Research review

Genetic transformation: a powerful tool for dissection of adaptive traits in trees

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Summary

Plant transformation and regeneration systems have become indispensable parts of gene discovery and functional characterization over the last two decades. Adoption of transformation methods in studies of plant adaptation to natural environments has been slow. This is a result of poor genomic knowledge and inefficient transformation systems for species dominating terrestrial ecosystems, and logistical difficulties in conducting field tests of genetically engineered organisms. In trees, where long generation cycles, high background polymorphism, large sizes and outcrossing systems of mating make production of near-isogenic lines and large experiments difficult, transformation is an attractive alternative for establishing direct linkages between genes and adaptively significant phenotypes. Here, we outline the capabilities, challenges, and prospects for transformation to become a significant tool for studying the ecophysiological adaptation of trees to the environment. Focusing on poplars (genus *Populus*) as model system, we describe how transformation-based approaches can provide insights into the genes that control adaptive traits. The availability of the poplar genome sequence, along with its large expressed sequences tag (EST) databanks, facile transformation and rapid growth, enable reverse genetic approaches to be used to test virtually any hypothesis of gene function.

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Genetic transformation for functional gene discovery and characterization

Since its initial application to plants two decades ago (De Block *et al.*, 1984), genetic transformation has become an indispensable tool in plant molecular biology and functional genomics research. More than 144 plant species have been successfully transformed, representing almost all major phylogenetic lineages of the plant kingdom (Birch, 1997). Although both biological and physical methods have been used, *Agrobacterium* is the predominant vector for all plant species today, including monocots. Its simpler, less fragmented pattern of gene insertions facilitates inheritance studies and, thus, interpretation of gene–phenotype relationships. Transformation is widely accepted as the 'gold standard' for demonstrating gene function; it is almost universally used for complementation of mutations or detailed dissection of gene function.

Agrobacterium T-DNA-based vectors have become a valuable research tool for insertional mutagenesis in model plant systems (Martienssen, 1998; Krysan *et al.*, 1999; Raizada *et al.*, 2001; An *et al.*, 2003). The development of efficient methods for transformation in *Arabidopsis* and rice (Bechtold & Pelletier, 1998; Sallaud *et al.*, 2003) has facilitated genome-wide T-DNA insertional efforts aimed at saturating their genomes with mutations (Speulman *et al.*, 1999; Parinov & Sundaresan, 2000; Sussman *et al.*, 2000; An *et al.*, 2003). *Agrobacterium*-generated insertions are usually simple (i.e. low copy number), facilitating isolation of the associated genes. In addition to gene disruption, elements harbored in the T-DNA have provided tools to induce mutations that cannot be generated using other mutagens. For example, activation tagging induces alterations of gene expression by inserting strong enhancers, usually positioned in close proximity to the binary-vector borders (Weigel *et al.*, 2000). This approach has been valuable for both functional dissection of gene families and generation of dominant/semidominant mutations (Nakazawa *et al.*, 2003). In addition, enhancer and gene traps use reporter genes to elucidate the expression patterns of nearby genes or regulatory motifs, thus helping to dissect the functional roles of newly identified genes. These functional genomics technologies have been used extensively in *Arabidopsis* and rice (Sundaresan *et al.*, 1995; Chin *et al.*, 1999), and are being successfully applied to functional genomics in poplars (Groover *et al.*, 2003; Fladung *et al.*, 2004).

Slow adoption of transformation-centered approaches in ecological research

By contrast to the widespread use of transgenic approaches in plant molecular biology and functional genomics, their incorporation into ecological genetics has been slow (Jackson *et al.*, 2002) for at least three reasons. First, many ecologically dominant undomesticated species and genotypes are still very difficult and/or costly to transform. For example, woody perennials, which dominate the terrestrial ecosystem and play a pivotal role in community and ecosystem dynamics, have been generally recalcitrant to transformation (Birch, 1997). Although major strides have been made in improving transformation efficiency and expanding the genotype spectrum, technical or cost constraints remain, and approaches for overcoming difficulties are largely empirical (Zaragoza *et al.*, 2004). Despite continuing efforts to improve our understanding of *Agrobacterium*-mediated transformation (Gelvin, 2000), the major constraint continues to be high-frequency regeneration of plants from transgenic cells, rather than gene transfer into cells (Han *et al.*, 1996).

Second, transformation is expensive. With the exception of *Arabidopsis*, where a simple *in planta* system is available (Bechtold & Pelletier, 1998), all other transformation systems use *in vitro* culture to regenerate transgenic plants. Unfortunately, traits such as wood structure and annual onset of dormancy cannot be adequately studied in *Arabidopsis* (Taylor, 2002). Because *in vitro* manipulations are labor-intensive and require substantial investment for infrastructure development, the costs of large-scale studies are prohibitively expensive for most investigators. In addition, the regeneration process is slow, ranging from several months to more than a year to produce and propagate plants for replicated studies. Combined with the time required for vector development and molecular analysis of transformants, the time investment often exceeds the life of most research grants.

Finally, but perhaps most importantly, there are often substantial logistical and social impediments to performing field studies with genetically modified organisms (GMOs). In many countries, the onerous and sluggish regulatory approval processes for field trials (Conner *et al.*, 2003; Nap *et al.*, 2003), as well as the risk of vandalism, present considerable obstacles to the use of GMOs in research. These difficulties appear to have discouraged many scientists from performing field tests (Strauss, 2003).

Map-based approaches dominate genetic dissection of adaptive traits in wild environments

The role of transformation in genetic dissection of adaptive traits in trees becomes even more important because of the difficulties associated with map-based approaches employing natural genetic polymorphism. For example quantitative trait locus (QTL) mapping has dominated efforts to genetically dissect adaptation in wild environments for many years (Frewen *et al.*, 2000; Howe *et al.*, 2000). Although QTL mapping has provided insights into the statistical architecture of adaptation, it has been of almost no value for identifying the genes controlling adaptive trait variation (Neale & Savolainen, 2004). Difficulties associated with these methods include: the multigenic nature of adaptive traits, low precision of experiments in uncontrolled environments, very low QTL mapping resolution, and a paucity of wild species with pedigrees and phenotypic data amenable to genomic dissection. In addition, the limited sample of segregating genes employed in QTL mapping of one or few families, and their low experimental power, make it unlikely that many of most important loci will be identified via marker-aided mapping. Linkage disequilibrium approaches (e.g. association genetics) hold promise for overcoming some of these limitations; however, they depend on identification of candidate genes and face very substantial logistical challenges for making strong statistical inferences from genome-wide scans (Neale & Savolainen, 2004).

The difficulties in constructing advanced pedigrees with trees for specific experimental goals are also daunting. Because these woody perennials have long generation cycles (years to decades) and are large at flowering, it is generally assumed that crosses from breeding programs, rather than those constructed for mapping, will be employed. These populations often lack

the size and structure needed for detailed mapping. Most trees species also have outcrossing mating systems and suffer from severe inbreeding depression (Williams & Savolainen, 1996). These complications make the development of near-isogenic lines, which provide a powerful means for converting quantitative trait loci to Mendelian traits for precise mapping, extremely difficult. In a large majority of poplars, selfing is biologically impossible, greatly slowing the development of inbred lines.

Transformation: a powerful genetic tool for dissecting adaptive traits in trees

Transgenic approaches can bring three major benefits to dissection of adaptive traits: (1) dominant mutations, (2) strong alleles and (3) facile gene identification.

Dominant mutations can be detected directly in primary transformants, alleviating the problem of long generation cycles and intolerance of inbreeding in trees. Dominant alleles can be generated using both forward (phenotype produced but no specific gene target) and reverse (specific gene mutated) genetic approaches. Ectopic expression and RNA interference (RNAi)-based manipulation of gene expression both produce dominant mutations for specific genes. For example, we produced dominant dwarfing phenotypes by overexpressing the *gai* gene, which inhibited native genes responsible for gibberellic acid (GA) signaling. This modification measurably affected plant growth within 6 months of when transformation was begun (Fig. 1). We have also produced forward dominant mutations via activation tagging and gene/enhancer traps (discussed later).

Strong alleles produced by transformation-based approaches can be identified unequivocally in few individuals, contrasting with the large field trials that are needed to distinguish the effect of a weak native allele segregating against a highly diverse genetic background. In a pilot study using activation tagging as a forward genetics approach in poplar, we were able to identify mutations using as few as four ramets of an independent event. For example, a mutation affecting leaf size was easily identified in a single pair of plants and was verified using a second pair that was randomly situated in an experimental field (Fig. 2). Such strong, dominant alleles are difficult to find in natural populations because of strong purifying selection, which keeps mutations that cause major developmental perturbations at very low allele frequencies.

T-DNA-based forward genetics vectors provide an efficient means, which is not map-based, for positioning inserts and cloning mutated genes. A variety of techniques can be used for these purposes, including plasmid rescue (Weigel *et al.*, 2000) and thermal asymmetric interlaced (TAIL) polymerase chain reaction (PCR) (Liu *et al.*, 1995). These methods recover a portion of the genomic DNA flanking the insertion site, which is then used as an anchor to position the site in the genome. The mutated gene is usually flanking the T-DNA or is in the immediate proximity of the tag (within 3–4 kb). Such resolution is impossible with any map-based QTL cloning methods, although it may be possible with association genetic approaches, given that sample sizes are sufficiently large and phenotypes are determined with precision.

We have recently demonstrated the power of activation tagging in poplar by cloning the first activation-tagged gene from a tree (Busov *et al.*, 2003). Using plasmid rescue, we recovered a genome sequence proximal to the insertion site of the tag in a dwarf mutant called 'stumpy'. A sequence showing high homology to *GA 2-oxidase* was identified approx. 300 bp from the right border. The gene tagging was validated via

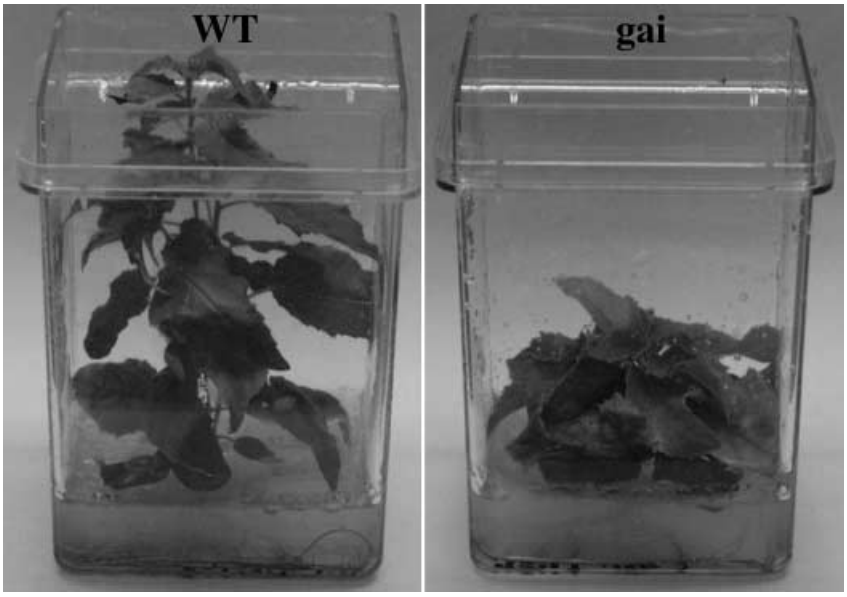

Fig. 1 Dominant dwarf phenotype observed in tissue culture approx. 6 months after transformation of poplar plants expressing the mutant *Arabidopsis gai* gene under the control of its native *Arabidopsis* promoter. The mutant gibberellic acid insensitive (GAI) protein has a truncation of the DELLA domain, which is important for mediation of GA responses. Complete truncation or nonsynonymous substitutions in this domain cause GA insensitivity similar to GA deficiency, which cannot be rescued by exogenous application of GA.

Fig. 2 Strong 'big leaf' mutant allele identified in an activation tagging field trial. Our field design employed two randomly assigned two-tree plots for identification of mutants. A tree's novel appearance was attributed to the T-DNA insert when the same mutant phenotype was observed in all ramets of the same unique line.

expression, analysis of endogenous GAs and phenotype rescue via GA_3 application and retransformation of the gene. The recent release of the draft of the poplar genome (http://genome.jgi-psf.org/Poptr1/Poptr1.home.html), and pending full annotation, will further facilitate the isolation and characterization of loci underpinning mutations found in this way.

The power of transformation-centered approaches can be leveraged by field studies

T-DNA tagging approaches have been successfully used in cloning genes important for adaptive responses in plants, including flowering time (Kardailsky *et al.*, 1999), cold tolerance (Ahad *et al.*, 2003) and stress response (Thompson *et al.*, 2000). However, these screens have been performed almost exclusively under controlled, laboratory conditions and the importance of many of these genes in wild environments has not been demonstrated. Recent progress with *Arabidopsis* has made it clear that genes identified in controlled environments can be very different from those identified in the wild (Weinig *et al.*, 2002). Thus, field studies are highly desirable for ecologically relevant dissection of adaptive traits.

We have shown that a dominant gene-tagging approach, combined with phenotypic analysis in a wild environment, can greatly enhance the ability to identify loci controlling adaptive trait variation. Screening an activation-tagged population of poplars in tissue culture and under greenhouse conditions yielded a mutant discovery rate of approx. 1%. This is equivalent to what has been seen with *Arabidopsis* in greenhouse screens. However, exposing our poplar population to three growing seasons in the field revealed many more mutant phenotypes, bringing the mutant discovery rate to almost 7%, approximately sevenfold higher than seen in the glasshouse or *in vitro*. Trees grew more than 4 m high, contributing to identification of mutants. However, they have still not flowered (most *Arabidopsis* mutants involve inflorescence/flower structure or timing of the transition to maturity). As trees mature, many more mutants are expected.

Remote sensing, using infrared (IR) aerial photography, can enhance the power of these approaches by making field phenotyping more efficient (i.e. identifying mutants by their reflectance signature). This approach may be essential for screening phenotypes in very large field trials (e.g. screens of thousands of transformants) that can cover many hectares, and may be required for ecologically relevant, large-scale gene tagging experiments. We obtained digitized IR images and extracted reflectance data in 7×7, 14×14 and 21×21 pixel windows centered on each individual tree in a field trial of 627 activation-tagged lines (Fig. 3). The transgenic lines were planted in pairs, replicated twice in a randomized design. Local indicators for spatial statistics were used to identify unusual lines and account for local patterns of spatial autocorrelation induced by heterogeneous field conditions (Anselin, 1995; Wilhelm & Steck, 1998). Lines that fell in the extreme tails of the distributions were classified as putative mutants and compared with mutant phenotypes identified by direct visual observation and measurement on the ground. Preliminary data indicate that more than 90% of mutants that were previously identified by field phenotyping could be identified by using spectral data from aerial photography (L. Ganio, 2004, unpublished).

Developmental vs eco-devo gene function

Although genetic and biochemical approaches have identified many genes controlling plant development, little is know about their ecophysiological functions in adaptation. Kessler *et al.* (2004) studied the effect of a mutation in jasmonic acid (JA) signaling, critical for herbivore defense, in a relative of cultivated tobacco that was grown in a wild environment. They found complex interactions not only on direct host–herbivore relations, but on higher-order trophic levels. Recently, we initiated studies in poplar to determine the roles played by various genes involved with gibberellic acid metabolic and signaling pathways in adaptive responses. As part of this work, we initiated transgenic studies to test the effects of different

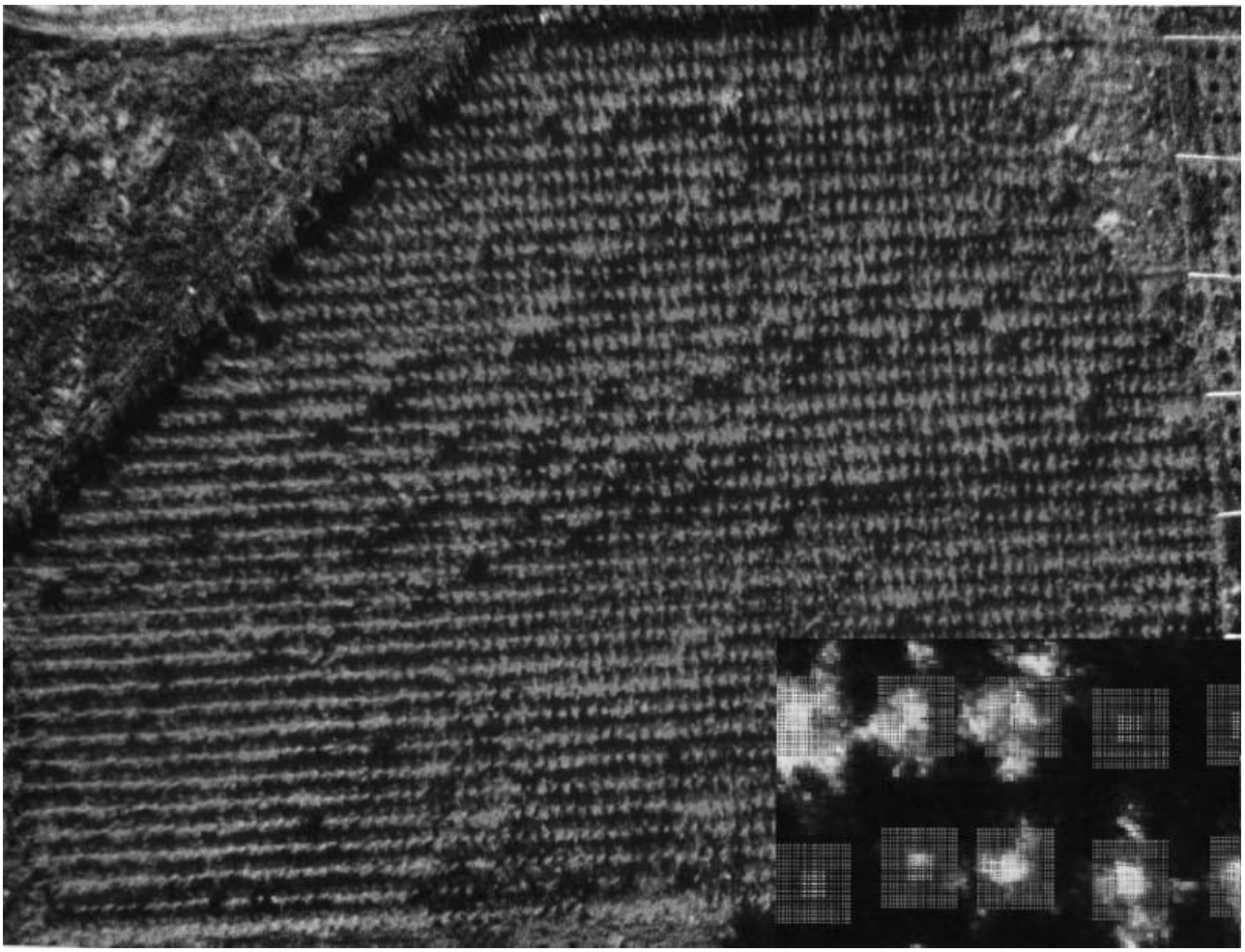

Fig. 3 Infrared image of the field plantation taken in October 2003. Inset indicates 7 × 7, 14 × 14 and 21 × 21 pixel windows centered on individual trees in the field test. These windows were used for digitizing the spectral data.

DELLA domain proteins on the regulation of dormancy, crown architecture, and elongation. Genes encoding DELLA proteins have been identified in many plant species, including *Arabidopsis* (Peng *et al.*, 1997; Silverstone *et al.*, 1997; Lee *et al.*, 2002; Wen & Chang, 2002), rice (Ikeda *et al.*, 2001), wheat (Peng *et al.*, 1999), maize (Peng *et al.*, 1999) and grape (Boss & Thomas, 2002). Gain-of-function mutations in *GA INSENSITIVE* (*GAI*), *REPRESSOR OF GA1* (*RGA*) and *RGA-LIKE1* (*RGL1*) cause a semidominant dwarf phenotype, while loss-of-function mutations are recessive and result in increased growth. *GAI*, *RGA* and *RGL1* share high sequence similarity with the other members of the GRAS gene family (Pysh *et al.*, 1999), but contain a DELLA domain that is absent in the other family members. This domain appears to be very important in mediating GA responses, and complete deletion or nonsynonymous substitutions in this domain cause a dominant gain-of-function mutation, resulting in constitutive inhibition of one or several GA responses (Peng *et al.*, 1997). Although these mutations have been well-characterized with respect to several developmental pathways, including height growth, flower development, and aleurone development, their role in adaptive responses in native environments are largely unknown. Our preliminary results from field studies containing transgenic poplars that overexpress various mutant and wild-type DELLA genes, suggest that they play important roles in control of ecophysiological traits, including timing of bud flush and leaf senescence, crown and root architecture, and secondary metabolism.

Transgenic 'allelic' series

Allelic series are sequence polymorphisms in a gene that generate a variety of phenotypic responses through changes in either protein structure or the expression level/pattern of the gene. Recent work suggests that variation in gene

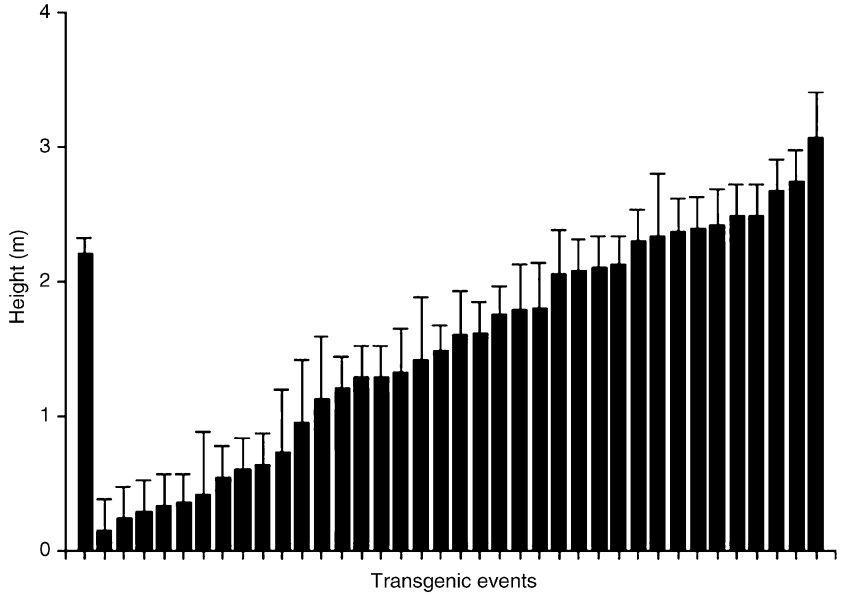

Fig. 4 Illustration of a transgene-imparted 'allelic series' in poplar. Variation in height among transgenic events in a *Populus tremula* × *Populus alba* clone that contains a 35S::*gai* transgene. Means and standard errors (bars) are based on heights of four trees, pairs of which were grown in two random locations for two years in western Oregon, USA. All trees were verified as transgenic based on polymerase chain reaction and a subset of extreme types was shown to vary in transgene expression level in proportion to the severity of stunting.

expression may be responsible for a great deal of natural morphological and physiological diversity (Mackay, 2001). For example, fruit size in tomato is regulated by the *fw2.2* locus, the product of which is involved in regulation of cell cycle (Frary *et al.*, 2000; Nesbitt & Tanksley, 2001). Associations of sequence polymorphism in the 5′ noncoding region and expression levels, not structural changes in the protein, controlled fruit size (Liu *et al.*, 2003). Therefore, generating allelic series that impart different expression levels could be useful for dissecting the genetic basis of quantitative trait variation.

Such series are difficult to identify in nature, requiring very intensive sampling, and are also difficult to obtain via artificial mutagenesis. A phenomenon in transgenic research known as 'position effect' provides a means for generating an 'expression allelic series' with relative ease. Position effects are associated with differential expression of transgenes inserted at various locations in the genome. A wide range of transgene expression levels and patterns can be obtained when a number of independent events are produced. In a study evaluating the effect of a dominant mutation in DELLA protein on tree growth and development (described above), we found an almost continuous gradient of tree stature (main phenotypic effect of this gene) across a large number of independent transformation events (Fig. 4). Transgenic expression allelic series can also be achieved by deliberately introducing multiple copies of a native gene (Liu *et al.*, 2003). This approach will generally be preferred to the commonly used knock-ins or knock-outs (i.e. over- or under-expression) for understanding how natural variations in expression level influence adaptive traits. Other variants of this method include the use of alternative alleles for transformation and insertion of native alleles with added enhancer elements upstream.

Future challenges

Development of sterile genotype to enable long-term studies

Concerns over transgene escape into wild populations are especially great for trees because they generally are not domesticated and have a propensity for long-distance movement of pollen and, sometimes, seed. Reproductively sterile genotypes might alleviate these concerns and facilitate regulatory approval for long-term field studies (Strauss *et al.*, 2004). The wealth of information about genes and regulatory pathways that control flowering in *Arabidopsis* (Boss *et al.* 2004; Wellmer *et al.*, 2004), and proven transgenic methods for inducing sterility in annual plants (Skinner *et al.*, 2003), indicate that reliably sterile poplars and other trees can be produced. However, validation that a sterility transgene is effective and durable in trees is slowed by their long juvenile periods (3–6 yr for poplars in the Pacific north-west) and the difficulties of measuring sterility in large, flowering trees. Because validation is a long-term project, our approach has been to develop multiple mechanisms for inducing sterility and field-test them in parallel. In addition, we are now using an early flowering poplar genotype (Meilan *et al.*, 2004) to speed assessment of sterility transgenes.

Methods for engineering sterility include: (1) ablation of reproductive tissues, (2) suppression of genes necessary for fertility and (3) prevention of flowering onset (Meilan *et al.*, 2001). Although our first field test of poplars with sterility transgenes revealed that stable sterility systems can be developed for trees via diverse means, this study also demonstrated the obstacles to achieving this goal. For example, a transgene consisting of a tobacco tapetal-specific promoter, TA29,

driving expression of the cytotoxin barnase (Mariani *et al.*, 1990) is effective in inducing male sterility in poplar (unpublished). However, this and other floral ablation transgenes negatively impacted growth in the field within a few years, which was not apparent under glasshouse conditions (Meilan *et al.*, 2001). An alternative method for engineering sterility is to target specific mRNAs for degradation via antisense or RNAi transgenes (Waterhouse & Helliwell, 2003). An antisense-*PTLF* (poplar *LEAFY*) transgene (Rottmann *et al.*, 2000) induced mutant floral phenotypes and highly reduced fertility in male poplars, but was ineffective in female trees (unpublished). With insights gained from this first study and advances in *Arabidopsis* research on the genetic control of flowering, we are currently evaluating more than 30 sterility transgenes that act via different mechanisms, including RNAi constructs that target multiple genes for suppression.

Development of transformation protocols for species of ecological importance

Few nonmodel, ecologically significant species have been used in ecological research (Taylor, 2002). One exception is the genus *Solanum*. A set of molecular tools, including *Agrobacterium*-mediated transformation systems, have been assembled to study pathogen–host interactions and community dynamics (Schmidt *et al.*, 2004). However, apart from studies of tobacco in wild environments (Kessler *et al.*, 2004), transformation has been used for relatively few taxa, other than poplar, to inform population genetics studies.

A number of species and genotypes of poplar, can be transformed at experimentally tractable frequencies (Fladung *et al.*, 1997). However, most are pure species and hybrids in the section *Populus*; the genotypes in other sections, though transformable (Confalonieri *et al.*, 1994; Jafari *et al.*, 1995), are considerably more recalcitrant. To date, only a limited number of genotypes in sections *Tacamahaca* and *Aigeros* have been successfully transformed (DeBlock, 1990; Huang, 1990; Confalonieri *et al.*, 1994; Wang *et al.*, 1996; Heuchelin *et al.*, 1997). Most notably the *Populus trichocarpa* genotype that was recently sequenced, Nisqually 1, which is in the section *Tacamahaca*, has been transformed, but at low frequency (Ma *et al.*, 2004). Further expansion of high-efficiency transformation in diverse species and genotypes in poplar and other taxa would allow use of transformation to address a wider range of traits controlling adaptive variation.

The array of transformation-associated and other tools available for poplar is rapidly expanding (Taylor, 2002). Inducible and tissue-specific expression are being developed, which will allow more precise manipulation of transgene expression for dissecting gene function. For example, there is an alcohol-inducible system that is controlled by elements of *alc* regulon of *Aspergillus nidulans* (Kulmburg *et al.*, 1992; Flipphi *et al.*, 2001). The *alc* system has been modified in various ways for use with plants (Caddick *et al.*, 1998; Roslan *et al.*, 2001; Chen *et al.*, 2003; Deveaux *et al.*, 2003). Padidam (2003) recently reviewed chemically induced gene expression systems used for plants. We have found the alcohol-inducible system to be effective for temporal regulation of reporter gene expression in poplar (Fig. 5). Ethanol induction does not appear to be toxic, and is effective when applied to living plants in pots or soil.

Integration with genomics approaches

Combining transformation-based approaches with transcriptome studies will facilitate our understanding of the regulatory networks associated with adaptive traits. For example, microarray expression analysis of mutants has been used to dissect developmental phase change, light and hormone signaling and legume-rhizobia symbiosis (Cluis *et al.*, 2004; Mitra *et al.*, 2004; Peragine *et al.*, 2004). In addition, microarray studies of environmentally induced changes in gene expression can provide similar insights into genetic networks. The position of genes within regulatory pathways, and the points of crosstalk between pathways, can help to identify genes for which variation may have adaptive significance. Changes in the activity of one gene can affect single or multiple pathways to varying degrees, and network structures might influence evolutionary dynamics (Cork & Purugganan, 2004).

Transformation-imparted perturbations of genes identified in microarray studies will be important to rigorously test hypotheses about their functionality. Inducible expression systems will be especially valuable for these studies because, unlike constitutive overexpression, they may allow identification of direct downstream targets of particular genes. Combining inducible systems with tissue-specific promoters provides an additional way to produce mutants with fewer pleiotropic effects, thus allowing more precise inferences about regulatory interactions. If naturally occurring alleles of adaptive significance can be identified by QTL or association studies, transformation can also provide a means to 'reproduce' the natural allele or a functionally similar version of it. This would allow comparisons between otherwise identical genetic backgrounds to reveal transcriptome changes induced only by the gene of interest, rather than comparing genotypes that contain different alleles at many loci.

Finally, genes identified via forward genetic screens, as well as those identified via microarray and/or transgenic studies, need to be subject to association tests to determine if genetic polymorphism in the pathways their products regulate is relevant to adaptation in wild populations. Although many genes affect adaptive traits, only a small subset may be useful in nature because of antagonistic pleiotropy or other factors that are difficult to observe in laboratory or short-term, single-environment, ecological studies. Ultimately, transcriptome analysis, together with proteomics and metabolic profiling, are likely to provide a flood of candidate genes and pathways.

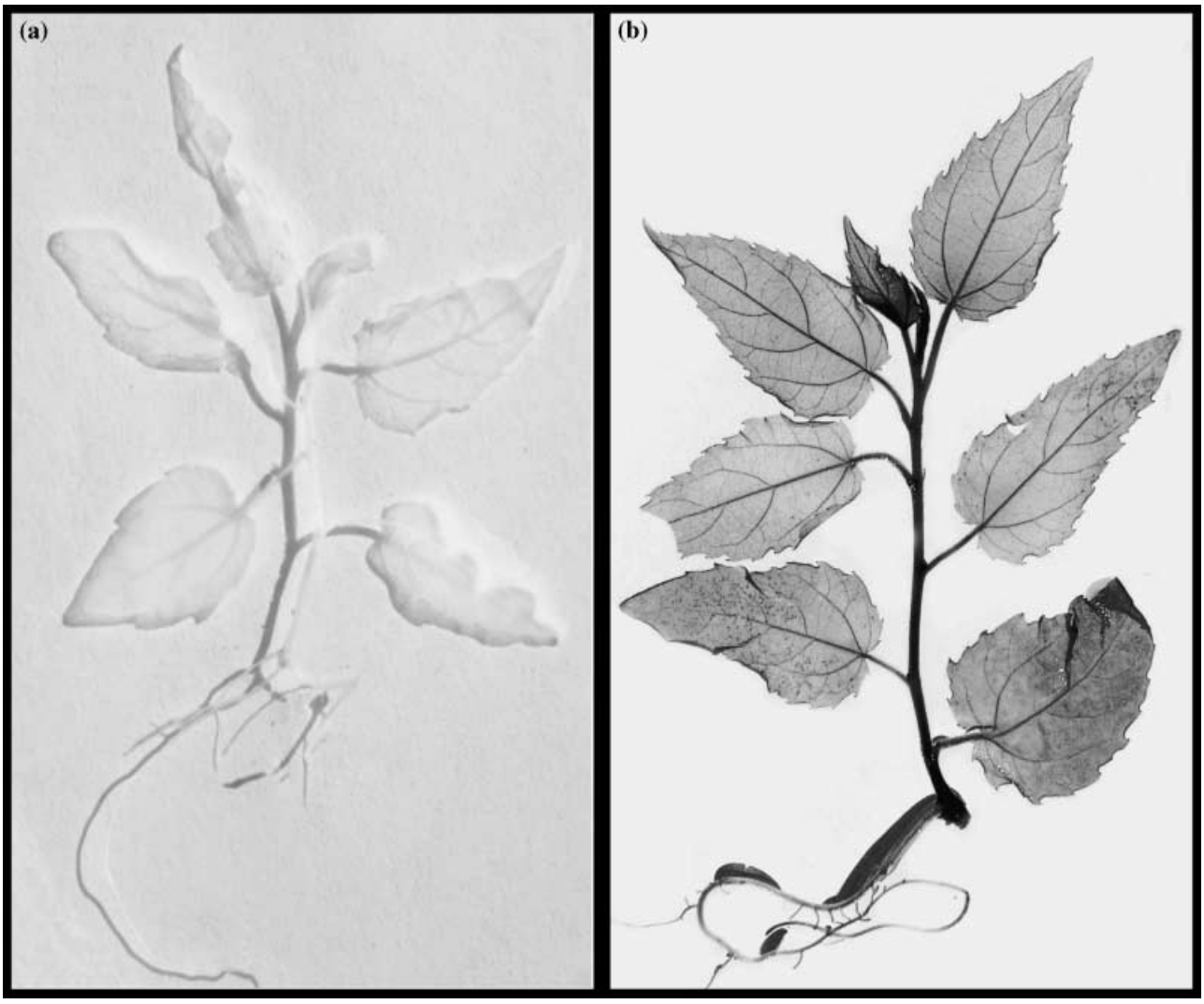

Fig. 5 Alcohol-inducible system in poplar. Induction of the β-glucuronidase (GUS) reporter gene was driven by a promoter from the *alc* regulon of *Aspergillus nidulans*. Transgenic poplar plants were treated in Magenta boxes with 4 ml of 2% ethanol using a combination of vapor and root drench methods. Both untreated (a) and treated plants (b) were stained for GUS activity 5 d after the start of alcohol induction. The histochemical staining of induced plants showed that the GUS activation occurred in leaves, roots and, to the lesser extent, in stems. Expression of GUS was also observed in vascular bundles, root hairs and leaf trichomes (data not shown).

Population genetics and transformation tests of wild alleles will act as filters to identify the subset of genes that are most likely to be of adaptive significance.

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