

Genetic Engineering Contribution to Forest Tree Breeding Efforts

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Abstract Forests have multiple roles, including traditional timber and fibre production, to sequestering global carbon emissions, preserving water systems and providing ecosystem services and shelter for a variety of plant and animal species. Genetically engineered (GE) forest trees can play a significant role in highly productive plantation culture for commercial products, where they would be beneficial for specific purposes. We review the knowledge of transformation technologies, and compare the role of GE technology in forest tree breeding to traditional tree breeding approaches. The role of genetic engineering in the climate change context is also covered. Finally, we speculate on the importance of tree genomics for future forestry, genetic engineering and forest tree breeding.

1 Introduction

Looking back at the history of mankind, domestication of the most important crop species has played an enormous role. Around 12,000 years ago, ancient people began a plant-breeding programme by transforming a number of wild plant species into domesticated crops by means of selective breeding. These crops also included the most highly productive crops, rice, wheat and maize, on which human survival is still dependent today (Doebley et al. 2006; Meyer and Purugganan 2013). Food grain production reached 1 billion tons in 1960 and it took almost 10,000 years, but to reach the additional 1 billion tons produced in the year 2000, it took only

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40 years. The recent increase in productivity was due to Dr. Norman Borlaug the 'Father of the Green Revolution' and recipient of the Nobel Peace Prize in 1970, and his many partners. In the Green Revolution, the genetically improved crop varieties were combined with the application of improved agronomic practices. The Green Revolution boosted crop yields especially in developing nations by introducing semi-dwarf genotypes of wheat and rice capable of responding to fertilization without lodging. However, population growth, increased standards of living, ongoing soil degradation and increasing costs of chemical fertilizer will make the second Green Revolution a priority for plant biology in the twenty-first century (Khush 2001).

Today, the global cultivation area of genetically engineered (GE) crops is already 175.2 million ha, which is 3 % (i.e. 5 million ha) more than in 2012, in 27 countries by 18 million farmers. Biotech (GE) crops, the fastest adopted crop technology, are considered to deliver substantial socioeconomic and environmental benefits (Clive 2013; Klümper and Qaim 2014). In the future, global growth of biotech crop hectares may be more modest due to the already high rate of adoption in all the principal crops in mature markets in both developing and industrial countries (Clive 2013); however, a much greater diversity of genes and traits is expected to be used.

Concerning forest trees and taking into account their specific biological properties such as the long generation interval/breeding cycles and lengthy juvenile period compared to crop species, it is obvious that breeding programmes are today only ongoing with commercially important species. Generally, forest tree domestication and breeding programmes mostly started at the earliest in the last half century and are, therefore, still in its infancy (reviewed by Campbell et al. 2003). Thus, the application of biotechnology/GE technology to forest trees offers a great potential to hasten the pace of tree improvement for desirable end uses. However, the challenges involved in predicted population growth and climate change including weather extremes (Nellemann et al. 2009) may increase the need for new cultivation areas and/or more effective food production. This means also increased pressure to accelerate forest tree domestication in a sustainable way to fulfil the future needs of wood, biomass and other bio-based products.

In this context, the traditional genetic improvement protocols, due to the long breeding cycles, alone may be too slow to achieve rapid domestication of forest trees. In these cases, both the lessons from the almost 25-year history of genetic engineering of forest trees combined with the experience on conventional breeding programmes as well as the information available and applications depending on the genetic and genomic knowledge, could speed up tree domestication. The cost-effectiveness, as well as the regulatory approval of the technology/end-products by the authorities, and last but not least, public acceptance is vital for the practice, as for the adoption of the technologies. However, even if the economic reasons favour the use of GE trees in plantation/production forests, it is also good to remember that the technology can be utilized also for conservation purposes of the species threatened with extinction (e.g. chestnut in USA or European elms).

In this chapter, the possibilities of GE tree exploitation are discussed in relation to the tree breeding efforts. This is done at the time when the European Council made an important step by reaching political agreement on 12 June 2014 towards allowing Member States to restrict or ban GMO cultivation in their territory and when simultaneously there are commercial cultivations of *Populus* species in China (Ewald et al. 2006; Hu et al. 2014) and ongoing efforts for deregulation of GE forest trees in South and North America. Transgenic trees are on the way from lab research to trait oriented uses in the next 25 years.

2 Tree Breeding from Its Infancy Towards Future Innovations

The breeding process for forest trees differs fundamentally from that of annual crops (Fladung 2008; Flachowsky et al. 2009). The reason simply lies in the special characteristics of trees, as these are predominantly out-crossing and have extensive vegetative phases as well as a high, individual age. Unlike crop plants, which have a high degree of domestication and often no wild relatives in Europe, many “bred” varieties of tree species are comparatively still with “wild plants” that are characterized by a high genetic diversity.

The breeding process of an agricultural crop is pure cross-breeding, i.e. after an initial cross between a wild plant (carrying e.g. a resistance gene) with an elite line (deficient in the resistance gene), repeated back-crosses and field testing ensures the presence of the resistance gene together with the accumulation of the genomic background of the elite line. On the contrary, in trees, the establishment of a second or third filial generation is practically excluded due to the long generation times of most forest tree species. In consequence, the concept of selective breeding has been coined in forest tree breeding. This means the inspection and selection of natural occurring forest trees (termed as elite or plus trees), followed either by simply collection of seeds and using them for the establishment of seed orchards (Langner 1957/58) or by an initial cross between elite or plus trees and evaluation of a hybrid F1 progeny in terms of forestry-relevant features, e.g. straight stem or vigour growth (hybrid selection; von Lochow 1929; Wolf 2003).

The forest tree breeding efforts were initiated between 1920 and the 1940s in many European countries including Finland, Germany, Norway, Portugal and Sweden. Since then, most of the European countries have had breeding programmes for a variety of hard- and softwood species, in which the general aim has been the improvement of traits considered economically or ecologically important (White et al. 2007; Mullin and Lee 2013; Pâques 2013). For instance in Germany, selective breeding of forest trees has a long tradition (Langner 1957/58; Wolf 2003). Examples are the breeding of fast growing hybrid poplar clones, micro-vegetative propagation of wild cherry clones, and selection of phenotypically superior hybrid trees in sessile oak, pine, alder or larch. However, many of the European tree

breeding programmes have already been suspended (Mullin and Lee 2013; Pâques 2013). Globally, the present breeding programmes include the following forest tree species: Douglas-fir (*Pseudotsuga menziesii*), *Eucalyptus* species, loblolly pine (*Pinus taeda*), Monterey pine (*Pinus radiata*), Norway spruce (*Picea abies*), *Populus* species, and Scots pine (*Pinus sylvestris*).

As reviewed by Neale and Kremer (2011) research in forest tree genomics lagged behind that of model and agricultural species. The ongoing next-generation sequencing efforts with several forest tree species is proceeding fast but the draft genomes of tree species still represent only 6 of the more than 100 plant families containing tree species, and thus, the possibilities for comparative genomic and genomic selection approaches are still limited (Neale et al. 2013). Before utilization in tree breeding or conservation or restoration purposes the reference genomes should be functionally annotated with omics approaches and preferably mapped with high density. This, together with feasible genotyping platforms would potentially in future enable marker-assisted selection (MAS) and genomic selection in forest tree species (Nieminen et al. 2012; Isik 2014; Zhang et al. 2014).

3 Genetic Transformation Technologies

Genetically modified organisms (GMOs) are officially defined in the EU legislation as organisms in which the genetic material (DNA) has been altered in a way that does not occur naturally by mating and/or natural recombination (Plan and Van den Eede 2010). In the Organisation for Economic Cooperation and Development (OECD), genetic modification is defining an organism whose genetic material has been altered by whatsoever method including, e.g. traditional methods such as selection breeding.

The first scientific peer-reviewed paper on GE forest tree was published by Fillatti et al. (1987). They introduced *AroA* gene coding 5-enol-pyruvate 3-phosphate and conferred herbicide resistance via *Agrobacterium tumefaciens*-mediated gene transfer to *Populus* hybrid (*Populus alba* × *grandidentata*). In the early days, the efforts were focused on the development of transformation technologies rather than trait-oriented transformation. Back in the days and actually still today, *Agrobacterium* (*A. tumefaciens* and *A. rhizogenes*) species-mediated gene transfer (Chilton et al. 1977; Schell et al. 1979; Zambryski et al. 1983; Bevan 1984; Wood et al. 2001) is the most common transformation technology. Biolistic transformation (particle/microprojectile bombardment) (Sanford 1988, 1990), in contrast to *Agrobacterium*-mediated transformation, is a physical process based on the delivery of DNA coated gold particles into the plant cells. Moreover, during the years, also other genetic transformation technologies were developed/used, such as electroporetic transfection (Ahokas 1989; Vik et al. 2001) and microinjection (Miki et al. 1989). In addition, DNA delivery to plant protoplasts was performed by electrophoresis and in some cases also via liposomes (as reviewed by Dan and Ow 2011). Furthermore, there are several so-called combination technologies where

particle bombardment or silicon carbide whiskers (Nagatani et al. 1997) were used prior to *Agrobacterium* transformation to wound plant tissue/cells to improve the transformation efficiency. Similarly, Trick and Finer (1997) used sonication before *Agrobacterium*-mediated transformation. Nevertheless, in spite of several possibilities, the most common transformation technologies within forest tree species are biolistic transformation and *Agrobacterium*-mediated transformation. For instance, Trontin et al. (2007) list in their review 30 *Pinus* species, including the important forest and plantation species *P. pinaster* in Europe, *P. radiata* in New Zealand, Australia and South America as well as *P. taeda* and *P. strobus* in North America, which have been targets of extensive research to improve the transformation methods. In the case of 17 pine species, foreign gene transfer and stable expression have been achieved either by co-cultivation with disarmed *A. tumefaciens* strains and/or biolistic transformation (Trontin et al. 2007).

The viral vectors have also been an advantageous alternative to the transgenic systems for the expression of foreign proteins in plants. There are two basic types of viral systems that have been developed for production of immunogenic peptides and proteins in plants, namely (a) epitope presentation where the sites of insertion are chosen so that the peptide is displayed on the surface of the virus particles and (b) polypeptide expression systems (Cañizares et al. 2005 and references therein). Plant viral vectors delivered by *Agrobacterium* are the bases of several manufacturing processes that are currently in use for producing a wide range of proteins for multiple applications, including vaccine antigens, antibodies, protein nanoparticles such as virus-like particles (VLPs), and other protein and protein-RNA scaffolds, but they are also important tools in research. Among the most often used viral backbones are those of the RNA viruses tobacco mosaic virus (TMV), potato virus X (PVX) and cowpea mosaic virus (CPMV), and the DNA geminivirus bean yellow dwarf virus (Gleba et al. 2014).

Virus-induced gene silencing (VIGS) is a transient RNAi-mediated gene silencing approach that facilitates rapid gene function assessment without the requirement of generating stable transgenes (Burch-Smith et al. 2004). Zhu et al. (2010) utilized *Nicotiana benthamiana* a well-proven platform for VIGS when studying plant cell wall formation. Sasaki et al. (2011) presented an effective VIGS system in apple, pear and Japanese pear using *Apple latent spherical virus* (ALSV) vectors. This was the first time when VIGS vectors were reported for *Rosaceae* fruit trees. Inoculation of ALSV vectors carrying a partial sequence of endogenous genes from apple [ribulose-1,5-bisphosphate carboxylase small subunit (*rbcS*), alpha subunit of chloroplast chaperonin (*CPN60a*), elongation factor 1 alpha (*EF-1a*), or actin] to the cotyledons of seeds by a particle bombardment induced highly uniform knock-down phenotypes of each gene from 2 to 3 weeks after inoculation.

In addition to transformation technologies, also a lot of experience has been gained concerning gene constructs used in transformation. Generally, the gene constructs include the gene of interest, promoter sequences (constitutive, inducible or synthetic, Venter 2007) guiding the expression or down-regulation of the target gene and selectable marker gene, which in most of the cases is *npII* (neomycin phosphotransferase II) providing resistance to kanamycin antibiotics or for instance

bar, which encodes for phosphinothricin acetyltransferase and which allow the selection of transgenic cells. With regard to the safety use of antibiotics in gene constructs EFSA (European Food Safety Authority) expressed already in 2004 and further confirmed in 2009, there is no rationale for inhibiting or restricting the use of category I antibiotic genes including *nptII* either for field experimentation or for placing on the market. Concerning the traits (genes of interest) in the research areas, multiple traits and species have been used during the 25 years, but looking at the existing field trials the traits could be grouped as recently presented by Häggman et al. (2013, 2014) marker genes, disease and insect resistance, abiotic stress tolerance, growth and wood properties and biosafety-related issues.

The new technologies to come such as zinc finger nucleases (ZFN), transcription activator-like effector nuclease (TALEN), LAGLIDADG homing endonuclease (LHE) (Curtin et al. 2012) or CRISPR-Cas systems where gRNA-directed Cas9 nuclease can induce indel mutations or specific sequence replacement or insertion (Sander and Joung 2014). The CRISPR-Cas systems can edit, regulate or target genomes with very specific outcomes. These new technologies may also cause very small changes in the genomes. Changes up to 20 bp are not considered as genetic modification as these changes cannot be distinguished from conventional mutagenesis or natural mutations (Cao et al. 2011). Therefore, it is at present under discussion whether or not these small changes will be regulated under the European gene technology directives. The result of this discussion will certainly guide the future technology choices in plant including tree breeding.

4 Genetic Engineering Can Accelerate Tree Breeding

The juvenility period of most of the forest trees are long, which also means long breeding cycles (White et al. 2007), usually >15 years for gymnosperms and for angiosperm species 7 or more years. Traditional tree breeding has principally focused on improving economically and ecologically important traits, including early stem growth and stem form, pathogen and disease resistance, adaptation and more recently wood properties (as reviewed by Häggman et al. 2013). These traits also show complex patterns of inheritance. The increase in genetic gain achieved by breeding cycle may also mean that applications for vegetative propagation are needed at the state of field testing. During the years, also MAS and discovery and use of quantitative trait loci (QTL) in breeding (as reviewed by Isik 2014) have been substantial and this has been done to reduce the long breeding cycles. However, MAS has not been successful in forest trees which generally lies in the fact that the QTLs discovered in experimental populations explain only a small percent of phenotypic variation and also the genotype by environment interactions make the MAS application more complicated (Isik 2014). The situation might, however, change together with the available high throughput genotyping platforms providing numerous markers to be utilized in high-density genetic maps.

By means of genetic engineering, the characteristics of forest trees could be improved faster by using “transgenesis” approaches. The tree characteristics of interested, including enhanced abiotic/biotic resistance, increased biomass production or modified/altered wood, could be achieved by altering the expression of specific (endogenous) gene/s or by introducing novel traits to trees. Another possibility could be reducing the vegetative period of trees from a few years down to one year, allowing breeding activities in a similar manner as in annual crop species. The shortening of the juvenile phase can be achieved by transgenesis with the transfer of “early flowering” genes leading to precocious flowering (Flachowsky et al. 2009) as described in detail in Sect. 4.2.

Besides being slow, traditional tree breeding can cause notable changes in the tree genome. In contrast, the mutagenesis based, genetic engineering technologies under development affect very moderately the genome organization—it may be that the changes are only present in a few bases. The new methodologies (ZFN, TALEN, LHE, CRISPR-Cas) hold promise that the genetic engineering of forest trees can be more precise in the near future in comparison to the biolistic and *Agrobacterium*-mediated transformation methods. However, the almost 25-year history of GE forest trees produced with these “old-school” transformation methods has proved that after the initial greenhouse characterization of GE forest trees, the foreign gene/s have stable expression under natural conditions causing no/minor unintended effects to the environment/other organisms (Häggman et al. 2013, 2014). Yet, we are lacking data on the performance of GE forest trees under long-term field trials crossing the border of juvenility and maturity.

We have a long experience on the genetic and phenotypic characteristics of the main forest tree species in conventional breeding programmes. The accumulation of genome sequence data is premise for better genetic maps needed for MAS. However, high-density genetic maps are not yet available and it will take time before they are for all the forest tree species involved in breeding programmes. The tree domestication can be speeded up by means of genetic engineering but the realization by genetic engineering requires public acceptance.

4.1 Genetic Engineering Versus Natural Genetic Variation

Natural genetic variation is the driving force of the evolution of all living individuals. Each spontaneously occurred mutation is checked for its value for the fitness and survival of each living individual. This is the principle of Darwin’s theory of selection and evolution. Along with the rapid development of new methods for genome sequencing, numerous genomes of different individuals already have been or will be totally sequenced and uncountable genetic variations are being or will be detected. Such genetic differences can be classified in different sections, small ones, i.e. single nucleotide variations (called single nucleotide polymorphisms = SNPs) including insertion/deletions (“Indels”) of up to 20 bp, rearrangement of intragenomic sequences or transposition events, or uptake of

foreign DNA (Arber 2010). Genetic variations can in consequence be beneficial or disadvantageous for living organisms, depending on the present and future environmental factors. When transmitted to the progeny, according to Darwin's theory, in the long-term end survival of the fittest will value the "best" genetic variation. Genetic variations can also be independent of environmental factors, e.g. when such variations inhibit necessary life processes or are even lethal in extreme cases (Arber 2010).

Molecular mechanisms behind genetic variations are manifold. Single nucleotide variations can occur spontaneously as in textbooks given at 10^{-6} frequency. However, the frequency of this type of mutation can be increased by induced mutagenesis by applying chemical mutagens or UV- or gamma-irradiation. Single nucleotide variations, however, can also be induced by the molecular machinery in each living cell, e.g. DNA replication mechanism. The larger genetic variations, i.e. DNA rearrangements or transposition events, can be triggered through homologous or site-specific recombination and mobile genetic elements (transposons, retro-transposons), respectively. Uptake of DNA is known as natural horizontal gene transfer or along with modern biotechnological methods such as genetic engineering.

Genetic variations up to 20 bp can occur when living cells are underlying all the mechanisms leading to small genetic variations as described above (Cao et al. 2011), thus these changes cannot be distinguished from genetic variations induced by genetic engineering (Arber 2010). Nevertheless, the larger genetic variations such as recombination and transposition leading to natural, spontaneous generation of genetic variants can also be observed along with genetic engineering. No differences in the amounts of nucleotides, but also in the molecular mechanisms are obvious, thus there is no scientific reason to assume that genetic engineering bears particular conjectural evolutionary risks (Arber 2010).

With transgenesis, the goal is to alter the expression of specific gene/s or to introduce novel trait to organisms in which case the introduced gene/genes are possibly absent from the genome of the recipient. If the introduced gene originates from a close, sexually compatible relative of the recipient or from the same species and the gene (or flanking regions) have not been modified, but include the T-DNA borders of *Agrobacterium*, term cisgenesis is applicable. Similarly, intragenesis refers to utilization of gene retrieved from a close relative or the same species of recipient organism but in this case, the gene is modified, that is, the recombinant version of the original gene. In contrast to transgenesis, intragenesis could, in theory, rise in plants naturally. Hence, EFSA (2012) concluded that the hazards associated to cisgenesis are the same as in conventionally bred plants. Thus, forest trees could be modified with constructs not dissimilar to the genetic content of the species; however, at present, the limited gene delivery methods usable for forest tree species hinder the accuracy of the approaches as the integration of constructs to the genome is random in biolistic and *Agrobacterium*-mediated transformation (Ahuja and Fladung 2014).

4.2 *The Use of Early Flowering Genes to Accelerate Tree Breeding*

The main factor hindering effective breeding in forest trees is the prolonged vegetative phase, variable lasting in some tree species until 40 years (e.g. *Fagus sylvatica* L.) (Meilan 1997). The use of a transgenic early flowering plant can accelerate the breeding of forest trees significantly (Flachowsky et al. 2009). The initial step in the breeding process is based on the cross of a transgenic early flowering plus tree with a tree carrying an interesting gene (“gene-of-interest”), e.g. for disease resistance. In the progeny of this initial cross, the early-flowering gene and the “gene-of-interest” will segregate following the Mendelian rules. By employing molecular marker, both genes can be detected in each plant in the progeny. The plants carrying the two genes will be submitted to back-crosses to increase the genomic background of the plus tree. In the next generation, again molecular marker can be used to select progeny plants carrying both genes. This cycle can be repeated several times. At the end, the early-flowering gene will be out-crossed, resulting in a disease-resistant plus tree that is not transgenic (Flachowsky et al. 2009). In this way, new, permanently improved tree varieties can be created in a reasonable time frame, which are also free of any “foreign” DNA sequences.

Very early, the idea came up to employ early flowering trees, either natural or transgenic ones, to accelerate tree breeding (Häggman et al. 1996; Meilan 1997; Fladung et al. 2000; Fladung 2008; Flachowsky et al. 2009). Most genes involved in regulation of flowering have been discovered in *Arabidopsis* (Theissen et al. 2000; Liu et al. 2009). Some of these genes have been tested for early flowering, mainly in poplar (Rottmann et al. 2000; Hoenicka et al. 2006; Hsu et al. 2006; Böhlenius et al. 2006; Hoenicka et al. 2012a), but also in apple (Wada et al. 2002; Flachowsky et al. 2007; Tränkner et al. 2011) and birch (Elo et al. 2001, 2007).

The first report on successful application of a flowering-inducing gene from *Arabidopsis* was the constitutive expression of the meristem identification gene *LEAFY* in *Populus* (Weigel and Nilsson 1995). The *Arabidopsis* gene under the control of the 35S promoter but also the poplar native gene reduces flowering time in poplars very effectively (Weigel and Nilsson 1995; Rottman et al. 2000). However, this early system had some disadvantages because of the dwarf phenotype of transgenic plants and the formation of single flowers instead of catkins (Hoenicka et al. 2006; Hoenicka et al. 2012b). Later, a very effective early-flowering system based on the *FLOWERING LOCUS T (FT)* could be developed (Zhang et al. 2010), however, also with some disadvantages, e.g. not inducible in tissue culture or in very young greenhouse-grown plants. An improvement of the system could be achieved by combining the *FT* with the *SUPPRESSOR OF CONSTANS (SOC)* gene (Fladung and Hoenicka, unpublished results).

The expression of the 35S::*BpMADS4* gene construct caused an acceleration of flowering time in birch, tobacco and apple (Elo et al. 2001; Flachowsky et al. 2007),

but not in poplar (Hoenicka et al. 2008). Similarly, genes inducing early flowering in *Arabidopsis* including *FLOWERING PROMOTING FACTOR 1 (FPF1)* and *FUL* showed no effect on flowering time in poplar (Hoenicka et al. 2008, 2012a). Induced early flowering was achieved using the genes located on the Ri plasmid of *Agrobacterium rhizogenes* in tobacco (*rolD*, Mauro et al. 1996) and poplar (*rolC*, Fladung et al. 2003). Unfortunately, constitutive *rolD* expression in transgenic poplars did not lead to precocious flowering.

5 Towards Trait-Oriented Approach

Today, there is a lot of information from genetic transformation technology and gene constructs both at the laboratory level and at greenhouse, the focus being on basic studies and biosafety issues. The European Commission has spent between 1982 and 2010 over 300 million € to study the biosafety of GMOs (EUR 24473 2010) and altogether 50 research projects were funded between 2001 and 2010 the specific focus of the projects being in biosafety issues. The main conclusion which was drawn from the efforts of more than 130 research projects, covering a period of more than 25 years of research, and involving more than 500 independent research groups, is that biotechnology, and in particular GMOs, are not per se, more risky than conventional plant breeding technologies (EUR 24473 2010).

Moreover, we have considerable experience on approved confined field trials from the United States (500 field trials), China (78), Brazil (68), Canada (45), EU (44) Japan (9) and New Zealand (5) according to Häggman et al. (2013). The following forest tree species have been approved for the trials: the hardwood species being *Betula pendula*, *Castanea dentata*, *Eucalyptus* spp., *Liquidambar styraciflua*, *Populus* spp., *Robinia pseudoacacia*, *Paulownia* and *Ulmus americana* as well as the following coniferous species *Larix* spp. *P. abies*, *Picea glauca*, *Picea mariana*, *Pinus* spp. *P. radiata*, *P. sylvestris* (reviewed by Häggman et al. 2013).

Genome resources are developing fast in forest trees but the completed and ongoing genome-sequencing projects in forest trees are today still limited to around 25 species representing mostly members of 4 families, i.e. *Pinaceae*, *Salicaceae*, *Myrtaceae* and *Fagaceae* (Neale et al. 2013) including species that have also been used in genetic transformation approaches. Comparative genomics tools may also be helpful in planning the genetic transformation approach and/or by comparing the GE tree functioning with the non-transgenic counterparts.

All the cumulative information on laboratory and greenhouse experiments as well as field trials and increasing amount of genome resources will support genetic engineering approaches to move from laboratory research to trait-oriented applications during the next 25 years. It will also influence and fasten the tree breeding efforts. However, also the general challenges of the society (population increase, food scarcity, deforestation and forest degradation, climate change) may emphasize the need for high yield fast growing GE production forests as one solution, but also the conservation of the species threatened with extinction such as chestnut in USA

(or the elms in Europe) might be good candidates for transgenic approach. Thus, the next steps will be to consider deregulation of forest trees in specific cases and from the legislation point of view—in all cases anyhow the changes in the forest tree genome will be minor compared to the traditional breeding or hybridization.

6 The Global Climate Change and Need for Wood

The sufficient production of wood without disturbing primary forests will be one of the most important issues for the near future. During the last 100 years, an increase in the CO₂ concentration in the atmosphere from 290 to 400 ppm has been recorded in middle Europe. During the same period, Europe's average temperature increased by 0.95 °C and the global average sea level has risen by 10–20 cm. These are strong indicators for the present ongoing climate change worldwide.

Besides increase in temperature and CO₂ concentration, other predicted consequences are an increase in the number of storms and other extremes as well as changes in precipitation patterns. For Europe, the expected consequences of the predicted climate change are dramatic. The melting of alpine glaciers and permafrost will lead to increased risks of flooding in central and northern Europe. Growth zones are shifting north, loss of important habitats such as wetlands and tundra will be the result (e.g. IPCC 2007).

6.1 Wood Production Should Be Ensured

The long-term goal for world forestry, under the scenario of climate change, is to ensure a sufficient wood production. This applies both for timber production in forests but also in plantations. However, the above described situations of increased number of storms, floods and droughts, heat waves and rising CO₂ concentrations towards 600 and 1000 ppm but also the spread of tropical diseases and parasites are severe threats for world forestry. Questions raised and to be answered are which tree species cope with climate change, which do not, and do native forest tree populations have a sufficient genetic variability to adapt to climate change? Is the existing natural adaptability of forests sufficient, since climate change is expected to take place very quickly? Or, is a forest conversion needed in a way that native tree species would partly be substituted with drought-tolerant exotic tree species? For the use of trees in plantation forestry, the question arises whether forest tree breeding is “fast” enough to meet a fast progressing climate change?

Wood production must be ensured without disturbing primary forests. Of course, stop of climate change by avoiding or reducing causing factors should have eminece. However, simply due to social responsibility and due to the possibility that climate change inexorably progresses, measures have to be taken to ensure survival of the world's forests in future, also in the sense of an adequately secured wood

production. Such measures should consider all kinds of approaches to accelerate tree breeding, and could also include the use of GE trees. From today's perspective, no possibility can be excluded per se to assure stable forests for an ecological-driven forestry and sustainable wood production.

A large number of foreign tree species from northern America and Asia have been introduced to European countries (Hoenicka and Fladung 2006). As many of the exotic species are naturally hybridizing with European tree species, they have been successfully included in classical tree breeding programmes (Wolf 2003). Unfortunately, due to the predicted changing climatic conditions, the appearance of new biotic pests is expected in a relatively short time span. To accelerate tree breeding, in the last 25 years, biotechnological techniques already widely in use for annual crops have been adopted to various tree species, e.g. tissue culture, transformation (gene technology) and genome analysis (Taylor 2002; Wullschlegler et al. 2002).

6.2 How GE Trees Can Help

The use of GE technology offers a unique opportunity to specifically improve desired traits of plants in a very sophisticated manner. Breeding objectives of tree species as well as acceleration of the tree breeding process to ensure reliable wood production also in future, in particular in regard to the predicted climate change, have been discussed some years ago in combination with domestication of poplar, and multiple targets were formulated (Bradshaw and Strauss 2001; Fladung 2008; Flachowsky et al. 2009). As the first important objective, maintenance and optimization of wood yield has to be considered. This goal could be achieved by increasing the fixation rate of atmospheric CO₂, enhancing the deposition of carbon in the stem or by diversion of photoassimilates into wood formation.

6.3 Efforts to Make Transgenic Trees Safer

6.3.1 Induction of Flower Sterility

To avoid uncontrolled spread of the mutated gene(s) within or outside the gene pool of the improved tree species should be avoided. Here, different molecular approaches have been developed to achieve gene containment in transgenic crops including trees, such as male and/or female sterility, and selection of non-GM plants by using recombination systems. The incorporation of sterility genes into transgenic lines of trees has been proposed early as a strategy to reduce or even avoid gene flow of transgenes into non-transgenic relatives (Brunner et al. 1998). However, evaluation of success concerning sterility in transgenic forest trees is difficult due to the long vegetative periods (Hoenicka et al. 2006). Strategies to

shorten reproductive phases in forest trees by using flowering-induction genes have been developed (Weigel and Nilsson 1995; Rottmann et al. 2000; Hoenicka et al. 2006; Zhang et al. 2010), and their usefulness to study fertility/sterility of flowers has been summarized in Hoenicka et al. (2012b).

Publications dealing with sterility induction in forest trees are still very rare (Meilan et al. 2001; Skinner et al. 2003; Hoenicka et al. 2006; Lemmetyinen et al. 2004; Wei et al. 2007). In most cases, sterility constructs successfully used in crops were simply tested in trees. As disadvantage, the heterologous floral-specific promoters often revealed “leaky” expression resulting in activity of cytotoxic gene in non-target, vegetative tissues, leading sometimes to a lower performance of transgenic plants (Meilan et al. 2001; Skinner et al. 2003; Lemmetyinen et al. 2004).

In an early study by Hoenicka et al. (2006), early flowering 35S::*LEAFY* poplar lines were used for evaluation of two different sterility constructs, TA29::*Barnase* and C-GPDHC::*VstI*, allowing evaluation of the sterility strategies only 2 years after transformation. It could be shown that CGPDHC::*VstI* promoted sterility in poplar (Hoenicka et al. 2006). A similar sterility system based on the *STILBENE SYNTHASE* (*STS*) gene under control of the gymnosperm promoter PrMALE1 led to reduced pollen number and very low pollen germination in tobacco (Höfing et al. 2001). In poplar, pollen development was disturbed in 68 % of catkins (Hoenicka et al. 2012b).

6.3.2 Selection of Non-GM Trees

To overcome the random integration of foreign gene(s) related to biolistic and *Agrobacterium*-mediated transformation (Kumar and Fladung 2001, 2003), site-specific recombination systems can be used (Kumar and Fladung 2001; Ow 2002). For the first time, Fladung et al. (2005) indicated the usefulness of two recombination systems in the tree species *Populus*. The idea of gene targeting is based on the action of the site-specific recombinase catalyzing an intragenomic mobilization of the randomly inserted gene(s) into a desired, previously characterized recipient locus (Fladung and Becker 2010).

An example of such successful cassette exchange has been provided by Fladung et al. (2010) by using a two-constructs approach. The first construct contained the FLP gene controlled by a heat-inducible promoter as well as a marker gene under control of the 35S promoter, while the second carried a promoterless second marker gene. Poplar plants were double transformed with the two constructs, and following heat induction of FLP, the promoterless second marker gene became active by exactly replacing the FLP gene/first marker gene and getting under control of the 35S promoter (Fladung et al. 2010). In the long-term, however, direct targeting of the new gene to a defined position is sought that should allow one to produce transgenic trees in which the incoming gene can be expressed in a predictable pattern.

The same system can be applied to remove the herbicide or antibiotic marker genes or even to select non-GM plants (Endo et al. 2002). A combination of two site-specific recombination systems in one gene cassette has been successfully

applied to remove the foreign genes from the pollen genome by having them still expressed in the plant body (Hoenicka et al. unpublished results). Here, the first recombination system was under the regulation of a heat inducible promoter, while the second recombinase was promoterless. A flower-specific promoter was also present but without a subsequent gene. Activation of the first recombination system led to *in vivo* assembling of the flower-specific second recombinase system. In consequence, when the transgenic tree starts to flower, the second recombinase becomes active and removes all transgenic sequences from the pollen genome.

Another possibility to reposition transgenes in the genome is based on transposon action (Cotsaftis et al. 2002). The functionality of transposons was first shown by Fladung and Ahuja (1997) and Kumar and Fladung (2003). Later, mobility of transposons across chromosomal barriers was confirmed by Fladung (2011) and Fladung and Polak (2012). 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. Such a method could be useful for quickly generating large populations of T-DNA site-free transgenic plants.

7 Concluding Remarks

The predicted increase in population size together with accelerated climate change including weather extremes, droughts, floods, etc., will also increase the need for food production. This will definitely also affect the forests of the world. Tree breeding efforts have so far been quite slow and, generally, forest trees can still be considered as undomesticated compared to crop plants. For crop plants, all of the most important species are already genetically engineered and include transgenes providing insect or herbicide resistance or both. In mature markets the adoption rate of some GE crops is also high (>80 %).

Concerning forest trees, there is also a need to improve growth, increase resistance to biotic and abiotic stresses, and other traits such as wood properties or chemical composition. The latter might serve the future bioeconomy-oriented society. To reach all these goals, also non-transgenic alternatives and new breeding technologies should be critically considered and compared to GE technology, keeping in mind the importance to hasten the breeding efforts. Today, we already have a lot of information about GE technology and it is an everyday research work in molecular biology laboratories. In the case of forest tree species, we have a long experience on GE forest trees at laboratory, greenhouse and field trial level and therefore a lot of information has been gathered during the last 25 years. Quite recently, also other so-called mutagenesis-based methods have been developed/under development and also information on tree genome sequences is cumulating fast. All these may provide interesting breeding possibilities for the future, which could include utilization of both new breeding approaches (phenotype-genotype correlation, MAS) and transgenesis. Transgenic trees are on the way from lab research to trait-oriented uses in the next 25 years.

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