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TRANSFORMACION GENETICA EN ARBOLES FORESTALES: ESTADO DEL ARTE Y PERSPECTIVAS FUTURAS

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Genetic Transformation in Forest Trees: State of the Art and Future Perspectives

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Summary

Genetic engineering offers prospects of generating novel genotypes at an accelerated rate in the forest tree species. By employing *Agrobacterium*-mediated gene transfer system, a number of alien genes have been transferred to forest tree species. In addition, gene transfer has also been accomplished in the woody plants by biolistic DNA delivery system. However, it is important tc determine the genetic stability and expression of introduced genes in long-lived forest trees on a short-term and long-term basis. In this direction, it would be desirable to isolate transgenic lines that are capable of moderate to high expression of the transgenes, and remain stable following vegetative and sexual reproduction. The transgenic plants must maintain a high degree of transgene expression during growth in the greenhouse, but most importantly under field conditions. Once stable transgenic lines have been established, the inheritance of the transgene can be determined by a progeny test. Thus genetic engineering can provide exceptional genotypes, which can then be incorporated in the clonal and zygotic forestry programs. Based on our experience and other published reports, I shall present strategies for isolating transgenic trees with relatively stable expression of transgenes.

Introduction

Various approaches have been employed for introducing foreign genes into woody plants. Of these, *Agrobacterium*-mediated genetic transformation and microprojectile bombardment DNA delivery system have been successfully used for transferring alien genes into forest trees. Forest trees have long generations cycles with vegetative phases extending from one to several decades. Therefore it is relevant to ask if the foreign genes would be stably integrated and expressed in the forest trees on a short-term as well as long-term basis (Ahuja, 1988 a, 1988b). Genetic stability of transgenes is important for subsequent utility in the commercial forestry.

Recent studies with several annual plant systems have revealed that transgene expression may be unpredictable and variable (see Finnegan and McElroy, 1994; Flavell et al. 1995; Matzke and Matzke, 1995; Stam et al, 1997). Therefore, it is important to find out how model promoters and coding sequences from phylogenetically unrelated organisms are stably integrated in the genome of the forest trees and are expressed at the biochemical and/or phenotypic levels. A number of recombinant genes are available that do not affect the phenotype of the transgenic plants in any detectable way, but their products can be analyzed only at the biochemical level. On the other hand, other chimeric genes exhibit a strong effect on the growth and phenotype of the transgenic plants, and their expression can be visually examined during the entire growth cycle of a plant.

In this paper I shall discuss the current status of genetic engineering and its prospects for genetic improvement of long-lived forest trees. In particular, I shall reflect on the stability and expression of transgenes in space and time.

Tree as a Genetic Engineering System

In order to accomplish successful genetic transformation in any plant, it is important to have: 1) an efficient *in vitro* regeneration system; 2) a vector system for the transport of the recombinant gene; and 3) and efficient gene transfer system. Of these, the first is critical, because in the absence of a high-frequency regeneration system, it would be difficult to produce adequate number of independent transformants and subsequently transgenic lines. And *in vitro* regeneration may be a bottleneck in a number of forest tree species, in particular conifers. Nevertheless, a number of regeneration systems are available in a number of forest tree species, including aspens (Ahuja, 1983, 1986, 1987, 1993), poplars (Chun, 1993; Ernst, 1993), yellow poplar (Merkle et al. 1993), radiata pine (Smith, 1997), white spruce (Attree et al. 1994), interior spruce (Roberts et al. 1995), sugar pine (Gupta, 1995), and Douglas fir (Gupta et al. 1995). In the angiospemic trees, mostly bud meristems have been used for micropropagation in clonal plantations. On the other hand, in the conifers, mostly regeneration via somatic embryogenesis has been developed reasonably well for testing and deployment of somatic embryos in clonal forestry programs (Timmis, 1998). And as time goes by, a number of other economically important but recalcitrant tree species, including eucalypts, are also in the pipeline for efficient micropropagation and their utility in the commercial forestry. This would mean that in a number of forest tree species, efficient regeneration systems are available for their utility in genetic engineering.

Genetic Transformation

Gene transfer in plants may be achieved by several different methods. These include:

- *Agrobacterium*-mediated gene transfer
- Electroporation
- Microprojectile-mediated DNA delivery
- In-planta transformation

Of these, two approaches involving *Agrobacterium* and microprojectile bombardment have been more frequently used in the forest tree species. Predominantly, *Agrobacterium*-vector system has been employed for genetic transformation of different tissues (leaf, stem) in the angiospermic trees (Fillatti et al. 1987; Klopfenstein et al. 1993; Nilsson et al. 1996; Leple et al. 1992; Faldung et al. 1997; Tzfira et al.1997), and microprojectile-mediated DNA delivery system for introduction of alien genes via somatic embryogenesis in conifers (Ellis et al. 1993; Charest et al. 1996; Walter et al. 1998). Since protoplast regeneration can be a problem in most forest tree species, electroporation has until now limited possibilities in trees. But it is possible that thin walled young cells might be amenable to gene transfer by electroporation.

Since regeneration of plants by tissue culture still remains a limiting factor in a number of forest tree species or their different genotypes, there is a possibility of genetic transformation without tissue culture. The first non-tissue culture protocol, called in-planta transformation, was developed by Feldmann and Marks (1987) in *Arabidopsis*. This procedure involves infection/transfromation of germinating seeds with *Agrobacterium* containing the marker gene. The progeny can be screened for the presence of marker, and the fate of the transgene can be followed in the progeny (Feldmann et al. 1994). The same protocol could also be tried in recalcitrant forest trees species, and the independent transgenic plants propagated by macropropagation (conventional vegetative propagation) for further study. Alternatively, the flowering shoots from mature trees could be transformed in-planta , and progeny tested for genetic transformants.

Recombinant Genes

A large repertoire of cloned genes from *Populus* (Jouanin and Pilate, 1997) and recombinant genes (Kim et al. 1997) are available for delivery into forest trees. The coding sequences and the promoters of these recombinant genes have been derived from viruses, bacteria, and plants. Some of these genes affect phenotypes, while others have no detectable effect on the phenotype. Some of these genes expressed in a tree model system, *Populus*, are categorized below.

Phenotypic expression:

- *rolC* gene from *Agrobacterium rhizogenes* alters growth, leaf color and size in aspens (Nilsson et al. 1996; Fladung et al. 1997)

- *LFY* (Leafy) gene from *Arabidopsis* alters growth and development, and induces early flowering in aspen (Weigel and Nilsson, 1995)
- *CAD* (cinnamyl alcohol dehydrogenase) gene from *Populus* in antisense orientation (*ASCAD*) for modification of lignin causes red coloration of xylem in poplars (Van Doorsselaere et al. 1995)
- *phyA* and *phyB* (phytochrome) genes from oats and *Arabidopsis* alters internode length and leaf color in aspens (Sundberg et al. 1997).

Non-phenotypic expression:

- GUS (*uidA*) gene from bacteria is detected by histochemical staining (blue color of cells), in poplars and aspens (Leple et al. 1992; Tsai et al. 1994)
- *Bt* gene from *Bacillus thuringiensis* confers insect tolerance in poplar (Leple et al. 1995; Wang et al. 1996)
- *aroA* gene from *Salmonella typhimurium* confers herbicide (glyphosate) tolerance in poplars (Fillatti et al. 1987; Donahue et al. 1994)
- *PIN2* (protein inhibitor II) from potato confers pest resistance in poplar (Klopfenstein et al. 1993).

The recombinant genes (also called chimeric genes) are in reality patchwork genes, consisting of genetic sequences derived from unrelated or related organisms. At least four components form a functional chimeric gene. The promoter region necessary for transcriptional regulation and start of the transcription may be derived from a viral gene (cauliflower mosaic virus, CaMV, 35S), or bacterium (NOS), or plant (light inducible promoter rbcS from potato, and EJCAD from eucalypt). The intron may be dissected out of another gene, and both the promoter and interon are then fused to a coding region derived from a third gene. The main coding sequence may be obtained from bacteria (many genes, eg. *rclC*, *aroA*, and *bt*), or plants (*A1* from maize, *Ac* from maize, *LFY* from *Arabidopsis*), or insect (*luc*, lucifrase from firefly). These three elements are finally linked to the poly(A) addition site, and the transcriptional terminator regions may be derived from a fourth gene, possibly bacteria. The normal genes also have the same genetic components for proper functioning. But it is remarkable, that these phylogenetically unrelated chimeric genes spliced by recombinant DNA technology can at all function in the higher organisms. But they do, with a remarkable degree, although their expression levels may be unpredictable and variable, and sometimes these recombinant genes are subjected to gene silencing. This might suggest that there is something still amiss regarding the expression of transgenes in plants.

Integration Sites of T-DNA

The integration of transferred DNA (T-DNA) can occur in any chromosome in the genome. There may be a single insertion on one chromosome, or several DNA single insertions or linked copies of a transgene on the same chromosome. In

other words, it is hard to predict how many copies of a recombinant gene will be inserted by genetic transformation in the primary transformants. The copy number could be different for different transformants. It is also not known at which region (heterochromatin or euchromatin) of the chromosome the chimeric gene integrates? The integration site would very likely affect transgene expression. If the T-DNA insertion occurs near the repetitive regions or heterochromatin (usually methylated regions), then the transgene may be subject to inactivation or silencing (Proels and Meyer, 1992). On the other hand; if the T-DNA is inserted in a transcriptionally active euchromatin region, the transgene may be influenced by the regulatory sequences of the host genes (Koncz et. al. 1989; Kertbundit et al. 1991). The site of T-DNA integration and the number of inserts would inevitably affect transgene expression and may be associated with gene silencing. Therefore, it is important to select transformants carrying a single copy of the transgene, and determine which of these shows moderate to high level of gene expression.

Transgene Expression

There is considerable variation in the expression of a transgene between individuals transfromants. This may be due to integration pattern or copy number of the transgene. Further, transgene expression is not solely determined by the type of promoter, as host's regulatory elements and epigenetic effects may also influence gene expression. A large number of studies involving gene transfer in plants, including trees, have used 35S promoter from CaMV for transcriptional control of the reporter genes. In most cases, transgenic plants carrying a single or multiple copies of the transgene, there was variation in expression of the reporter gene. For example, transgenic *Populus* carrying 35S-*rolC*, or 35s-*uidA* (GUS) respectively showed variation in the expression of the transgene at the phenotypic level (*rolC*) and biochemical level(GUS) during different periods of the growth cycles (Ahuja and Fladung, 1996; Fladung et al. 1997; Nilsson et al. 1996). Variation in the expression of reporter genes transcriptionally regulated by other promoters has also been observed (Ahuja and Fladung, 1996; Fladung et al. 1997).

Transgene Inactivation

Although mechanisms of transgene inactivation or silencing are incompletely understood at the present time, there are a number of factors which might influence gene expression. These include:

- Type of recombinant gene construct
- Type of promoter
- DNA delivery system
- Extent of homology between a transgene and endogenous host gene
- Plant genotype

- Hormone regimes for *in vitro* transformation and regeneration of plants

Before gene silencing became recognizable in the transgenic plants, many of the transformants in early experiments with little or low transgene expression were probably discarded so that the investigator could focus on the well functioning transgenic genotypes. Once it was established that gene inactivation could be produced predictably (Matzke et al. 1989; Matzke and Matzke, 1993), experimental evidence from the plant model systems revealed that gene silencing is more commonly prevalent in transgenic plants than previously expected (Finnegan and McElroy, 1994; Flavell et al. 1995; Matzke and Matzke, 1995; Meyer, 1995; Stam et al. 1997; Ahuja, 1997; Caplan et al. 1998). Before considering the mechanisms of gene silencing, it might be useful to point out that somaclonal variation found in tissue culture derived plants may also contribute to the instability of transgenic plants (somatoclonal variation; Ahuja, 1988a, 1988b, 1988c) during growth and development (Ahuja, 1997, 1998).

Gene silencing may occur at the transcriptional level or post-transcriptional level. The first type called transcriptional gene silencing (TGS) probably results from promoter inactivation. The transcriptionally silent genes produce less than expected RNA or no RNA at all, or produce in some cells, but not in others. This type of gene inactivation may result from the location of the transgene near a methylated region of the chromosome, or by methylation of the promoter region (Proels and Meyer, 1992; Neuhuber et al. 1994; Park et al. 1996). However, it is not clear whether methylation is the real cause or consequence of gene inactivation.

The second type of transgene silencing called post-transcriptional gene silencing (PTGS) occurs when the promoter is active and the transgenes are transcribed in the nucleus, but there is little or no mRNA accumulation in the cytoplasm (Van Blokland et al. 1994; English et al. 1996). The post trancriptionally silent transgenes can also suppress the transcription of a homologous endogenes: the latter process called co-suppression (Napoli et al. 1990; Jorgensen, 1995). Although TGS and PTGS suggest two distinct types of mechanisms for gene silencing, there is some overlap when there is interaction between homologous DNA sequences of the transgene and the endogenous genes. At least one mechanism involving methylation that seems to be associated with PGS, may also be operational in some cases of PTGS (Hobbs et al. 1993; Ingelbrecht et al. 1994; English et a. 1996). Therefore, the two types of gene silencing may share some common mechanisms before the transgenic plant system has reached a steady-state functional condition.

Forest Tree have long life cycles, with extended vegetative phases that range from one to several decades. Because trees are firmly anchored in one location for 20 to 2000 years, depending upon the species, they are exposed to changing environments over long periods that may influence their physiological

and morphogenetic processes. For long-term survival, tree must adapt to the new challenges posed by the global climate changes. The gene pool of forest trees has an adequate reservoir of genetic variability to cope with the changing environments. However, most genetic changes in their stable genome structure, whether caused by classical mutations or phylogenetic leap-frogging (Regal, 1994), by genetic engineering, are not well tolerated and may be subject to genetic and/or epigenetic instability. Under such conditions, mutant genes conferring low fitness in trees, for examples some transgenes, may be silenced. However, some genetic changes may have adaptive value over periods of time.

Stability of Transgene Expression in Trees

Although genetic engineering has progressed well during the past decade, there are still problems with the expression of transgenes in transgenic plants. Because of long life cycles, trees may require special consideration before they are genetically transformed. On the other hand, genetic engineering in trees may offer perspectives for long-term studies on expression of transgenes. In any event, recent advances in biotechnology have paved the way for an understanding of gene expression and development in trees. Transgenic research will undoubtedly contribute considerably towards this goal. Before we delve into stability of transgenic trees, there is another aspect of transgenes that needs to addressed. This relates to the functional utility of a transgene in space and time (Ahuja, 1997).

Transgenes conferring herbicide tolerance should be active during the major part of the life cycle of an annual transgenic crop so that they have a competitive edge over the weeds when sprayed with a herbicide. On the other hand, in the forest trees, expression of a herbicide tolerant transgene would be required only in the first and the second year of tree growth, when herbicide is sprayed to kill the competing weeds. After a year or two, herbicide tolerant transgene may not be expressed in the absence of stress, the herbicide. What will happen to that transgene during the next 10 to 50 years of the tree growth remains an open question. Will the herbicide tolerant transgene become non-functional, or inactivated, or perhaps discarded from the tree genome during the long vegetative phase of a tree? Future transgenic research in tree will answer such questions.

Recombinant genes conferring disease or pest resistance, or those involved in the growth and development, on the other hand, must remain active throughout the life cycle of an annual crop or a long-lived forest tree species. Therefore, expression of such transgenes would, to a large extent, depend on the functional utility during the long life of a tree. In the long run, it is the fitness and adaptability of a transgene that would determine its long-term survival in the forest tree species.

Improving transgene stability under field conditions is particularly important for commercialization of transgenic crops or forest trees. Following field trials, a

number of transgenic crops, including tomato, potato, tobacco, corn, soybean, and canola, have already been released in the marketplace (Ahl Goy and Duesing, 1995; Birch, 1997). Trees may be next. But before that we have to do extensive research in the production of transgenic trees and test them under greenhouse and field conditions.

In this direction, we have carried out a European Union project "Stability and Expression of Foreign Gene in *Populus*". The objectives of this project were to investigate whether there were any instabilities of the recombinant genes transferred to *Populus* by *Agrobacterium*-mediated gene transfer which could be detected, on a short-term and long-term basis, at the genomic and/or transgene expression levels. After four years of research, involving three independent groups from Sweden (Goteborg University), France (INRA, Ardon), and Germany (Institute of Forest Genetics, Grosshansdorf), and employing five different *Populus* geneotypes and some 15 different recombinant gene constructs, we have generated thousands of transgenic plants and have tested them under greenhouse and field conditions. Both types of recombinant genes, having phenotypic and non-phenotypic affects, were employed in the investigation. Our combined results indicate that constitutive 35S promoter from CaMV causes variation in the expression of the transgene under different set of conditions. Other promoters, such rbcS from potato, and rol promoter from *Agrobacterium rhizogenes* seemingly were less affected by stresses. On the phenotypic level, *rolC* expression was variable during growth cycles. There were a few cases of gene silencing as well. Minor to major T-DNA arrangements can occur during the integration process. However, once a single copy of the transgene was integrated in the genome, there were seemingly no further detectcable changes in the structure of the transgene. However, this aspect requires further scrutiny (Ahuja and Fladung, 1996; Fladung et al. 1997; Nilsson et al. 1996; Olsson, Pilate, Fladung, and Ahuja, unpublished project data). The field trials on transgenic *Populus* have been started in France (1990), Sweden (1995), and Germany (1996), and they are still continuing.

Based on our experience and other published reports, I can make some recommendations on the genetic engineering of trees. The following guidelines would be useful for isolating transgenic trees in which the transgenes are stably expressed. However, occurrence of some variation in the expression of transgenes even in the apparently stable transgenic lines is not completely ruled out.

- Develop moderate to high-frequency micropropagation system, by organogenesis or somatic embryogenesis
- Minimize use of high concentration of hormones, such as 2,4-D, which give excessive callus in tissue culture
- Use media and hormones which give direct differentiation of microshoots or somatic embryos *in vitro*
- Minimize excessive callus formation and the time tissues remain in culture to avoid somaclonal variation

- Try in-planta transformation of germinating seeds/mature branches of forest genotypes that are recalcitrant in tissue culture
- Select gene constructs with promoters that are less prone to methylation; promoters cloned from plants/trees may function better
- Select uniform (non-chimeric) transformants following genetic transformation (by *Agrobacterium*, or microprojectile bombardment, or in-planta)
- Propagate transformants by tissue culture technology, or by macropropagation
- Select transformants carrying a single copy of the transgene, having no extra border sequences
- Produce a large array of transformants carrying a single copy of the transgene, and from those select the ones that give stable and less variable expression of the transgene
- Test the selected transgenic lines under greenhouse, and later under field conditions for monitoring stable expression of the transgene
- Perform progeny test, if possible, to check the transmission of the transgene in the offspring, and its stable expression
- Deploy exceptional transgenic genotypes in the clonal forestry programs. Those exceptional genotypes could also be eventually integrated in the breeding programs.

Outlook

Genetic engineering has come of age in forest trees. Hopefully, transgenic trees with relatively stable gene expression would become available in due course of time. However, transgenic research should not lose sight of some concerns regarding genetic stability, containment of transgenic pollen, and ecosystem disturbances. The expression of the transgene may be stabilized by flanking transgenes with matrix attachment regions (MARs) (Mlynarova et al. 1994; Spiker and Thompson, 1996). Genetic engineering of reproductive sterility may be one approach for the containment of transgenic pollen (Strauss et al. 1995). Above all, it is necessary to follow guidelines provided by the national and international regulatory and biosafety agencies for conducting transgenic research. In the final analysis, it would be the public perception and acceptance of genetically engineered trees that will determine their future in forestry.

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