Tree Genetics & Genomes (2005) 1: 135–142
DOI 10.1007/s11295-005-0019-8

OPINION PAPER

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Insertional mutagenesis in *Populus*: relevance and feasibility

Received: 19 July 2005 / Revised: 20 September 2005 / Accepted: 21 September 2005 / Published online: 10 November 2005

Abstract The recent sequencing of the first tree genome, that of the black cottonwood (*Populus trichocarpa*), opens a new chapter in tree functional genomics. While the completion of the genome is a milestone, mobilizing this significant resource for better understanding the growth and development of woody perennials will be an even greater undertaking in the years to come. In other model organisms, a critical tool for high-throughput analysis of gene function has been the generation of large mutagenized populations. Some mutagenesis technologies and approaches cannot be applied to trees because of their typically outcrossing breeding systems, high heterozygosity, large body size, and delayed flowering. In contrast, gene-tagging approaches that use insertional mutagenesis to create dominant phenotypes are ideally suited for trees and, especially, *Populus*. Both activation tagging and enhancer trap programs have been successful in identifying new genes important to tree development. The generation of genome-wide insertional mutant populations, which provide direct functional links between genes and phenotypes, should help to integrate *in silico* analyses of gene and protein expression, association studies of natural genetic polymorphism, and phenotypic analyses of adaptation and development.

Keywords Functional genomics · Woody perennial development · Gene tagging · Trees

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Introduction

There have been extraordinary developments in the molecular genetics and genomics of trees in recent years These advances were most striking in *Populus* species and hybrids[1], a result of a broad international consensus regarding its value as a scientific and technological model for woody perennial plants (Wullschleger et al. 2002; Brunner et al. 2004). International efforts have established large expressed sequenced tag (EST) databases, high-density genetic maps, microarrays, and efficient transformation systems (reviewed in Boerjan 2005). The growth of genomic resources in *Populus* culminated in the recent sequencing and annotation of the first tree genome (*Populus trichocarpa*; http://genome.jgi-psf.org/Poptr1/Poptr1.home.html). This creates an unprecedented opportunity to understand development of woody perennial plants and thus the evolution of all modern angiosperms (Groover 2005).

A powerful tool for functional genomic analysis of model organisms has been the creation of genome-wide mutagenized populations (Alonso et al. 2003). Except for a few pilot studies, such populations are not available in *Populus* or any other tree species. The large physical size, reproductive biology, and long generation cycles of many forest trees make generation, maintenance, and distribution of mutant germplasm a difficult task. Here, we summarize the status, constraints, and prospects for insertional mutagenesis to become a significant tool in poplar functional genomics.

[1] *Populus* species and hybrids are referred to in this paper as poplars, and include aspen and cottonwoods.

Insertional mutagenesis in model plants

Insertional mutagenesis using transposons or T-DNA is an extremely valuable research tool for model plant systems (Martienssen 1998; Krysan et al. 1999; Raizada et al. 2001; Jeong et al. 2002). Disrupted genes are molecularly "tagged," meaning that the known sequence of the insertion element can be used to rapidly clone the flanking genomic sequence associated with the tagged gene. In addition, a gene encoding a selectable marker (e.g., antibiotic or herbicide resistance) is typically included in the insertion element and can also be used in cloning strategies or to follow inheritance of the insertion element. This greatly facilitates the cloning of genes and circumvents laborious map-based cloning typically associated with chemically induced mutations. Newly developed TILLING technology has made identification of chemically induced gene mutations much easier but, to date, has been largely limited to model plants and reverse genetics approaches that require the production of homozygous mutants (McCallum et al. 2000). In addition to facile gene cloning, insertional mutagenesis provides opportunities for generation of different types of mutations (e.g., activation tagging and gene/enhancer trapping) where the mutagen can enhance the gene function or provide clues about gene expression patterns at a tissue/cellular level (further discussed below). Such mutations have proven essential in the dissection of complex gene families, where functional redundancy impedes the expression of phenotypes from single gene knockouts (Nakazawa et al. 2003). Discovery of efficient transformation methods in both *Arabidopsis* and rice has greatly contributed to the growth of insertional mutagenesis techniques (Bechtold and Pelletier 1998; Sallaud et al. 2003). Because, for many of these techniques, the only prerequisite is the availability of one transformable genotype, insertional mutagenesis has been growing in popularity in other species of plants as well (Zubko et al. 2002; Furini et al. 1997; Mathews et al. 2003; Busov et al. 2003).

T-DNA tagging for insertional mutagenesis in *Populus*

In addition to excellent genomic resources, a number of poplar genotypes have the unusual ability among trees that large numbers of transgenic trees can be regenerated at low cost (Busov et al. 2005). This feature has spurred interest in applying insertional mutagenesis in a manner similar to that used in *Arabidopsis* (Groover et al. 2004; Busov et al. 2003; Fladung et al. 2004). However, beyond transformation, trees have many important differences from model annual plants that require careful consideration when considering insertional mutagenesis programs.

Activation tagging

Most knockout mutations produced by either ethyl-methane sulfonate (EMS)-generated base substitutions or T-DNA insertions result in recessive mutations. This means that mutant phenotypes can be assayed only in plants homozygous for the mutation. For most forest trees, the long generation times and limited degree of self-fertility make such types of mutagenesis unworkable. For poplars, which are predominantly dioecious, producing insert homozygosity by inbreeding would require at least two generations of sibling matings.

Activation tagging is an insertional mutagenesis approach that circumvents this problem by producing dominant and semidominant mutations that can be identified in T1 (primary) transformants. Activation tagging uses a T-DNA vector that contains strong enhancers positioned near the left or right borders (Hayashi et al. 1992; Weigel et al. 2000). The insertion of the vector in a genome location near a gene can cause up-regulation of that gene, resulting in a gain-of-function, dominant mutation (Hayashi et al. 1992). The resulting dominant phenotypes can be screened in primary transformants, obviating the need for sexual reproduction. Generally, only one to a few percent of transgenic events produce a discernible phenotype, requiring that large populations are produced and screened (Weigel et al. 2000). The position of the vector is established by recovering a genomic sequence flanking the insertion sites by using either thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR; Liu et al. 1995) or plasmid rescue techniques (Weigel et al. 2000). Examples of genes cloned in *Arabidopsis* via activation tagging include the histidine kinase gene, whose overexpression bypasses the need for cytokinin in shoot regeneration (Kakimoto 1996); the flowering time gene *FT*, whose overexpression causes early flowering in *Arabidopsis* (Kardailsky et al. 1999), and *SOC1*, whose overexpression suppressed the late flowering phenotype of plants with functional *FRI* and *FLC* alleles (Lee et al. 2000). Because of the utility of this method, a special activation-tagged, sequenced-indexed stock center was created for *Arabidopsis* (Ichikawa et al. 2003).

Activation tagging is most efficient in species where full genome sequence is available. Because the activation tag usually acts by insertion into the promoter, intron, or other noncoding region near to or within genes, for which there is no sequence information from EST projects, isolation of the activated gene requires laborious genome walking, making characterization of large mutagenized populations impractical. Sequencing of the *Populus* genome has fully enabled activation tagging as a means for functional gene discovery on genome-wide scale.

In *Populus*, to the best of our knowledge, two activation tagging populations have been created. One activation tagging population of 627 lines was generated and planted in a field trial in western Oregon, USA. The first gene tagged via insertional mutagenesis in any tree was reported from this pilot population (Busov et al. 2003), demonstrating the feasibility of this approach in *Populus*. This population was used to uncover many more mutant phenotypes, which were identified only after 2 years of field testing, with the identified trait abnormalities related to woody perennial growth and with no corresponding

phenotypes in annual systems (Busov et al. 2005). Generation and analysis of additional *Populus* activation-tagged lines is in progress (Busov and Strauss, unpublished data). Recently, a population of more than 2,000 new transformants, using the same genotype and vector, has been generated at Queens University, Canada (Regan, personal communication).

Because it is generally not practical to observe co-segregation of the tagged gene and phenotype in sexual progeny from activation-tagged trees, recapitulation of the phenotype via retransformation is required to confirm gene identification. In addition, because there have not yet been careful analyses of somaclonal variation carried out in association with the generation of activation-tagged populations (i.e., using the same transformation method and an enhancer-less isogenic T-DNA), it is not yet possible to specify the final rate of gene-tagging efficiency. Large-scale studies of tagged and control populations, together with recapitulation of phenotypes, are needed to fully evaluate the value of activation tagging for gene discovery in poplar.

Enhancer and gene traps

While mutant phenotypes resulting from loss of function or misexpression provide a great deal of information about gene function, most plant genes do not result in an obvious phenotype under standard laboratory- or greenhouse-based phenotypic screens (Martienssen and Irish 1999). Genetic redundancy inherent to many aspects of developmental networks can mask the effect of loss of function mutations. This is expected to be a larger obstacle for species with large and complex functional genomes, such as may exist in conifers because of high levels of gene duplication (Kinlaw and Neale 1997). Physiological compensation is most likely to mask loss of function phenotypes in short-term studies, especially when screening in single environments. In addition, many genes are expressed only during specific points in development or in response to specific environmental factors and may not condition a phenotype under a single screening regime.

An alternative insertion-based strategy is based on screening gene expression, rather than mutant phenotypes (Springer 2000). Enhancer trapping makes use of a recombinant DNA vector carrying a reporter gene. 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 green fluorescent protein (GFP) and luciferase have also been employed (Alvarado et al. 2004; Yamamoto et al. 2003). 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. The enhancer trap vector is inserted into the genome at random locations, and if it lands in or near a gene, the reporter is expressed in a pattern that reflects the normal expression pattern of the tagged gene. The endogenous gene driving expression of the reporter gene is molecularly "tagged" by the enhancer trap insertion and can be identified by PCR amplification and sequencing of chromosomal DNA flanking the insertion site. Because any expressed gene can be detected through gene or enhancer trap insertion, the percentage of genes that can be identified through expression screens is generally much larger than that by screens based on production of developmental or metabolic phenotypes, as is done in activation tagging.

In practice, large numbers of plants, each carrying a unique enhancer trap insertion somewhere in the genome, are generated and screened for reporter gene expression patterns. Screening strategies can be devised to identify genes expressed in specific tissues or cells (e.g., Groover et al. 2004), genes expressed at specific points in development (e.g., Springer et al. 1995), genes expressed in response to experimental treatments (e.g., Alvarado et al. 2004), or genes encoding proteins targeted to specific subcellular locations (e.g., Groover et al. 2003). Many variations of enhancer trapping have been employed in species ranging from prokaryotes to plants and animals. A common variation is "gene trapping," in which a vector carrying a reporter gene must be inserted into the transcribed portion of a gene to be expressed (Springer 2000). Recently, gene and enhancer trapping has been used to identify *Populus* genes involved in vascular development and wood formation (Groover et al. 2004). New populations of *Populus* gene and enhancer traps are currently being established and are available for screening (http://www.fs.fed.us/psw/programs/ifg/genetraps.shtml).

Transposon-based systems for insertional mutagenesis in *Populus*

The use of naturally occurring transposons for plant gene tagging has its roots in the early days of maize genetics. The use of engineered, heterologous transposon systems for insertional mutagenesis was first introduced in *Arabidopsis* (Springer et al. 1995; Parinov et al. 1999; Marsch-Martinez et al. 2002; Raina et al. 2002) and subsequently in rice (Wang et al. 2004; Sallaud et al. 2003). These studies, which mainly exploited the heterologous *Ds* transposon system from maize, led to the identification of many new and uncharacterized genes (Kubo et al. 1999; Dodds et al. 2001; Borner et al. 2000). Transposons provide several advantages compared to the T-DNA as insertional mutagen. First, the transposon-based system allows convenient reversion from mutant to wild type by remobilization of the transposable element (Nishal et al. 2005). This circumvents the need for complementation or recapitulation of the mutant phenotype required in T-DNA tagging systems. Second, transposons preferentially integrate into intragenic regions, whereas T-DNA, while preferentially inserting into gene-rich regions of the genome, shows preference for intergenic, usually promoter or other flanking regions (Pan et al. 2005; Alonso et al. 2003; Schneeberger et al. 2005). Third, *Ds* transposons tend to transpose to linked loci and can be remobilized to

provide a system for local genome coverage in species where transformation is problematic (Nishal et al. 2005).

Transfer, excision, and reintegration of the maize transposon, *Ac*, have been reported in *Populus* (Fladung et al. 2004; Fladung and Ahuja 1997; Fladung and Ahuja 1997; Fladung et al. 1997; Howe et al. 1994; Howe et al. 1991; Fig. 1). Preferential reintegration of the element in the aspen-*Populus* genome near coding regions led to the suggestion that transposon mutagenesis could be an important tool to discover gene functions in trees using reverse genetics strategies (Kumar and Fladung 2003). A loss of function strategy using the autonomous maize *Ac* element was tested in haploid poplar (Deutsch et al. 2004; Fladung et al. 2004). However, in addition to the difficult handling of haploid tree material (slow growth, spontaneous diploidization), the inability to control transposon activity limited the use of an autonomous transposon. Inducible transposon systems combined with dominant mutagens, like activation tagging, can overcome these difficulties (Nishal et al. 2005) and is being tested in *Populus* (Strauss and Fladung, personal communication).

Novel modifications of "model" trees

Some of the same unique features that make forest trees interesting for biological study also make them unwieldy study subjects. For some studies, it could be advantageous to utilize trees that have been modified to allow researchers to grow more trees in a given space, reach sexual maturity at a younger age, or allow loss-of-function mutations to be uncovered in haploid lines. All of these modifications have been explored for *Populus*.

Controlling tree size

Poplar trees grow fast, reaching up to 5 m in a single year, exceeding the greenhouse and space limitations of most research facilities, and rapidly exceeding field site capacities for many research programs (e.g., Fig. 2). Several reports have demonstrated control of tree size in *Populus* via different means (Busov et al. 2003; Olsen and Junttila 2002). For example, overexpression of gibberellin (GA) 2-oxidase (the main plant GA-catabolizing enzyme) generates GA deficiency, resulting in reduced stature (Busov et al. 2003). The main substrates for GA 2-oxidase are GA_1 and GA_4, but the enzyme cannot catabolize GA_3 (another bioactive form of GA; Sakamoto et al. 2001). Trees with genetic manipulations of this enzyme survive well in the field, retain their phenotype over multiple (at least 4) years, and do not display acute abnormalities in wood formation (e.g., produce xylem, phloem, and have nearly normal lignification; Busov, unpublished data). Their size varies widely among transformation events; trees that are fourfold less in all dimensions and thus occupy much less space than their wild-type relatives can be easily recovered (Fig. 2). However, strong constitutive expression of this gene causes numerous pleiotropic effects. Lower levels of transgene expression using tissue-specific or weaker promoters should enable trees to be produced that have fewer abnormalities and more limited dwarfism. Alternatively, natural dwarf genotypes with normal wood formation have been found and could be used in functional genomic endeavors requiring large populations (Fladung, unpublished data; Fig. 3). It remains to be established whether such dwarf genotypes would represent suitable models with respect to all criteria for a mutagenesis program, including rapid transformation and regeneration of transgenic plants. However, by spraying plants or supplementing the growth media of GA 2-oxidase-overexpressing dwarfs with GA_3, wild-type growth can be temporarily restored (Busov et al. 2003). This should allow their rapid transformation, regeneration, and development to the needed size for greenhouse or field studies.

Fig. 1 Leaf from 35S-*Ac-rolC* transgenic aspen. Transposition of *Ac* is indicated by the pale green leaf area at *top*, which is caused by restoration of *rolC* activity. For details, see Fladung et al. (1997)

Early-flowering trees

Acceleration of generation cycles via precocious flowering has been explored in poplar and other tree species (Meilan et al. 2001). The main avenues investigated have been various environmental and chemical treatments, selection of early-flowering genotypes, and transgenic manipulation of flowering time. Environmentally and chemically induced precocious flowering has shown various levels of success in different species, but has been largely unsuccessful in poplar (Meilan 1997). The only naturally occurring early-flowering tree studied for this purpose, a female *Populus alba* genotype, can produce flowers in approximately 1 to 2 years (Meilan et al. 2004), though flowering efficiency can vary widely from year to year (Ma and Strauss, unpublished data). Early-flowering poplar genotypes were also generated using transgenic manipulations of poplar flowering time genes, such as *LEAFY* orthologs (Rottmann et al. 2000; Nilsson et al. 1998). However, *LEAFY* is ineffective in many genotypes, the flowers produced are highly abnormal, and fertile pollen or stigmas have not been recovered (Rottmann et al. 2000). The use of early-flowering genotypes for insertional mu-

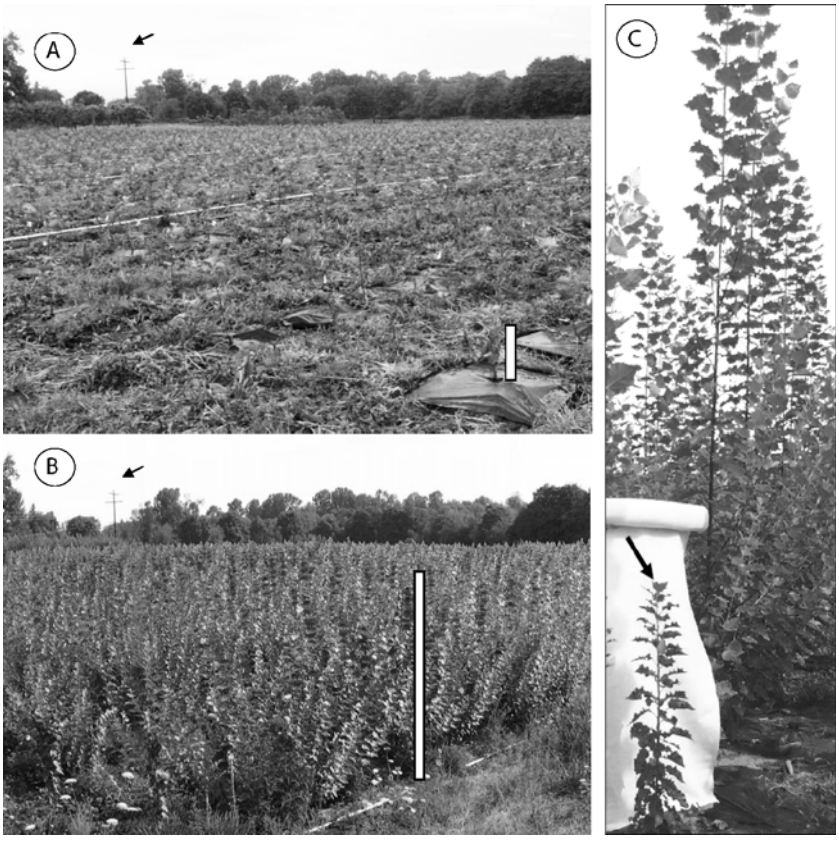

Fig. 2 Rapid growth of activation tagging field trial in USA. *Arrows* in **a** and **b** point to a common landmark. *Bars* in **a** and **b** indicate the approximate size of the trees when pictures were taken. **a** Plantation at the beginning of the growing season (bar size ~0.3 m). **b** Plantation at the middle of the same growing season as show in **a** (bar size ~3 m). **c** Comparison of wild type-like tree (~4 m) to the right with the activation mutant *stumpy* to the left (~0.7 m) after 2 years of growth in the field. *Arrow* points to the mutant plant

tagenesis is also limited because poplar is dioecious, intolerant to inbreeding, and the transformability of most poplars, especially those outside the aspen group (section Leuce), is insufficient for large scale, genome-wide efforts.

Fig. 3 Dwarf genotype in 4-year-old *Populus alba* × *Populus tremula* hybrid (~0.8 m)

Haploid trees for detection of recessive mutations

The use of haploids can facilitate the detection of mutations, most of which are recessive and difficult to detect (Bajaj 1983). The natural occurrence of haploid plants in the kingdom of vascular angiosperms was first recorded for *Datura stramonium* (Blakeslee et al. 1922). Artificial generation of haploid plants following embryogenesis from anther culture was also first described in *Datura* (Guha and Maheshwari 1964), but most of the early progress with this technique was made in *Solanaceous* species (Hu and Zeng 1984). In poplar, the first and only naturally derived haploid plants were described by Tralau (1957).

In woody angiosperms and gymnosperms, different approaches have been followed to produce haploids (Baldursson and Ahuja 1996). Anther culture was the most commonly used method to produce haploid plants for *Populus* species and their hybrids (Ho and Raj 1985; Mofidabadi et al. 1995). Methods included cross-pollination with heat-treated pollen (Kopecky 1960; Winton and Einspahr 1968), irradiated pollen (Stettler and Bawa 1971), or pollen treated with toluidine blue (Illies 1974). Haploid poplar lines produced via microspore culture (Deutsch et al. 2004; Fig. 4) exhibit very low viability, when compared to their doubled haploid tissues (Fig. 5). Early spontaneous chromosome doubling and only a few surviving specimens are on record (Deutsch et al. 2004). Unfortunately, with present technology, the low viability and frequent spontaneous

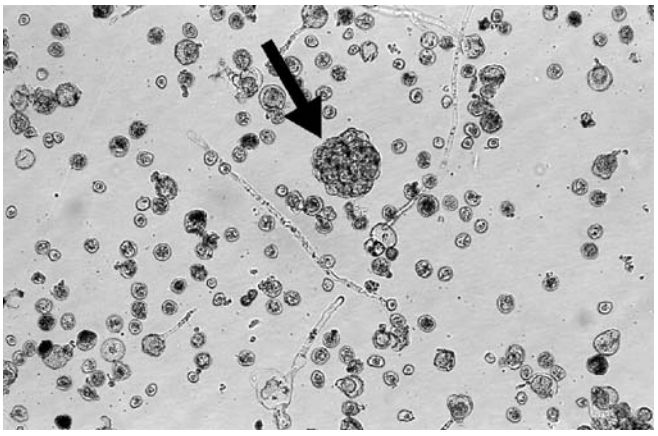

Fig. 4 Cultivated *Populus* microspores. *Arrow* points to a microcallus. For details, see Deutsch et al. (2004)

reversion to the diploid state severely limit the use of haploid plants in large-scale functional genomics efforts in poplar.

Populus stock centers

A common feature of large-scale functional genomics programs is the existence of stock centers that contain propagules harboring mutations in most genes of the genome. This model was first developed in *Arabidopsis*, where stocks of mutagenized seeds can be stored in a small area, and then disseminated to the research community upon request at low cost. Genome-wide T-DNA insertions for approximately 70% of *Arabidopsis* genes are now available and can be ordered over the phone or worldwide web (Alonso et al. 2003). This model cannot be directly applied for poplar because obtaining seeds is impractical due to the time period required (5–10 years) for seed production. In addition, poplar seeds rapidly lose viability (precluding long-term storage), seeds from heterozygous trees are highly variable in their properties due to genetic segregation, and obtaining seeds from genetically modified trees under field conditions is problematic as many countries have regulations that make it difficult, or very costly to allow transgenic trees to flower. Therefore, the most feasible model for storage and dissemination of *Populus* genetic material is via vegetative propagules. These vegetative propagules can be stored reliably by three methods:

- *Clonal archiving under field conditions.* This model has already been applied for commercial clonal propagation of elite ornamental and fiber-producing cultivars of poplar. It is relatively easy and safe with respect to somaclonal-induced variability. However, this method requires large amounts of land, annual maintenance, and long-term funding commitments for the costly annual management operations such as frequent pruning to prevent flowering and dissemination. It is also problematic due to regulatory restrictions and risk of vandalism in a number of countries. Finally, in most countries, there are serious quarantine restrictions on international shipment of both sterile and nonsterile vegetative propagules due to the risk of spread of tree pests.
- *Cold storage under low temperature and nutrient deficiency.* Although this method does not require field maintenance and is relatively more efficient than continued subculture in normal in vitro programs (Son et al. 1991), it is suitable mainly for short-term storage (e.g., 1 to 3 years).
- *Cryostorage under subfreezing temperatures.* Cryopreservation emerges as the favorite for storage because it is the most space- and cost-efficient and can be applied for indefinite amounts of time (reviewed in Tsai and Hubscher 2004). However, acclimating and cryostoring large multiple ramets from large numbers of transgenic events is costly.

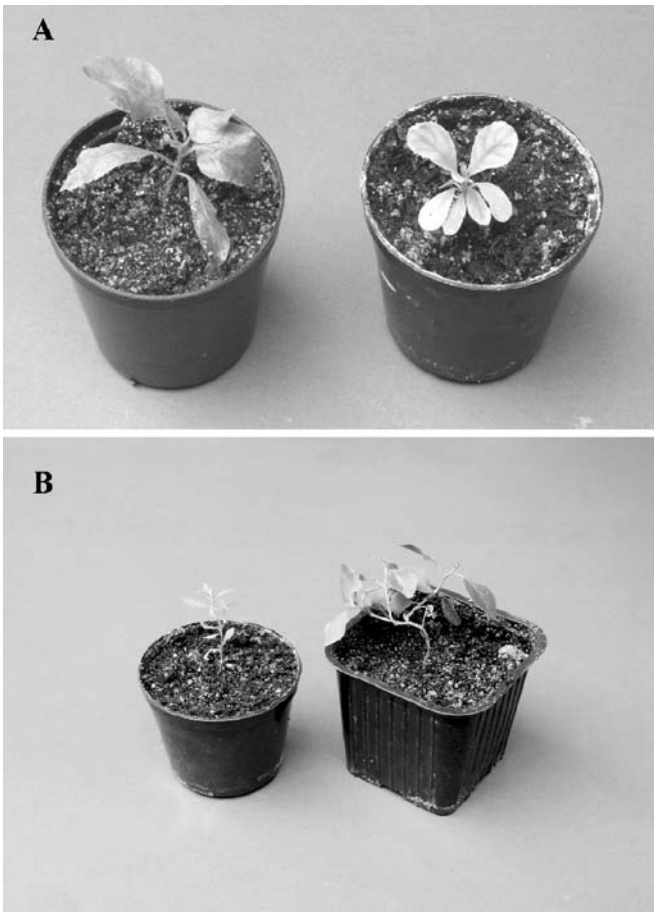

Fig. 5 Decreased viability of haploid poplars. **a** Haploid (*right*) and FD25 double haploid (*left*) lines. **b** Haploid (*left*) and FD83 double haploid (*right*) lines. Haploid plants are slow growing and chlorotic. For details, see Deutsch et al. (2004)

Towards an integrated functional genomics platform in *Populus*

Once genes of interest are identified in a tagging study, they must be further characterized. Silencing via RNAi, or

misexpressing the gene of interest by use of strong promoters, or creating novel dominant alleles based on selective modification of protein structure, can all provide insight into function. It is also possible to identify naturally occurring variant alleles in populations, using association genetic approaches (Neale and Savolainen 2004). The growing genomics and expression databases for many model and crop species makes the placement of a newly discovered gene into a larger context with regard to molecular function and evolutionary history rapid and highly informative.

In pilot studies, the feasibility and power of the forward genetic approaches (activation tagging and gene/enhancer traps) have been demonstrated in *Populus*. However, for insertional mutagenesis to become a significant tool for functional gene discovery, large populations that contain mutations affecting most regulatory genes are needed. Such efforts will require significant investments and long-term commitments to generate the resource and make it available to the research community. Thus, a coordinated international effort, similar to that with *Arabidopsis* and rice, is needed. As discussed elsewhere (e.g., Brunner et al. 2004), this needs to have (1) transformation centers to produce and distribute transgenic plants; (2) multiple laboratories, glasshouses, and field environments capable of screening for different traits and recovering insertion site sequences; (3) stock centers where all mutants—or at least those considered valuable for scientific research—are kept and from which sterile (i.e., pathogen-free) plants can be distributed at cost; and (4) an informatics/computer center where information on all mutants, including their molecular phenotypes (e.g., array-based expression phenotypes), is organized and made available to the international community. A coordinated international effort is needed to move poplar genomics into the functional, postgenomic era.

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