, long delayed onset of reproduction, and annual cycles of dormancy acquisition and release. Despite trees' economic, ecological, and biological importance, the genetic and physiological control of these important traits remains poorly understood. This is in no small part due to the difficulties of standard genetic approaches in studying gene function. Although natural mutants have been identified for some traits (e.g. Gill et al., 2003), routine genetic segregation and inheritance studies are problematic in trees due to their long generation time and strong inbreeding depression.

Knock-out or knock-down manipulation of gene activity is widely accepted as the “gold standard” method for studying gene function. The two major approaches taken for altering gene expression are generalized mutagenesis and gene-directed transgenesis. Transformation, because it inserts DNA in an approximately random manner into euchromatic portions of the genome, is useful for both approaches. Genes can be specifically knocked-out or -down by delivering specific transgenes that trigger one or more forms of RNA interference, which act in trans to their gene targets (e.g., antisense, RNAi, synthetic microRNAs). T-DNA can also act as a random mutagen because, when inserted in the sequence of a gene, it generates lesions that usually result in a loss-of-function allele. The known sequence of the T-DNA, now present in the mutated gene, facilitates gene isolation – a method generally known as gene tagging (Parinov and Sundaresan, 2000; Sessions et al., 2002; An et al., 2005). The importance of T-DNA tagging for genetic research in model organisms is demonstrated by the generation of many collections that reach near to genome saturation (Alonso et al., 2003).

2 Value of Transformation as a Genetic Tool

Reviews of both approaches for mutagenesis that focus on trees have been published elsewhere (Fladung et al., 2004; Busov et al., 2005a, b). We will therefore only briefly highlight how transformation mitigates some of the major obstacles to performing genetics experiments in trees. A key element of these approaches is the use of transgenic methods that impart, or employ, dominant alleles. This is critical because it allows modifications in gene function to be observed in primary transformants. In contrast, most classical mutations have their manifestations via loss-of-function mechanisms, and thus show recessive inheritance. They require strong inbreeding, usually selfing, to produce homozygotes that express a physiological effect from the lesion. This makes the process of mutational analysis impractical in most tree species. Efforts to trigger precocious flowering to shorten generation cycle via transgenic approaches are underway (Bohlenius et al., 2006; Hsu et al., 2006; Flachowsky et al., 2009) (Fig. 3c, d). However, they have not led

to the production of viable gametes and seeds in many tree species tested, including poplar.

Most trees, including poplars, are largely undomesticated and carry large genetic loads (Bradshaw and Strauss 2001; Bradshaw et al., 2001). Inbreeding to reveal recessive mutations is therefore poorly tolerated, and the expression of this load among progeny would make it difficult to distinguish the effects of specific gene lesions from the large number of additional loci whose mutant alleles will also be expressed. In addition, most poplar species are dioecious, thus producing homozygous plants via inbreeding requires at least two generations of close sibling mating. This would require a decade or more for a single mutagenesis experiment.

Finally, genetic redundancy is a major problem in all plant species. Plant genomes, including *Populus*, have very large number of repeated, functional genes. The genome of the model Arabidopsis plant is significantly duplicated (Vision et al., 2000), and poplar has an additional ~30% more duplicated genes (Tuskan et al., 2006). Therefore single loss-of-function mutations often do not have obvious phenotypic effects in most experimental environments. In Arabidopsis, this problem can be circumvented by identification of individuals carrying mutations in individual genes, followed by controlled crosses bringing two or more mutations in related genes together in one individual. This strategy, which is not a trivial undertaking in Arabidopsis, is impractical in trees. Multiple gene knock-downs therefore can be only addressed by transgenic approaches (discussion below).

3 Postgenomic Challenges and Opportunities

Recent sequencing and annotation of the poplar genome sequence identified more than 45,000 gene models (Tuskan et al., 2006). Understanding even a significant fraction of these putative genes in the context of woody plant development and adaptation presents a daunting challenge. Sequence comparisons have identified a substantial number of putative homologs of previously described genes from herbaceous model systems. However the *in silico* predictions for many genes warrant verification as they are based on limited domain homologies. Moreover, an even larger part of the poplar gene space encodes unknown, hypothetical or putative proteins, as well as RNA regulatory molecules like microRNAs. There are also likely to be many genes, or presumed pseudogenes, that were not identified, have incorrect gene models, or have important splicing variants that were misannotated. It is therefore highly desirable that a high throughput functional transformation pipeline be developed that can evaluate the roles of the many uncharacterized, hypothetical genes in poplar.

In developing a functional genomics pipeline, the list of genes could be prioritized based on expected functions in woody plant processes, tissue level expression databases, scientific novelty, or other criteria. Genes can be fed into the pipeline efficiently through the use of recombination-based cloning systems optimized for plant expression/suppression vectors (Hilson, 2006). Among others, Gateway cloning technology allows the development of such resources (see for example

Dubin et al., 2008). The goals may include over/ectopic expression, RNAi, promoter studies, localization studies (GFP fusions) or *in planta* bimolecular fluorescence complementation (Karimi et al., 2002; Weiste et al., 2007; Dubin et al., 2008). Fusion proteins with GFP can be used to study precise protein localization and co-localization of potentially interacting proteins (Koroleva et al., 2005). An alternative strategy is to insert in bulk whole cDNA libraries in RNAi or ectopic expression binary vectors, transform and screen for phenotypic alterations in traits of interest. The use of normalized, full-length cDNA libraries would help to minimize redundancy (Ichikawa et al., 2006; Weiste et al., 2007).

4 Transformation and Poplars

Populus was the first woody plant to be transformed (Parsons et al., 1986), and several genotypes – particularly in section *Populus* – can be transformed at high rates (Confalonieri et al., 2003). For transformation to be a useful tool for functional gene dissection it must be reasonably rapid, produce transgenic plants with stable transgene expression, and not induce a high frequency of transformation-associated mutation. These requirements are largely met for *Populus* (Strauss et al., 2001; Brunner et al., 2007; Li et al., 2007).

The transformation procedure, from inoculation to rooted transgenic plants carrying the transgene (see for details below), can be accomplished within 4–8 months depending on the genotype, skill of personnel and transgene used in the study. Approximately 20 independent transformation events per transgene can be generated within this period in a typical experiment.

Stable transgene expression and RNAi silencing is important if transgenic phenotypes are to be expressed over the months and years needed for phenotypic evaluation in *Populus*. Several studies have reported highly stable transgene expression in *Populus* (reviewed in Brunner et al., 2007). In a study of 40 independent transgenic hybrid cottonwood (*P. trichocarpa* × *P. deltoides*) plants that expressed herbicide resistance and GUS reporter genes and were monitored for four years under field conditions, only one showed instability of the transgene expression (Meilan et al., 2002). Similar results were obtained in a study of 20 transgenic events that expressed the GUS reporter gene under 35S and xylem-specific promoters (Hawkins et al., 2003). In the same study stable transgene expression was found under *in vitro*, greenhouse and field conditions and the expression was not affected by stress treatments. Although studies on efficiency and stability of gene suppression approaches in *Populus* are still scarce, in a recent study of 56 independent events, RNAi-induced gene suppression was found to be stable over 2 years and during the annual growth cycle. This occurred despite the use of an *rbcS* promoter, whose activity varied widely during transitions from active growth to dormancy, driving the RNAi transgene (Li et al., 2007). Similarly, antisense gene suppression appears also to be stable over time in transgenic *Populus* with silenced cinnamyl alcohol dehydrogenase (CAD) or caffeate/5-hydroxy-ferulate O-methyltransferase (COMT) genes (Pilate et al., 2002).

For genetic studies in species where sexual inheritance is required, single transgenic loci are greatly preferred as they give simple inheritance patterns. They are also usually less prone to co-suppression, may produce stronger gene suppression, and are less likely to cause unintended gene disruptions than multicopy insertions. However, in poplar gene copy number has no association with level of RNAi suppression (Li et al., 2007), no relationship with instability, and a positive association with level gene expression (Li et al., 2008a). Nonetheless, it is easy to recover single copy transformants in poplar. *Agrobacterium*-mediated transformation typically produces 1–2 insertions (Li et al., 2008a, b). A study of 45 independent events produced a mean of 1.5 insertions (Groover et al., 2004). Similarly among 53 independent poplar transgenic events with RNAi transgenes, 45 contained a single copy, 5 had two copies, and 3 harbored three copies (Li et al., 2007).

Morphological abnormalities induced by transgene insertion are rare for *Populus*. A main reason for this is likely that *Populus* is diploid, and gene disruption during transformation would typically allow the second allele to cover the mutation. In a large number of transgenics, morphological abnormalities were observed in only three events (~0.06%) among several thousands studied (Strauss et al., 2004). Other studies appear to have observed higher levels of somaclonal variation where highly sensitive reporter genes are employed (Wang et al., 1996; Kumar and Fladung, 2001). However, none appear to be so high as to pose a significant constraint on functional genomics studies (where several events are studied for each experimental treatment).

5 Approaches for Transgenic Modifications of Gene Function

Transgenic modifications can involve both gain- and loss-of-function like modifications. Gain-of-function is generally studied via ectopic gene expression, and usually imparts abnormal but physiologically informative phenotypic changes. Ectopic approaches are especially important in functional dissection of gene families, where functional redundancy often obscures the phenotypic effects of specific gene knock-outs.

Transgenic loss-of-function like modifications are usually achieved via post-transcriptional gene silencing (PTGS), targeting specific RNAs for degradation (Brodersen and Voinnet, 2006). PTGS is triggered by double stranded RNA (dsRNA) produced when an enzyme known as Dicer (with a RNase III domain) recognizes and cleaves the dsRNA into short 21–26-nucleotides. These fragments, termed siRNAs for “short interfering RNAs,” remain in double stranded duplexes and act as templates for the RNA induced silencing complex (RISC) that targets and destroys the homologous mRNA messages. Historically, antisense technology, which represents one of the first used forms of PTGS, was successfully used in poplar to study several key enzymes in the lignin biosynthetic pathway (Baucher et al., 1996) and genes involved in control of dormancy (Rohde et al., 2002). Because of the relatively low efficiency and inability to discriminate between closely-related paralogs, antisense-mediated gene suppression as a tool for gene

suppression is rapidly being replaced by RNA interference (RNAi) (Matthew, 2004), a more potent inducer of gene silencing. Studies in plants and other eukaryotic organisms have shown that inverted-repeat transgenes (especially if they are separated by an intron) provide a reliable and highly efficient means for suppression of gene expression (Chuang and Meyerowitz, 2000; Smith et al., 2000).

The substantial gene redundancy in many plant gene families poses specific challenges to dissecting individual gene function(s) using RNAi approaches. An alternate approach is generation of “dominant negative” mutations. Typically dominant negative mutation involves modifications in the coding sequence of the protein (reviewed in Veitia, 2007). The modified gene encodes mutant polypeptides that when over-expressed will disrupt the activity of the wild-type protein. For example, mutation in proteins that oligomerize can lead to inhibition of the protein complex. Many cell surface receptors typically form di- or multimers upon binding an extracellular ligand. The dimerization leads to activation of a cytoplasmic domain (e.g., kinase). Expression of protein that lacks the cytoplasmic domain reduces or typically abolishes signal transduction by sequestering native WT proteins from productive complexes. Similarly, dominant negative mutations in the Arabidopsis actin 2 gene impaired the oligomerization of the actin fiber and allowed functional characterization of the gene with respect to growth of root and aerial organs (Nishimura et al., 2003). Another example of a dominant negative mutation is production of transcription factors with truncated activation but fully functional DNA binding domains. Such truncated proteins compete for the same DNA binding sites with WT proteins, thus reducing the activation/repression of the target gene(s) (Veitia, 2007). This strategy can also produce less severe phenotypes for regulatory genes, where loss-of-function can be lethal.

6 Other Approaches for Generation of Knock-Down Gene Modifications

Several novel approaches that show promise for generation of loss-of-function gene modifications have been recently developed. Although their utility in poplar is untested, they have been applied successfully to other non-model plants. Therefore we briefly review the technologies and their potential applications in poplar.

6.1 Artificial miRNAs and Overexpression of siRNAs

Micro RNAs (miRNAs) are non-coding small (~20–28nt) RNA molecules that negatively regulate gene function via transcriptional or translational repression (Jones-Rhoades et al., 2006). They represent a native regulatory mechanism widely-distributed among eukaryotic organisms (Zamore et al., 2000). The mode of action and regulatory function of miRNAs very much resembles the siRNAs generated during the process of RNAi-mediated gene suppression (described above). However in contrast to RNAi, where multiple 20nt siRNAs are produced from approximately

200–300 bp sequence, miRNA biogenesis results in only one 20nt regulatory fragment. This is possible because a precursor RNA molecule forming a hairpin loop structure with a specific sequence and secondary structure features are recognized and processed by specialized recognition and nuclease machinery. The progress in identification of many precursor RNA molecules allowed generation of a “designed” miRNA precursors, now known as artificial or synthetic miRNAs (Ossowski et al., 2008).

Artificial miRNAs act in a similar manner as to RNAi molecules in causing post transcriptional gene silencing (PTGS), but are highly gene-specific – thus allowing discrimination of closely related gene family members. Genome-wide microarray analysis showed that like the native miRNAs, amiRNAs are similarly highly gene specific (Schwab et al., 2006). The utility of this approach was demonstrated in Arabidopsis, rice, tomato and tobacco (Alvarez et al., 2006; Warthmann et al., 2008). Initial reports indicate a high rate of silencing, similar to that of RNAi (~90%), and expression of amiRNAs can be driven by any *pol II* promoter (Ossowski et al., 2008). An interesting feature of amiRNAs is that the level of expression conferred by the promoter corresponds to the level of gene suppression (Schwab et al., 2006; Alvarez et al., 2006). Thus it is possible to generate quantitative knockouts that differ in the degree of gene suppression.

Artificial miRNAs use a functional precursor miRNA as a backbone, but the native miRNA sequence is replaced with a user-defined 20nt targeting sequence (Alvarez et al., 2006). Selection of the 20nt is important for the efficiency and specificity of gene silencing, and must follow specific guidelines to be properly processed and function (Ossowski et al., 2008). The selection of miRNAs has been automated in a web-based application that helps ensure specificity to the desired target gene and adherence to guidelines for functionality (<http://wmd2.weigelworld.org/cgi-bin/mirnatools.pl>). The engineering of the selected 20nt into the precursor can be accomplished by overlap PCR where the endogenous miRNA is replaced by the selected 20nt and the resulting amplified fragment is cloned into a vector of choice. Although heterologous precursor miRNA backbones have been successfully used, to ensure correct processing it is preferable to use a native pre-miRNA (Ossowski et al., 2008). Several poplar pre-miRNAs have been already found and their processing experimentally verified (Lu et al., 2005) (Fig. 1).

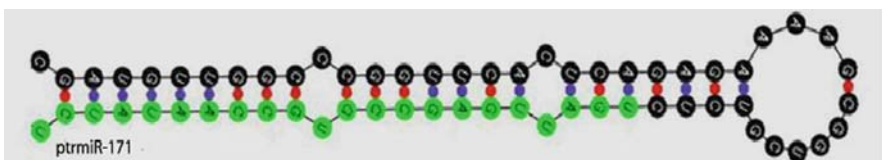


Fig. 1 Secondary structure produced by poplar *premiR171*. Sequence was amplified using RT-PCR and secondary structure generated using MFOLD. The *miR171* sequence is highlighted in green. Graph courtesy of Rewati Potkar, Michigan Technological University

Small RNAs have also been used to induce transcriptional gene silencing (TGS) (Aufsatz et al., 2002). In this case, a sequence homologous to the promoter of a gene is employed in the hairpin loop structure. The derived siRNAs cause DNA

methylation and chromatin modifications. Li et al. (2007) reported a much lower rate of gene silencing with dsRNA directed at transgene promoter compared to coding sequences in poplar. We know of no systematic studies of the success rate of PGS vs. TGS in any plant species.

6.2 Zinc-Finger Nucleases (ZFN)

ZFNs have been developed to: (i) increase the extremely low rate of homologous gene targeting (GT) in plants and most mammalian systems (excluding mice) and (ii) introduce mutations in a target locus by the imperfect repair of the cleaved target DNA by the ZFNs (Durai et al., 2005). ZFNs are synthetic proteins that can introduce double-stranded breaks (DSB) in DNA. The protein consist of a DNA binding domain (DBD) composed of 3–4 (Cis2-His2) zinc fingers fused to the DNA-cleavage domain of a *FokI* restriction endonuclease (Durai et al., 2005). The zinc fingers provide targeting and binding to a particular specific sequence, and the cleavage domain introduces a DSB. The break can be repaired by either the process of homologous recombination (HR) or non-homologous end joining (NHEJ) (Ray and Langer, 2002). The HR is precise and requires extensive homology between the repaired strands. In contrast, NHEJ does not require extensive homology and the repair often results in a mutation at the repaired site. In plants NHEJ is the predominant repair mechanism, thus DSBs introduced by the ZFNs result in a high rate of mutations at the target locus (Lloyd et al., 2005).

A ZFN that will introduce DSBs in a target locus requires design of a combination of fingers that will recognize and bind to both strands of a target sequence. Each finger consists of 30aa that recognize a 3 bp nucleotide sequence. The process of matching finger-to-sequence is still imperfect, and two approaches are widely used. One is based on a selection process, where different zinc finger combinations are screened for affinity to target sequence via library (phage or bacterial two-hybrid system) screening procedures (Durai et al., 2005). An alternative strategy is using computer-generated matches that use a domain library (<http://www.scripps.edu/mb/barbas/zfdesign/zfdesignhome.php>). Delivery of ZFNs can be accomplished via genetic transformation under an inducible promoter. This strategy was demonstrated in Arabidopsis using a heat shock promoter (Lloyd et al., 2005). Alternatively, in tobacco the DNA encoding a ZFN was successfully delivered into protoplast cells via electroporation (Wright et al., 2005).

6.3 Virus Induced Gene Silencing (VIGS)

VIGS has been developed to alleviate some of the problems associated with the time required for traditional mutagenesis and transgenic approaches. By inducing gene silencing in regenerated plants, it can also bypass the problem of lethality during embryonic development (Robertson, 2004; Burch-Smith et al., 2004). VIGS provides for the rapid reduction, but not elimination, of gene expression by taking

advantage of natural PTGS mechanisms used for viral defense. It is thus fundamentally similar to RNAi technologies (Ossowski et al., 2008). Typically, VIGS vectors include the bulk of the viral genome, but where a sequence of the gene that is the target for silencing has been inserted (Robertson 2004; Burch-Smith et al., 2004). Inoculation with the recombinant virus triggers the plants' silencing machinery, to suppress both the virus and the mRNA of the gene corresponding to the inserted fragment. The silencing signal spreads systemically in RNA viruses, imparting a phenotype similar to what would have been caused by PTGS for the target gene (Robertson, 2004).

Several VIGS vectors have been developed and their utility proven in functional analyses of genes involved in diverse processes (reviewed in Robertson, 2004). The tobacco rattle virus (TRV) vector is of wide applicability in the Solanaceae, as it has a wide host range, high rate of silencing, mild viral symptoms, and acts within meristematic tissues (Ratcliff et al., 2001). In poplar, the best candidate for development of a VIGS vector is the poplar mosaic virus (*PopMV*). *PopMV* is a RNA carlavirus that naturally infects species and hybrids in the genus *Populus*. Recently a VIGS vector based on the genome sequence of *PopMV* (Smith and Campbell, 2004) was developed (Naylor et al., 2005) and successfully tested in *Nicotiana benthamiana* for suppression of a GFP reporter gene. Information on effectiveness of this vector on transgene suppression in poplar, however, is unavailable.

Delivery of VIGS vectors to plants can be accomplished in several ways (Burch-Smith et al., 2004). In vitro transcribed RNA (most VIGS vectors are RNA) or DNA can be rubbed into the leaves (Ratcliff et al., 2001). This method is labor and time intensive but yields high infections. Alternatively, the vector sequence can be cloned into a binary vector and delivered by injection of *Agrobacterium* cultures into parenchyma cells (Schob et al., 1997). Microprojectile bombardment has also been successfully used for DNA-based vectors (Redinbaugh et al., 2001).

7 Transformation Methods in *Populus*

A variety of transformation methods have been successfully used in *Populus*, including biolistic approaches (McCown et al., 1991) electroporation of protoplasts (Chupeau et al., 1994), and cocultivation with *Agrobacterium*. Protoplast transformation is very rarely used, and biolistics is mainly used in specialized applications such as for rapid assessment of promoter::reporter expression in different tissues, transformation of highly recalcitrant genotypes, and transformation of plastids. For routine nuclear transformation using organogenic systems – the prevalent means for poplar transformation – *Agrobacterium* is by far the most widely used method. Although many poplar species have been transformed at least once (discussed below), each species and genotype tends to require detailed customization of regeneration procedures for successful and efficient transformation. The reasons for the extraordinary genotype specificity of regeneration and transformation procedures remains unknown, but imposes a major obstacle to efficient transformation of all plants.

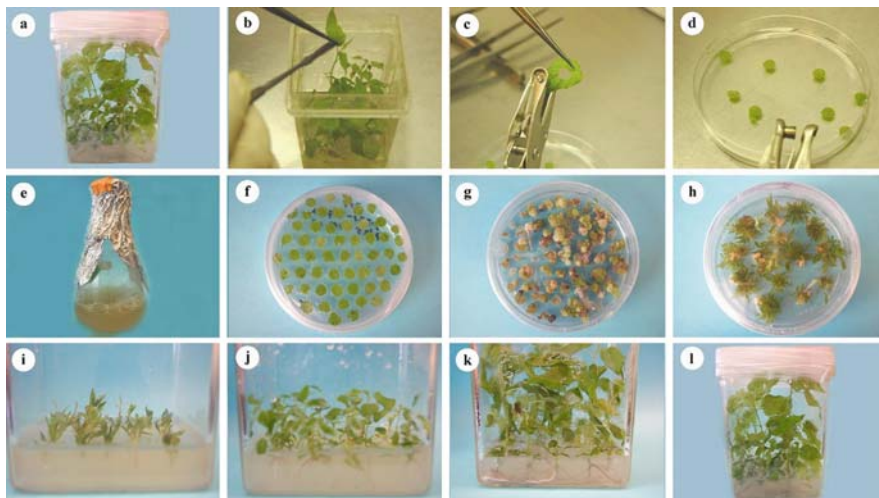


Fig. 2 Transformation in *Populus*. Explants preparation (a–d); co-cultivation (e, f); callus and shoot induction (g, h); shoot rooting (i–l). Photos courtesy of Cathleen Ma, Oregon State University

Most of transformation procedures using *A. tumefaciens* follow a classical protocol upon which there are many variations (e.g., Leple et al., 1992; Han et al., 2000; Confalonieri et al., 2003) (Fig. 2). These protocols involve the following steps in chronological order: (1) Co-cultivation of native or pre-induced tissues with *Agrobacterium* carrying the T-DNA of interest; (2) Callus induction if indirect shoot regeneration is employed, in the presence of a selective agent such as an antibiotic; (3) Shoot induction also in the presence of a selective agent; and (4) Rooting in the presence of a selective agent. Selective agents can be used in many ways and concentrations; they can be employed immediately after cocultivation, or their use delayed until after shoots have regenerated. Preculturing of explants before co-cultivation activates cell division, and can thus increase cell competence for T-DNA integration. Three major factors determine the success of transformation procedure – genotype of the host plant, *Agrobacterium* strain, and the regeneration procedure (plant physiological state, in vitro medium composition, environment, selection, and hormone treatments). We will briefly discuss each of these three factors below.

Successful transformation has been reported for many poplar species, with the majority from section *Populus* (reviewed in Confalonieri et al., 2003). For example, transformation protocols have been developed for *P. alba* (Okumura et al., 2006), *P. tremula* (Fladung et al., 1997; Tzfira et al., 1997), *P. tremuloides* (Cseke et al., 2007), *P. tremula* × *P. alba* (Leple et al., 1992), *P. tremula* × *P. tremuloides* (Fladung et al., 1997), *Populus canescens* × *P. grandidentata* and *P. tremuloides* × *P. davidiana* (Dai et al., 2003). Successful transformation have been reported for species and hybrids from sections *Aigeiros* (cottonwood) and *Tacamahaca* (balsam poplar) including *P. deltoides*, *P. trichocarpa* “Nisqually-1” (Ma et al., 2004; Song et al.,

2006), *P. trichocarpa* × *P. deltoides* (De Block, 1990; Han et al., 2000), *P. deltoides* × *P. nigra* (Heuchelin et al., 1997), *P. alba* × *P. grandidentata* (Fillatti et al., 1987) and *P. nigra* × *P. trichocarpa* (McCown et al., 1991), *P. sieboldii* × *P. grandidentata* (Matsunaga et al., 2002), *P. nigra* “Italica” (Nishiguchi et al., 2006) and *P. ciliata* (Thakur et al., 2005). The most widely used transformation “laboratory rat,” has been the *Populus tremula* × *alba* hybrid clone known as INRA 717-1B4 (Leple et al., 1992).

As the main vehicle for delivering the transgene, selection of an appropriate *Agrobacterium* strain is essential. Although both *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* have been successfully employed for poplar transformation, *A. tumefaciens* is the species of choice. The interest in *A. rhizogenes* transformation has been driven by the ease of production of transformed hairy roots, which self-select and regenerate, and those provide for more rapid generation of transgenic tissues (Cseke et al., 2007). Nopaline strains of *Agrobacterium tumefaciens* often have a higher transformation efficiency in tree species including poplars (Fladung et al., 1997). However, exceptions to that rule have been reported where octopine strains like EHA105 are more successful than nopaline strains C58 and LBA4404 (e.g., of two *Populus trichocarpa* × *Populus deltoides* clones: Han et al., 2000). Because knock-down RNAi manipulations often include sequence repeats that are prone to recombination, use of recombinant-deficient strains like AGL1 can help to avoid rearrangements during storage and transformation. AGL1 is also a hypervirulent strain and thus is believed to provide a higher level of transformation efficiency.

Most protocols in poplar transformation use direct or indirect in vitro shoot organogenesis, followed by rooting. One of the main determining factors affecting transformation efficiency is the origin and physiological condition of the explants. Although explants from leaves, petioles and stems have been successfully employed, the success rate with different types is highly variable and species-dependent. For example, transformation efficiencies with aspen are highest when leaf explants are used. In contrast petioles and stems seem to produce best results for cottonwood hybrids (Han et al., 2000). In vitro grown plants are generally used as a source for explants because they do not require sterilization prior to co-cultivation, however, protocols involving non-sterile greenhouse/growth chamber-grown plants have also been successfully used, and are sometimes found to be superior to in vitro materials (Song et al., 2006).

Co-cultivation conditions also affect transformation efficiencies. Optimum *Agrobacterium* concentration, precise timing, media and light conditions can affect the success of the T-DNA transfer. Callus induction usually requires presence of auxin (e.g., 2,4-D, NAA or IBA) and is performed under dark or very low-light-intensity conditions. The propensity of different types of explants (e.g., leaf, stem or petiole) to produce calli is highly genotype-specific. The callus induction step is necessary in some genotypes but not in others, and should be omitted whenever possible to reduce the risk of somaclonal variation and shorten the transformation cycle. Direct or indirect shoot organogenesis requires high levels of cytokinins such as BA, zeatin, thidiazuron (TDZ) and others. The presence of auxin in combination with

cytokinins has also proved beneficial in some species. Rooting is accomplished in auxin-dominant media and is generally a non-limiting step in poplar transformation and regeneration.

8 Using Transformation for Mutagenesis

As discussed above, insertional mutagenesis using T-DNA is widely used in plant biology to create recessive mutations revealed in homozygous individuals (Parinov and Sundaresan 2000; Sessions et al., 2002; Jeong et al., 2002; Alonso et al., 2003; An et al., 2005), but is impractical to apply in trees. However, methods that cause dominant mutations are applicable to trees. For example, activation tagging vectors allow generation of gain-of-function mutations via upregulation of genes positioned in the proximity of the insertion (Weigel et al., 2000). This is accomplished by cloning an array of strong enhancers near one of the T-DNA borders. The presence of the enhancers can cause upregulation of genes proximal to the insertion site, and thus a dominant mutation. In the majority of the cases the native expression of the gene is retained but at a higher expression level – giving a mild but clear and easily regenerated mutant phenotype. Specific gene upregulation also avoids the problem of gene redundancy that can obscure loss of function mutations.

Results from two pilot studies in *Populus* have demonstrated the effectiveness of activation tagging (Busov et al., 2003; Harrison et al., 2007). A population of 627 events was used to identify the first tagged gene in a tree (Busov et al., 2003). This population is under further investigation and results to date indicate a high rate (~7%) of mutant discovery based on (1) morphological inspection; (2) successful positioning of the tag in the genome for several dozen events; (3) activation of proximal genes up to 10 kbp; and (4) numerous successful recapitulations of the original phenotypes when the candidates are re-inserted under the control of a strong promoter. In the study reported by Harrison et al. (2007), a similar mutant discovery rate was found and different types of mutations were identified. In both cases, the majority of tagged genes are functionally novel to science, and many affect traits of particular interest for tree biology (e.g., wood quality, frost hardiness, phenology).

Enhancer and gene traps insert reporter genes that indicate the expression patterns of nearby genes. They provide high resolution (cell/tissue type) level information about the expression patterns of tagged genes (reviewed in Springer, 2000). Different reporter genes have been used in gene and enhancer traps, including the widely used GUS system. Reporters that allow imaging of live plants such as GFP (green fluorescent protein) and luciferase have also been employed (Szabados et al., 2002; Yamamoto et al., 2003). In the enhancer traps the reporter gene is preceded by a minimal promoter, which typically contains basal sequences required for transcription and translation, but is not sufficient to drive expression of the reporter gene. Insertion of the enhancer trap sequence proximal to a gene results in an activation of the reporter gene by neighboring regulatory enhancer *cis*-elements. Gene traps are variations of the enhancer traps; the reporter gene coding sequence is preceded by a splice acceptor sequence (SA). When the T-DNA sequence carrying the gene trap

inserts in the coding region of a gene, a novel splice variant between the native and reporter gene is produced that encodes a fusion protein that serves as marker of the expression pattern of the tagged gene. Enhancer/gene traps are useful in isolation of promoters and regulatory elements, as well as the identification of candidate genes that can be further manipulated by gene-silencing or ectopic expression approaches. Studies demonstrating the utility of this approach have been performed in *Populus* (Johansson et al., 2003; Groover et al., 2004; Filichkin et al., 2006b).

9 Future Prospects and Challenges

Transformation is a major tool for genetic research in poplar that, based on its frequency of use in scientific publications, is unequaled by any other taxon of trees. This valuable tool for leveraging the genome sequence, and for linking physiology to gene function, will continue to empower poplar research for many decades to come. However, there remain some substantial obstacles to the broader use of transformation and transgenic trees to improve biological research.

First, transformation remains a costly and slow procedure, requiring a laboratory with well developed means for *in vitro* culture, growth chambers, greenhouses, and generally more than a year from cocultivation to physiological analysis. Although there has been some progress on *ex vitro* methods for transformation in poplar – such as the development of a method that allows generation of transformed somatic cambium sectors (van Beveren et al., 2006) – there are no general methods that are widely used or reliable. The development of *in planta* methods, as have revolutionized *Arabidopsis* research, would be highly beneficial in poplar. The long, costly and facility intensive process emphasizes the need for centralized transformation facilities, as well as investment in the generation and maintenance of large transgenic collections that have broad value to the research community.

Second, transformation in poplar and many other species remains highly idiosyncratic, with its efficiency varying widely between tissues, genotypes, and species. Even in *Arabidopsis*, most of transgenic research is performed in a single variety (Columbia) that is highly amenable to transformation. The identification of a single reference genotype for poplar transformation would be highly desirable as it would allow more direct comparison of results across different laboratories. Advances in resequencing technologies allow near complete genome sequences for one or more standard genotypes to be readily produced. Nevertheless, in the near term the slow rate of molecular evolution, and thus the high sequence conservation found in both coding and non-coding sequences in poplar (Tuskan et al., 2006), will allow gene constructs based on the Nisqually-1 genome sequence to be productively analyzed in a variety of transformed poplar genotypes. Unfortunately, although transformation procedures have been reported for Nisqually-1 (Ma et al., 2004; Song et al., 2006) a robust technique is still not available.

In the long term, in addition to sequencing one or more transgenic models, it will be necessary to develop methods that are effective on a wide variety of genotypes chosen for their importance based on biological or breeding criteria. Breakthroughs

are needed that employ genes, such as the *rol* gene cassette from *Agrobacterium rhizogenes*, which promote the regeneration of transgenic cells (Arias et al., 2006). These genes could then be removed via methods such as recombinase mediated excision to produce normal plant phenotypes. A prototype was developed some years ago (e.g., Matsunaga et al., 2002), but does not appear to have been developed into a broadly effective method. Much more work along these lines is needed, perhaps exploring a much wider variety of types of regeneration-inducing genes.

Third, because poplar is an emerging model species, the transgenic “toolkit” is still very limited. There are only a few promoters whose tissue and cell-specific expression properties have been demonstrated in transgenic plants (e.g. Wu et al., 2000; Johansson et al., 2003; Hao et al. 2005; Filichkin et al., 2006b). Although gene excision has been used in poplar, most notably as part of the MAT system (Ebinuma et al., 1997; Matsunaga et al 2002; Zelasco et al., 2007), a number of other useful and well studied excision systems such as CRE/LOX (Marjanac et al., 2008) and FLP/FRT (Sonti et al., 1995) have not, to our knowledge, ever been characterized for their effectiveness in poplar. Nevertheless, technological advance in other species seem to be transferable to poplar (Fig. 3) (Filichkin et al., 2006a).

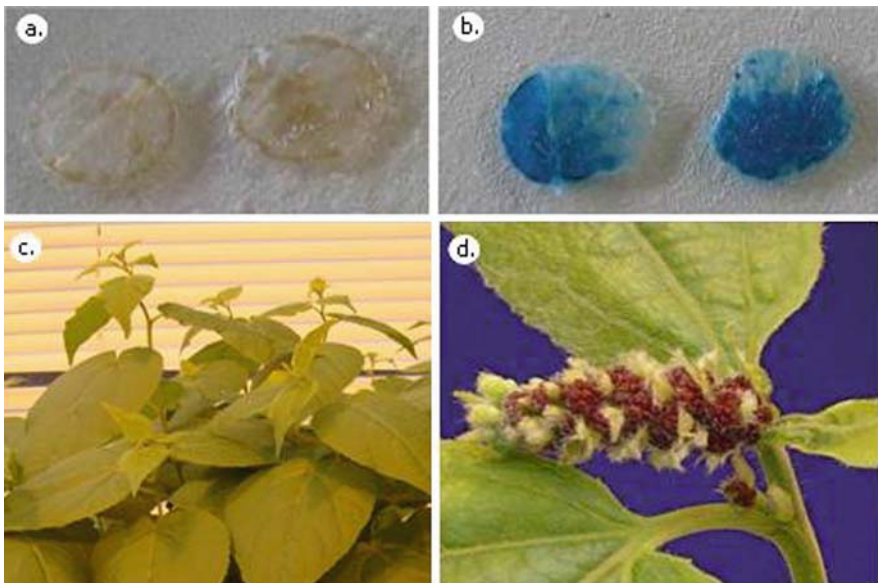


Fig. 3 Inducible expression in poplar. Photos on *left and right panels* represent explants and plants before and after induction respectively. *Top*: Induction of a GUS reporter gene under the control of the ecdysone induction system (construct provided by Metabolix Co. and based on the construct used in (Kourtz et al., 2007)) (a, b). Photo courtesy of Cathleen Ma, Oregon State University. Heat induction to induce flowering in poplar. *Bottom*: Plants were heat shocked by placing them in a growth chamber at 37 °C for 1 h/day (2–4 weeks). Flowers were observed after 2 weeks from the initiation of the treatment. Transgenic male poplar (*P. tremula* × *P. tremuloides*, INRA 353-53) containing the Arabidopsis *FT* gene under the control of the soy heat shock promoter were employed. Photos courtesy of Huanling Zhang, Oregon State University

Fourth, the capacity for forward genetics is limited in poplars. A worldwide, public investment in high quality QTL/association mapping resources that can identify specific causative genes or polymorphisms, and a much larger investment in gene tagging populations such as the activation tagging populations discussed above, would seem to be logical avenues for progress. Such a systematic gene discovery effort for traits important to woody species is likely to identify genes that would have been entirely missed based on selective reverse genetics approaches – as work to date for activation tagging has suggested (discussed above). The high costs of phenotyping in such programs, and the lack of capacity for maintenance/sharing of these collections without costly *in vitro* or cryogenic facilities, are the main limitations to progress (reviewed in Tsai and Hubscher, 2004).

Fifth, the inability to efficiently target genes for mutagenesis, and the associated “position effect” variation in transgenic populations associated with individual gene insertion events, remain major obstacles to efficient dissection of gene function in poplar and all other plant species. Recent advances in zinc-finger nucleases and related technologies appear to hold significant promise (Tovkach et al., 2009), but to our knowledge have not yet been studied in poplar.

Finally, a major deterrent of using transformation as a research tool is the difficult regulatory requirements for planting of genetically modified organisms (Strauss, 2003). Tree phenotypes must ultimately be studied in the field if they are to be relevant to normal physiology and breeding. However, the wide potential dispersal of pollen and seeds pose large obstacles to field trial approval in most countries. Flowering is problematic as most research plots are within the range of wild or feral species, and the female test trees themselves could produce progeny that establish in the field as they are not fully domesticated (though some hybrids and triploids have very low fertility). However, if fully sterile trees could be produced using transgenesis or other means, and the stability of the sterility trait verified over a number of years, these genotypes might serve as hosts within which a wide variety of genes could be tested over a normal rotation length. Transgenic research toward this end will clearly take many years, but a number of strong genome-enabled options exist for producing robust sterility systems (Brunner et al., 2007). The main limitation is the regulatory system itself, which even in the USA does not allow the dispersal of genes from small research plots even if their only effect is to reduce fertility, though this would appear to pose no significant environmental risk (controls and some transgenics will not be perfectly sterile during the research phase, and it is practically very difficult to remove all flowers/fruits from large flowering trees). Unfortunately, there are no established means to accelerate the normal flowering of poplars in the greenhouse to provide an effective first screen; as discussed above the *FT* and *LEAFY* genes only appear capable of producing abnormal, partially functional flowers. We know of no public research programs anywhere that are adequately organized and funded to be able to deal with this purely regulatory hurdle to flowering and biosafety research.

Despite these challenges, transformation in its current form will clearly remain the major genetic research tool for dissection of gene function in poplar for many years to come. The natural amenability to transformation in many

genotypes remains the key biological attribute that makes poplar the most powerful model organism for genetic analysis of woody plant growth, adaptation, and development.

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