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Forest Tree Transgenesis and Functional Genomics: From Fast Forward to Reverse Genetics

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Summary

Genomics has become an integral part of the forest tree improvement, and gene structural and expressional data are being produced at an unprecedented rate. However, biological resources in the form of tagged mutants are still lacking in forest trees, which at present is a missing part of tree genomics. The potential bottlenecks here are the steps involving plant transformation, which is instrumental both in reverse and forward genetics strategies aimed at to determine gene function. With few exceptions, genetic transformation is an obligatory final step by which traits are engineered into plants. For basic research transgenesis is the method of choice to confirm gene function, after deductions made through comparative genomics, expression profiles, and mutation analysis. The biological features of long-lived tree species create obstacles as well as provide opportunities to design new approaches to overcome the barriers associated with forest tree genomics.

To understand how a cell works we need to know the function of almost every gene in its genome. Genome sequencing provides a tremendous amount of information for the development of global approaches towards this goal, complementing and enhancing the more traditional (single-gene) approaches. Genome sequencing has been completed in model plant *Arabidopsis* (The *Arabidopsis* Genome Initiative, 2000) and in rice (GOFF et al., 2002; YU et al., 2002), a model cereal. Similarly, forest biologists have given enough justification to sequence *Populus* genome as a model for trees and woody perennials (WULLSCHLEGER et al., 2002; TAYLOR, 2002). A 6X coverage of the black cottonwood (*Populus trichocarpa*) genome as first tree genome will be available in public domain by the end of the year 2003 (International *Populus* Genome Consortium). Once whole-genome information is available for an organism, the challenge turns from identifying the parts to understanding their function as well as to improving genome structure, thus ushering in the 'post-genomic' era. In the short term, the first goal is to assign some element of function to each of the genes in an organism also referred to as 'functional genomics', and to do this with high-throughput, systematic approaches.

Major challenges of functional genomics in trees are to assess tree growth and wood yield. These parameters are important in terms of wood quality like strength and fibre length, and renewable energy resources (CHAFFEY et al., 2002; CAMPBELL et al., 2003; CONFALONIERI et al., 2003). With most of the *Populus* genome still to be assigned function, the notion of accumulating this information one gene at a time is hard to contemplate. This knowledge gap has been the crucial impetus for developing 'whole-genome' approaches that can acquire functional information, in the form of expression profiles, protein–protein interactions, computational approaches and the response to loss or gain of function by mutation.

Key words: Forest trees, genetic transformation, genomics, gene tagging, transposon, gene silencing, site-specific recombination.

Functional genomics of forest trees: A missing link?

Forest tree genomics is now undergoing a transition or expansion from the mapping and sequencing of genomes to an

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emphasis on genome function. Functional genomics represents a new phase of genome analysis, which refers to the development and application of global experimental approaches to assess gene function by making use of the information and reagents provided by structural genomics. Depending upon the methods used there are two major routes for the functional analysis of a genome. The first category is the *in vitro* analysis, which is based on the high-through-put analysis of gene products, and includes techniques like expression profiling and proteomics. These methods being technological once developed are applicable across different species and can be adapted from one system to another. *In vivo* analysis is the second approach for functional genomics, which being biological in nature rely on modifying expression of endogenous genes unravelling their function within plant system. Efficient use of *in vitro* technology eventually relies on the interconnection of diverse and accurate expressional profiling of resources developed by *in vivo* method. Transcription profiles gathered together into a reference database or 'compendium' will allow the matching of expression patterns of uncharacterised mutants with known profiles in the database. The utility of the 'compendium' approach has been validated in yeast *Saccharomyces cerevisiae* by examining 300 genome-wide profiles caused by deletions in both characterised and uncharacterised genes, as well as treatments with compounds with known molecular targets (HUGHES et al., 2002). A similar functional genomics approach making multiple conditions including tagged mutants has been used in the model plant *Arabidopsis thaliana* to analyse complex processes or pathways during systemic acquired resistance (SAR) to disease (MALECK et al., 2000). Such a 'compendium' could also result in a comprehensive identification of co-regulated transcript groups, which may lead to function identification of genes based on their regulatory characteristics.

By the time genomics reagents were made available in annual plants the scientists had already developed enormous resources of biological material in the form of tagged mutants. In *Arabidopsis,* it is already feasible to acquire a mutant of every second open reading frame in the genome by using publicly available populations of insertional mutagenesis lines (PARINOV and SUNDARESAN, 2000) and we are reaching to the stage where almost whole genome of *Arabidopsis* is enriched with T-DNA (SESSIONS et al., 2002) or transposon tags (MARSCH-MARTINEZ et al., 2002). Similarly efforts are underway for large scale gene tagging in rice (GRECO et al., 2001), maize (COWPERTHWAITE et al., 2002) and other crop plants (MEISSNER et al., 2000, VAN ENCKEVORT et al., 2001).

A significant progress has been made in large scale functional analysis of number of tree species including conifers, and thousands of ESTs are available from poplar, eucalypts, pine and other tree species (WULLSCHLEGER et al., 2002; STRABALA, 2003; HERTZBERG et al., 2001). Similarly, proteomics has been developed in maritime pine (DUBOS and PLOMION, 2002) and metobolomics has been initiated in poplar (WULLSCHLEGER et al., 2002) for genome expression analysis. While these resources are available for high-throughput functional genomics, *in vivo* resources in form of tagged mutants are still lacking in trees, which is indeed a missing link in tree functional genomics. Only handful of T-DNA tagged mutants are available in *Populus* (KUMAR and FLADUNG, 2003; BUSOV et al., 2003) and such a research program has not yet been initiated in other tree species. BUSOV et al. (2003) have recently demonstrated potential of activation tagging in forest trees overexpressing a dominant gibberellin catabolism gene using T-DNA tagging. However, large-scale efforts are needed to match the amount of data produced by tree genomics. Given the long generation cycles and recalcitrant nature of many tree species both for *in vitro* regeneration and genetic transformation, the present scenario is quite understandable. The potential bottleneck therefore is the lack of transformation technology that can match other fast track functional genomics tools. This clearly raises the need to device alternate methodologies to address problems associated specific to trees.

Forest tree transgenesis: A new perspective

Since the pioneering transformation of a model tree species (FILLATTI et al., 1987) much of research efforts have been devoted to a horizontal spread of the technology making it possible to transform a wide variety of forest tree species (PENA and SEGUIN, 2001). Less emphasis has so far given to advancing the efficiency of the transformation process itself. With the transformation methods currently available, the transgene incorporated into the plant genome is integrated randomly and in unpredictable copy numbers, often in the form of repeats abolishing the expression of transgene (KUMAR and FLADUNG, 2001). To obtain transgenic plants that harbour stably expressing single intact copy of the introduced transgene, the conventional approach has been to screen molecularly for the rare individuals among a large pool of transformants. Such a time-consuming molecular screening might be feasible with fewer trait genes at hand. However, with increasing magnitude of data provided by tree genomics, such screening efforts would be hard to manage.

To overcome the problem of expression variability and gene silencing systematically, the ability to precisely modify or target defined locations within the genome is required. This can be obtained by using site-specific recombination systems (OW, 2002). The targeting strategy relies on site-specific recombinase-catalysed intra-genomic mobilization of the randomly inserted target DNA into the previously characterised recipient locus (*Figure 1*). Although site-specific recombination system has been used in a tree system for the removal of the marker gene (ENDO et al., 2002), it has not yet been tested for the precise insertion of transgenes into tree genome. Successful targeting of a new gene to a defined position should allow one to produce transgenic trees in which the incoming gene can be expressed in a predictable pattern.

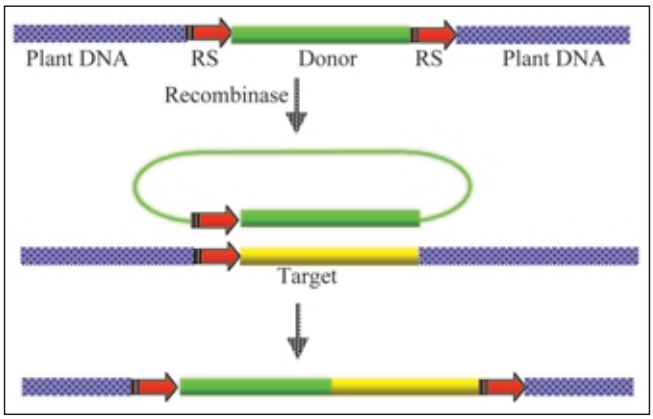

Figure 1. – A site-specific recombination (SSR) system. Recombinase-mediated excision of randomly inserted donor DNA (donor) between directly oriented recognition sites (RS). This leads to an extrachromosomal circle containing a single RS, which can then recombine with the RS of recipient locus (target) that was previously characterised for the stable expression free from position effect variegation. The SSR reaction requires the action of a site-specific recombinase that can be supplied either by stable or transient transformation. Once a genomic target is shown to confer suitable expression, subsequent deliveries of any transgene can be made to that site.

An alternative strategy that needs to be tested in trees is the transposon-mediated repositioning of transgenes (COTSAFTIS et al., 2002). This limits the initial effort required for production of primary transformants in species or cultivars poorly amenable to transformation. A relocated transposon-borne transgene is free from unwanted T-DNA sequences and may be less prone to gene silencing than at the original integration site. As maize Ac element has proven to be active in aspen (KUMAR and FLADUNG, 2003), such a method could be useful for quickly generating large populations of T-DNA site free transgenic plants.

Forward genetics in forest trees: Taking a shortcut

Forward genetics strategies aim to clone genes that have been defined by a mutant phenotype or function. This approach continues to be an extremely successful one, yielding mutations that results in overt phenotypes reflecting the function of the corresponding gene. However, it has limited application in forest trees because these methods tend to generate recessive mutant alleles needing an obligatory step of selfing. Another limitation of loss-of-function screens is that they rarely identify genes that act redundantly or those that are required during multiple stages of the life cycle and whose loss of function results in early lethality (SPRINGER, 2000). Genes that are not absolutely required for a certain pathway can still be identified, if such genes are sufficient to activate that pathway. Similarly, genes that are essential for early survival may be identified if ectopic activation of the pathways they regulate is compatible with survival of the organism (WEIGEL et al., 2000). The key in either case is the availability of gain-of-function mutations. Gain-of-function phenotypes can either be caused by mutations in the coding regions that lead to constitutive activation of the resulting protein or by mutations that alter levels or patterns of gene expression. This can be achieved using T-DNA or transposon tagging. T-DNA tagging is useful only for the species that can easily be transformed using *Agrobacterium* and thus has limited application in trees. An attractive alternative is the transposon tagging.

We have investigated the somatic activity of the maize *Activator (Ac)* element in aspen with the objective of developing an efficient transposon-based system for gene isolation in a model tree species (KUMAR and FLADUNG, 2003). In this report it was shown that *Ac* is actively reinserted, frequently into or near coding regions in aspen and, therefore, can be used for gene tagging studies. While this work has given a fine proof of concept, the method needs further modification for making practical gene tagging. The autonomous *Ac* element used in this study needs to be stabilised to obtain stable tagged events. Traditionally two element *Ac*/Ds system is used in annual plants where *Ac* element is segregated out after selfing thereby stabilising the non-autonomous Ds element after it has been excised from its original position and reinserted into the genome. These conventional two-element *Ac*/Ds systems are impractical for forest trees that have long generation cycles. Because of dioecious nature of poplar trees these approaches are difficult to achieve in a model tree, even if the prolonged time to flowering (6-8 yr) were not a deterrent.

An elegant approach that could circumvent the need of these conventional breeding practices is based on inducible *Ac* system that makes the Ds element stable in the absence of the induction. The method uses either a negative selection marker or phenotypic marker for the transposition events. The transposon tagged lines can be can be regenerated from the primary transgenic lines and screened in tissue cultures thus overcoming the barriers of long generation cycles in forest trees. The inducible *Ac* system (BALCELLS et al., 1994) has successfully been applied in model and crop plants. They have so far not been tested in a tree system. The tagged plants can be screened for the dominant gain-of-function mutant phenotypes in the same generation. Alternatively, it is possible to use same strategy for reverse genetics, sequencing tagged genomic regions in high-throughput manner (SESSIONS et al., 2002). Lines containing insertions into coding regions or in the genomic regions of interest can be determined and retained for further analysis. This method can potentially be extended to tree species that are recalcitrant to transformation because a few reagent transgenic lines are needed for subsequent transposon tagging. The reagent lines can be raised either via Agrobacterium or through particle bombardment.

Reverse genetics: A promising tool for tree genomics

Reverse genetics aims to determine the function of gene for which the sequence is known, by generating and analysing the phenotype of the corresponding knockout mutant. With the recent expansion of sequence databanks, locus-to-phenotype reverse genetics strategies have become an increasingly popular alternative to phenotypic screens for functional analysis. In plants, the most common methods for producing reduction-of-function mutations is RNA silencing or RNAi (RNA interference; WATERHOUSE and HELLIWELL, 2003). RNA silencing is based on a principle where a fragment of a gene is introduced into a cell as dsRNA (double stranded RNA) or as DNA that will give rise to dsRNA. The dsRNA activates the dicer-containing complex to generate siRNA (small interfering RNAs). An endonuclease-containing complex (called the RNAi silencing complex, RISC), is guided by the antisense strand of the siRNA to cleave specific mRNAs so that the properties of the affected cell reflect a loss of function in the corresponding gene (BAULCOMBE, 2001; WATERHOUSE and HELLIWELL, 2003). The dsRNA constructs can be made in high-throughput manner using GATEWAY destination vectors compatible to *Agrobacterium tumefaciens* (KARIMI et al., 2002). In trees, gene knockouts using RNAi techniques are being established to modify flowering or lignin biosynthesis (MEILAN et al., 2001; WAGNER et al., 2003), however, no successful application has been reported so far.

A parallel approach using virus induced gene silencing (VIGS) is being carried out in plants. The plants are infected with viruses carrying inserts corresponding to the genes under investigation. The function of the gene can be inferred from the symptoms that develop in the infected plant (RATCLIFF et al., 2001). RNA silencing does not require complete sequence identity in the dsRNA and the target RNA and silencing can be obtained even if there is 5% or 10% mismatch in the initiator of silencing and the target RNA (BAULCOMBE, 2002). Thus it is possible to investigate the function of multigene families in which different members have overlapping and redundant roles. Since these reverse genetics techniques do not require any genetic crossings, they have a big potential for fast track functional analysis of forest tree genome. Therefore, as the amount of sequence data grows for poplar and other tree species, it is important to develop genome-scale reverse genetics strategies that are broadly applicable and capable of creating wide range of mutant alleles that is needed for functional genomics.

Conclusions and future prospects

The long generation interval of trees and their recalcitrant nature for transformation preclude most forward genetic strategies that are used in annual plants for defining gene

function. Consequently, alternate methodologies should be devised to overcome these inherent problems associated with forest tree species. Reverse genetic approaches via transgenics or RNA silencing appear to be promising, however, these methods need to be adapted for trees before their efficacy can be tested in forest tree functional genomics.

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