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Review

A review on transgenic approaches to accelerate breeding of woody plants

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With 6 figures

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Abstract

The long juvenile period of trees delays the breeding of new varieties. Flowering begins within 5–10 years in most cultivated forest trees under intensive management, but can take up to 40 years in some species and environmental conditions. To accelerate the breeding process several agrotechnical and biotechnical methods have been developed. Knowledge about genes controlling flower initiation in model plants like *Arabidopsis thaliana*, and identification of homologous genes in trees, have led to new possibilities for early-flower induction. Overexpression of MADS-box and other floral regulatory genes resulted in early flowering in some tree species and/or varieties. However, these methods have not yet been shown to enable the production of fertile, viable or normal gametes and progeny; developmental research towards these ends is therefore of high priority. A breeding scheme has been developed to use early flowering trees for the introduction of genes from wild species that would allow several backcrosses to occur in only a few years, and to produce at the end a non-transgenic improved variety. Research to develop practical early flowering methods could lead to several new methods for breeding and biotechnology.

Key words: Early flowering — transgenic — forest — fruit trees — mutation — GMO — genetic engineering

Breeding of both fruit and forest trees has a long tradition in Germany (Lochow Von 1929, Rudloff 1931, Langner 1957/58, Wolf 2006), although aims and progress differ between these two types of woody plants. Forest tree breeding is mainly focused on improvement of efficiency, quality and robustness. Following crosses between selected individuals, it is important to select robust and diverse populations as well as novel trait combinations and elite genotypes. For forest trees, breeding efforts are slowed by the long periods of evaluation and delayed onset of reproduction (Fladung 1998). Even though substantial breeding progress has been made with intensively bred pines, poplars, eucalypts and a few other taxa, forest tree 'cultivars' can still be considered as nearly 'wild plants' with few if any of the hallmarks of crop domestication (Fladung 2008).

On the other hand, cultivated fruit trees show a much higher degree of domestication, and feral wild relatives of most varieties are rare in Europe. One of the major objectives in fruit tree breeding is increased fruit marketability (Janick et al. 1996). Therefore, the attention of breeders is mainly focused on attractiveness and fruit compositional quality, represented by traits like skin colour, size and shape of the fruit, flesh texture and flavour. Additionally, improvement of resistance to plant diseases is always one of the most important breeding goals.

Even though the goals of fruit and forest tree breeding are significantly different, their breeding strategies have much in common. Breeding trees is much more time consuming than for annual plants, requires substantial space, and is much more expensive. Producing a new apple cultivar takes at least 15–20 years and costs approximately €400 000 (Ken Tobutt, personal communication cited in Fenning and Gershenzon 2002).

One of the major impediments is the long-lasting juvenile stage in which plants are not able to develop floral organs and fruits. Unlike annual herbaceous plants which flower within a few months, juvenility in tree species can last 5 to 40 or more years until the reproductive stage is reached. During juvenility no further genetic development of the plant material, such as backcrosses, inbreeding or production of new hybrids, is possible. During this time only selection based on juvenile traits can be done. In addition, trees reach a considerable height during the long juvenile period which makes the crossing procedure difficult. This is especially true for commercial scale breeding, where hundreds or thousands of crosses are normally made. This requires the investment in major infrastructure, equipment and requires a great deal of land area.

To flower, plants have to undergo a transition phase in which plants switch from the juvenile to the adult stage. Poplars, for example, usually reach the reproductive phase after 7–10 years (Hsu et al. 2006). Similar periods of time are known for fruit trees. In apple the juvenile stage can take 5–10 or even 12 years (Visser 1964, Fischer 1994), and in citrus up to 20 years (Peña et al. 2001). Therefore, shortening the juvenile stage is always an important breeding objective, and has been the subject of intensive horticultural research, generally using chemical or physical methods (Meilan 1997).

Many dozens of genes whose expression influences the structure and onset of flowering have been characterized in recent years. Most of these studies have been in the model *Arabidopsis*, whose entire genome sequence is known, and

which completes its life cycle in approximately 1 month in the greenhouse. There are a number of different developmental changes that occur during the transition to flowering, starting with the re-programming of the apical shoot meristem, through to the development of flowers and fruits (Henderson and Dean 2004, Putterill et al. 2004). Different endogenous and environmental signals are known to contribute to the transition of the apical meristem into an inflorescence and then a floral meristem. These include photoperiod and light quality, temperature (vernalization), hormone levels (especially gibberellic acid) and autonomous factors (Putterill et al. 2004, Roux et al. 2006).

Detailed models for control of flowering onset in *Arabidopsis* have been proposed by several groups (Putterill et al. 2004, Corbesier and Coupland 2006, Roux et al. 2006). Environmental signals, photoperiod and temperature, have two different targets (Corbesier and Coupland 2006), but both indirectly influence the *FLOWERING LOCUS T* (FT). The FT protein has been proposed as the long-postulated, mobile flower-inducing factor 'florigen' (Corbesier et al. 2007, Jaeger and Wigge 2007). Photoperiod is largely tracked by the action of *CONSTANS* (*CO*). Inactivation of *CO* causes late flowering, while its overexpression induces early flowering (Putterill et al. 2004, Wellmer et al. 2006). During long days, *CONSTANS* positively regulates the activity of the flowering-time gene *FT*. Low temperature (vernalization) strongly reduces transcription of *FLOWERING LOCUS C* (*FLC*), a negative regulator of *FT* transcription, so that *FT* transcription increases. *FLC* is also the target for the autonomous pathway leading to higher *FT* levels. *FT* activates genes like *APETALA 1* (*AP1*) and/or *SUPRESSOR OF CONSTANS* (*SOC1*). The hormone gibberellic acid directly induces the expression of *SOC1* which activates the *LEAFY* (*LFY*) gene. *AP1* and *LFY* are both flower meristem identity genes. After their activation floral meristem formation and organ differentiation begins.

A number of genes that regulate flowering time in woody plants have been isolated based on the results from model plant species. In experiments, testing the *Arabidopsis* genes or homologues isolated from trees via transformation, promising results were obtained that the juvenile stage of trees might be substantially reduced and tree breeding thereby accelerated (Elo et al. 2007, Flachowsky et al. 2007). Unfortunately, there are no studies to date that have shown that the breeding cycle has truly been reduced, mainly because, with the exception of *Citrus*, the precious flowers that have been induced have largely been abnormal, malfunctional or did not lead to fully mature pollen or seeds. We discuss the results to date, and how the development of effective early flowering technology could impact tree breeding programmes.

Limitations of Conventional Tree Breeding Programmes

Improvement of crop species is based on breeding large numbers of individuals, either as groups via mass pollination or in specific combinations. Varieties are then be obtained following repeated selection and backcrossing to homogenize and fix desired traits. In many forest tree species, however, the production of backcrossing is practically excluded because of the long generation cycle. Hence, in forest tree breeding, the concept of 'selection breeding' has been created. This concept is based on the assessment of an F_1 progeny of two elite or 'plus'-trees with respect to forestry relevant traits (hybrid breeding) (Lochow Von 1929, Wolf 2006), or via making crosses between groups of individuals whose pollen has been mixed. Practically 'plus'-trees are well-chosen from natural or planted stands according to phenotypic criteria. After mature flowering twigs are harvested and grafted onto rootstocks, they can produce seeds within one to several years after grafting. These grafted plants are used in seed orchards where the trees mate randomly. Because of the lower height of the 'stopper bushes' (rootstocks), seeds can be harvested much easier than from large old trees (Langner 1957/58). Flowering is also often stimulated using chemical and physical treatments, and the trees pruned to keep them of manageable height.

A number of advances have been made during the last 100 years of forest tree breeding. Marked improvements in growth rate have attributable to interspecific crosses. This includes hybrids between Japanese and European larch, *Populus deltoides* × *Populus nigra* (*Populus* × *canadensis*), and between North American and European aspen species. Specific full-sib families and clones in different pine species (e.g., *Pinus radiata*, *P. pinaster*, *P. taeda*), usually not hybrids, have shown greatly improved wood production, straightness in their stems and biotic resistance. In Germany, the production of frost-hardy Douglas firs from crosses between the two major American varieties, the selection and micro-vegetative propagation of rowanberry clones, and 'cross breeding' in sessile and pedunculate oaks and black alder are also considered important successes (Wolf 2006). Nonetheless, the breeding effort has been very slow, having been restricted in part by the long-lasting juvenile stage of trees.

With the recent increasing importance of *Populus* and *Salix* (willow) breeding for bioenergy purposes in recent years, it is highly desirable to speed up their breeding cycles. Members of the two genera are fast growing with low demands for soil quality and fertilization, and therefore are good candidates for short rotation plantation forestry. Tools are already established that can help to improve the success of poplar and willow breeding, like effective morphological characterization as well as the discovery of molecular markers for early selection (marker-assisted selection) (Fladung 1998, Fladung and Muhs 2001, Butcher and Southerton 2007). The juvenile period remains a constraint on breeding, especially for poplar, and especially for the application of advanced methods such as in introgression of novel alleles from other species.

A similar situation exists for fruit tree breeding. The selection of apple trees with improved traits was formerly done by collecting open-pollinated seeds from attractive fruits. These seeds were sown and interesting phenotypes from seedlings were selected. This long-established method was replaced by deliberate hybridization about 200 years ago (Gardiner et al. 2007). The first crosses using controlled pollination were performed by Thomas A. Knight in 1806 (Brown 1975). Since that time the performance of controlled crosses between selected parents and the selection of the best seedlings from full-sib families has been the most common breeding method (Noiton and Shelbourne 1992). The success of such a breeding strategy is limited by the fact that apple has a long-lasting juvenile stage which can take 5–12 years (Fischer 1994).

Accelerated selection in the juvenile stage is only realistic for traits such as simply inherited disease resistance such as resistance to apple scab caused by *Venturia inaequalis*, which can be evaluated on seedlings and which are not related to the fruit. A complete evaluation of the progenies can only be started when the trees have reached the adult stage in which

they develop flowers and fruits. Likewise, for forest trees, traits related to wood quality generally require several years before accurate selection is possible. Fortunately, for some species and varieties, there are effective conventional treatments that can help to accelerate onset of flowering (Longman and Wareing 1959, Chalupka and Cecich 1997). However, these often still a few several years, or are ineffective in specific taxa or genotypes.

Genes for quality, adaptability and diseases resistance are often present in wild relatives of cultivated genotypes. However, the time until release of a new variety using such genes is especially long as a result of the additional breeding needed to remove the unwanted traits that also come along during interspecific hybridization. A good example is the introduction of the scab-resistance gene *Vf* from the crab-apple *Malus* × *floribunda* 821. 'Prima' (Dayton et al. 1970), the first scab-resistant cultivar, which was introduced about 30 years after the Purdue–Rutgers–Illinois (PRI) breeding programme was started specifically to develop scab-resistant varieties (Crosby et al. 1992, for review see Gardiner et al. 2007). Roughly one hundred scab-resistant cultivars have been released (Gessler et al. 2006), but most of them are not of sufficient quality to compete with the global-leading varieties. Among the current top 10 world cultivars, there are no scab-resistant varieties, yet we have seen more than 50 years of scab-resistance breeding. Only 4 of the top 10 cultivars were derived from advanced breeding programmes at all (O'Rourke et al. 2003), the others being derived from open-pollination. It is clear that the benefits for fruit tree breeding from accelerated flowering could be large.

Early Flowering Mutants in Woody Plants

If there is sufficient genetic diversity, it may be feasible to breed for early flowering using conventional means. However, this appears to be very difficult in most species. Very few natural early flowering mutants are known in woody plants. For *Betula verrucosa*, Stern (1961) proposed that the generation cycle could be shortened down to 2 years but would require very intensive selection. The author also described that for practical reasons breeding for very rapid onset of flowering might be accompanied by reduction of the fertility of female flowers.

In *Populus alba* an early flowering genotype, which forms female flowers within 1 year after sowing, was identified after intensive selection within a breeding programme (Meilan et al. 2004). However, the very early flowering of this line, after vegetative propagation, was not repeated in a field trial in the USA (Strauss, unpublished results). Extensive flowering did occur, however, in the second growing season. Flowering in the first year for this genotype was also not observed in Germany (Hönicka and Fladung, unpublished results).

For *Eucalyptus*, an early flowering mutant found in a nursery in northern Brazil was described by Missiaggia et al. (2005). This mutant started to flower approximately 90 days from germination. Mendelian segregation of 1 : 1 of the early flowering character was found among progeny following crossing with normal flowering trees. Missiaggia et al. (2005) successfully located the early flowering locus in an existing microsatellite reference map, and efforts to identify the causative gene are underway.

An early flowering mutant in spruce was described in *Picea abies* 'acrocona' in Sweden in ~1890 (cited in Langner 1954). The growth habit of the tree is short and compact (Fig. 1a), needles are fresh-green in the flushing shoot and become dark green later. The special characteristic of the mutant is the formation of mostly female cones at the end of the shoot, but sometimes male flowers are also formed. In 1993, selfings and controlled crosses were carried out with a single *P. abies* 'acrocona' plant. In total 301 seedlings were obtained and planted in 1995, and in 1998, nine of about 300 3-year-old plants formed female cones. In extreme cases 4 to 5-year-old plants had a terminal cone in almost every branch. In another population from this cross a female cone was observed even in 1-year-old plants (Fig. 1b). The 'acrocona' mutation was confirmed as dominant based on Mendelian ratios (Langner 1954, Fladung, unpublished results). A family with a total of 81 individuals was used to map the 'acrocona' locus, and it was located on linkage group VI of *Picea* (Acheré et al. 2004). The molecular basis for this mutation is still unknown; however, based on other research candidate genes for 'acrocona' are the *DEFICIENS-AGAMOUS-LIKE* (*DAL*) and the two *LEAFY* genes detected in conifer species (Mouradov et al. 1998). Because of the large genome size and difficult transformation of conifers, chromosome walking to the locus and transgenic confirmation will be difficult. However, fine mapping of SNPs is possible if the progeny size can be increased substantially.

Seedlings with a dramatically shortened juvenile stage were also described for trifoliate orange [*Poncirus trifoliate* (L.) Raf]. Three seedlings with precocious flowering were found in 1975 at the Central Horticultural Experiment Station in Chethalli, India (Yadav et al. 1980). These seedlings produced

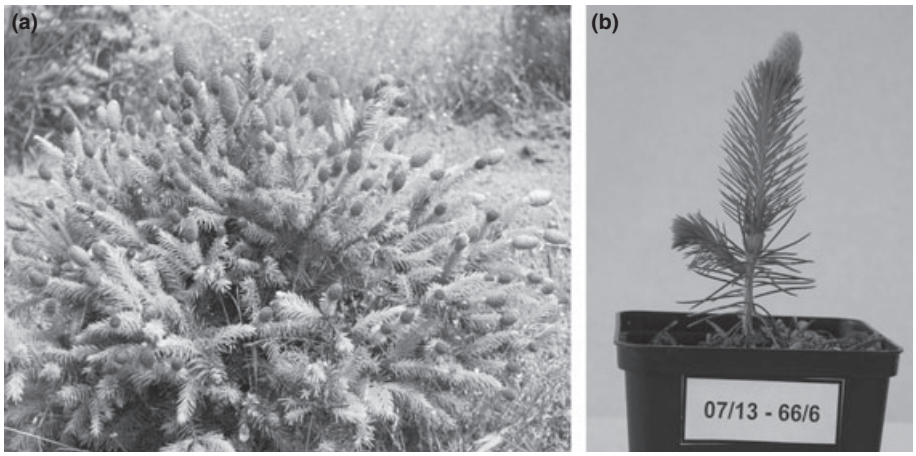

Fig. 1: (a) *Picea abies*, acrocena' F_1-seedling, nine years old. Flower formation occurred at age three to four for the first. (b) One year old seedling revealing a terminal female cone

their first flowers only 9 months after germination and seedling growth had begun, whereas 7–8 years of juvenility are normal in *Poncirus*. Two seedlings reverted back to the juvenile phase in 1976, but one seedling continued flowering. Seeds of this *Poncirus* genotype were raised and the F_1 seedlings exhibited the same phenotype. Early flowering was also reported for grape fruit (*Citrus paradisi*), pummelo (*Citrus grandis* L.) and their respective hybrids, as well as for yamamikan (*Citrus intermedia* Hort ex Tanaka) and ponkan (*Citrus reticulata* cv. 'Blanko') (for review see Holland et al. 1995). For these species, precocious flowering could also be obtained after application of inductive treatments. However, in these cases, after flowering the plants reverted back to the juvenile stage for several years.

Differences in the duration of the juvenile stage of seedlings were also described for the temperate fruit tree species apple, pear, apricot and cherry (Visser 1964, 1965, 1967, 1970, Hjeltnes 2004), but mutants flowering within months after seed germination were not described. Zielinski (1963) and Visser (1976) showed that early flowering was an inherited trait in apple and pear.

Agrotechnical Approaches to Induce Early Flowering in Trees

A range of agrotechnical approaches have been tested on different tree species with the aim of reducing the duration of the juvenile stage. For poplar it was reported that the application of hormones or growth retardants (daminozide, paclobutrazol) induces early flowering in grafts from mature trees. Yuceer et al. (2003) described the application of a combined treatment of water stress, root pruning and paclobutrazol to 3-month-old rooted cuttings from mature *P. deltoides*. All treated cuttings started to flower whereas non-treated controls formed only flower buds. However, 1-year-old rooted cuttings from juvenile plants did not form flowers when treated similarly. These results were confirmed in investigations by Chalupka and Cecich (1997) and Fladung and Hoenicka (2004). Early flowering was described in 13 to 16-week-old seedlings of *Eucalyptus occidentalis* (Southerton 2007) when cultivated under high fertility and irrigation with a long-day photoperiod (16 h of light daily). In 2 to 3-year-old *Pinus banksiana* plants, Cecich et al. (1994) described early flowering after external application of gibberellic acid. Following extensive investigations in *Pinus nigra* on genetic variation in flowering, Matziris (1994) concluded that the time of the first flowering is under strict genetic control.

Similar studies were performed on fruit trees (for review see Hanke et al. 2007). Based the work of Visser (1964, 1965, 1970) and others, it is widely known that the juvenile period of apple can be shortened by using early flowering genotypes as parents. A variety of techniques can promote induction including grafting of seedling scions onto appropriate rootstocks, extension of the growing season via light/temperature supplementation, trunk ringing, bark scoring and inversion, root pruning and fertilization. Defoliation and placing shoots in a horizontal position can also have promoting effects (Longman et al. 1965, Tromp 1967, 1968, Taylor et al. 1984).

One of the most common methods for accelerating the onset of flowering in fruit tree breeding is the application of growth promoting cultural practices, so that seedlings pass from the germination stage to the transition to flowering rapidly (Visser 1964, Aldwinckle 1975). Rapid growth and grafting of seedling scions onto a dwarfing rootstock (Hackett 1985) can reduce the juvenile stage dramatically, thus accelerating breeding (Fig. 2, as first presented by Fischer 1994). This system starts in January with sowing apple seeds in the greenhouse. After selection for scab-resistance in March/April the seedlings are cultivated in the greenhouse for 2 years by application of 16 h total light in the first months of growing. In the spring of the third year the seedlings are pruned at a height of 180 cm above soil level. The part above 180 cm stem height in general is adult and used as scion and directly grafted onto precultivated interstem Hibernal/M.9 rootstocks in the field. Using this method, some of the trees begin to flower in the same year (3 years after seeding instead of 5–12 years). If no screening for scab-resistance is conducted, collecting adult wood can be done 1 year following sowing of seeds, but only a segment of each population will directly flower following grafting on to planted interstem Hibernal/M.9 rootstocks growing in the field in the following spring. A further reduction of the juvenile stage appears to be impossible because most of

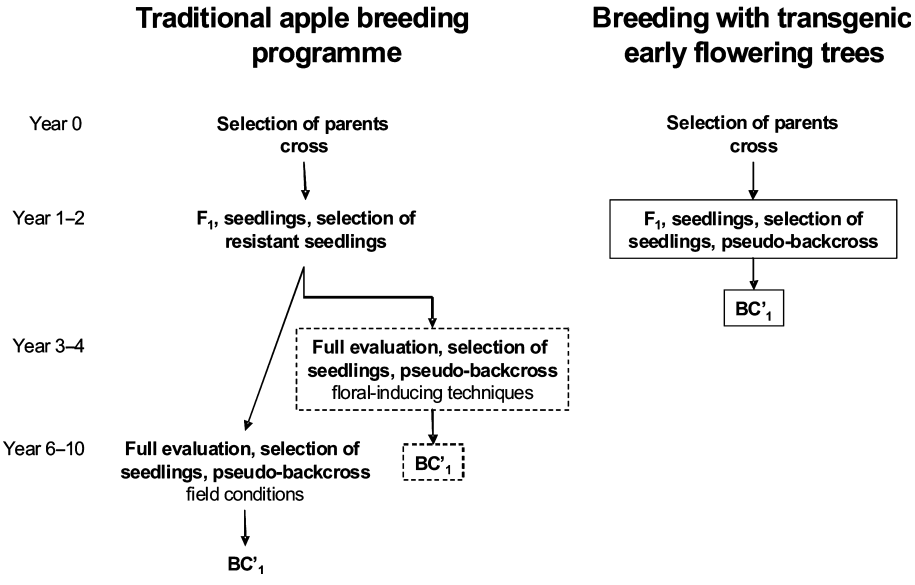

Fig. 2: Comparison of different breeding strategies. Time scale of traditional breeding programmes using natural conditions in the field (left) and using floral-inducing techniques (dashed box) in comparison with a breeding programme which is based on the use of transgenic early flowering plants (right); F_1 first filial generation; BC'_1 first pseudo-backcross generation; dashed box - reduction of one breeding cycle up to seven years by using floral inducing techniques; black framed box – further reduction of one breeding cycle by using transgenic early flowering plants

the floral-inducing techniques can be successfully applied first only after the plant has reached the transition phase (Hanke et al. 2007).

To summarize, in fruit trees it does not appear possible to get flowers earlier than in the second year after sowing. Nevertheless, the use of floral-inducing techniques for fruit tree breeding was a first step to reduce the breeding cycle dramatically (Fig. 2), although not all genotypes respond well to treatment. In contrast to many fruit trees, where flower formation was successfully achieved using such agrotechnical approaches, many forest tree species are recalcitrant (Chalupka and Cecich 1997).

Biotechnological Approaches to Induce Early Flowering in Trees

Because early flowering genotypes are often unavailable or infrequent, and agrotechnical approaches are not always successful, a variety of transgenic approaches have been tested. Some have involved genes with general physiological effects, and others were directed at genes known to be involved in floral development. In the former case, the *rolD* gene from the Ri plasmid of *Agrobacterium rhizogenes* was shown to induce early flowering in transgenic tobacco (Mauro et al. 1996). However, this effect did not occur in transgenic poplar (Hoeicka and Fladung, unpublished). However, another gene from the Ri-Plasmid of *A. rhizogenes*, the *rolC* gene, under control of the constitutive CaMV35S promoter, led to a clear reduction of the juvenile phase in transgenic poplar. However, the use of the *rbcS* promoter with the *rolC* gene had no effect on flowering in transgenic poplars. However, all of these genes have also undesired pleiotropic effects on plant growth and physiology, so would be difficult to apply in breeding programmes.

More promising is the transfer of genes that are specifically involved in the flowering pathway. Several of these genes are members of the MADS-box gene family that is characterized by a highly conserved protein motif, the MADS-box. MADS-box genes are found in many eukaryotic organisms and encode transcription factors which are involved in diverse biological functions. This includes the transition from vegetative to reproductive growth, determination of floral-organ identity, development of vegetative tissues, senescence, winter dormancy and many others (reviewed in Theissen et al. 2000, Saedler et al. 2001). The majority of basic studies on MADS-box genes have been carried out in *Arabidopsis* (Theissen et al. 2000), however, increasing information is appearing from fruit and forest tree species (reviewed in Cseke and Podila 2004).

The overexpression of several MADS-box genes in deciduous tree species and other plants has been described in a number of papers. In birch (*Betula pendula*) various MADS-box genes have been identified and overexpressed in tobacco and birch (Elo et al. 2001). The overexpression of the *FRUITFULL* (*FUL*)-homolog *BpMADS4* has been found to induce early flowering in birch (Elo et al. 2007) and apple (Flachowsky et al. 2007) with a dramatic reduction of the juvenile period. In contrast to apple and birch, no early flowering was obtained in transgenic poplar constitutively overexpressing the *BpMADS4* gene (Hoenicka et al. 2008). In *BpMADS4* transgenic poplar, bud-set was delayed, an effect also seen in *BpMADS4* transgenic apple (Flachowsky et al., unpublished).

The induction of early flowering in other forest tree species has had highly variable levels of success (Meilan et al. 2001). The non-MADS-box meristem identity gene *LFY* from *Arabidopsis* has been successfully used for the induction of early flowering in poplar (Weigel and Nilsson 1995, Rottmann et al. 2000, Hoenicka and Fladung 2006). However, transgenic poplar plants transformed with the poplar homologue *PTLF* show early flowering only seldomly, varying widely among genotypes (Rottmann et al. 2000). Interestingly, female poplar clones overexpressing *LFY* sometimes revealed a gender change from female to male. Transgenic male clones with down-regulated *PTLF* have also shown a sex change to female (Rottmann et al. 2000, Hoeicka and Fladung, unpublished). The *Arabidopsis LFY* gene was also tested in fruit trees, but with different results. In transgenic citrus plants the juvenile phase was reduced (Peña et al. 2001). However, the same gene was less successful in transgenic apple (reviewed in Hanke et al. 2007). Identical results were obtained by Kotoda et al. (2003), who overexpressed the apple *LFY*-like genes *AFL1* and *AFL2* in transgenic apple without observing precocious flowering.

The MADS-box gene *FT* from *Arabidopsis* and the poplar homologues *PtFT* and *PtFT2* induced early flowering in poplar with high efficiency (Böhlenius et al. 2006, Hsu et al. 2006, Hoenicka and Fladung, unpublished). *FT* transgenic plants were shown to form catkins with what appeared to be fertile male or female organs, while *LFY* transgenics produced only single flowers and have never been shown to produce viable pollen or seeds in poplar. In combination with *CO* the *FT* gene plays a key role in flowering time in response to variations in daylength, short-day induced growth cessation and bud set occurring in fall (Böhlenius et al. 2006). The citrus *FT* homolog (*CiFT*) described by Hisada et al. (1997) was successfully used to accelerate flowering in trifoliate orange *P. trifoliata* L. Raf. (Endo et al. 2005) and in pear *Pyrus communis* L. (Matsuda et al. 2006). In pear the overexpression of *CiFT* led to *in vitro* flowering from transgenic shoots. The suppression of the apple *TERMINAL FLOWER 1* homolog *MdTFL1* resulted in a significantly reduced juvenile period as recently reported by Kotoda et al. (2006). The first solitary flowers were detected 8 months after grafting on glasshouse plants (Kotoda et al. 2003).

Other genes involved in early flowering in *Arabidopsis,* like *AP1* and *CAL* which act downstream the flowering signal (Henderson and Dean 2004) were also tested in woody plants. Peña and colleagues found that the constitutive overexpression of the *Arabidopsis AP1* gene induces early flowering in transgenic citrus plants (Peña et al. 2001). Contradictory results were obtained in transgenic apple plants overexpressing the *AP1* gene of *Arabidopsis* and the apple homologue *MdMADS5*/*MdAP1*. Kim et al. (2006) recently reported that the overexpression of the *AP1* homologue *MdMADS5*/*MdAP1* in 'Fuji' resulted in a dramatic reduction of the juvenile stage. First flowers were already developed on transgenic shoots during *in vitro* cultivation. However, in the apple variety 'Orin' the overexpression of *MdMADS5*/*MdAP1* did not result in early flowering (Kotoda et al. 2003) and accelerated flowering was not observed after constitutive overexpression of the *Arabidopsis AP1* gene in the apple rootstock 'M.26' (reviewed in Hanke et al. 2007). In poplar, *AP1* did not promote precocious flowering, nor did the other genes tested apart from *LFY* (*OsMADS1*, *CONSTANS*, *AGL20*) (Strauss et al. 2004). The promoter of *PTD* (an

Arabidopsis AP3 homologue) has been successfully used in poplar to express sterility genes without any impact on yield traits in a greenhouse trial (Skinner et al. 2003).

Experiments Using Early Flowering Transgenic Trees in Cross-Breeding Programmes

For poplar the process by which a flower-inducing transgene like *FT* might be employed is straightforward. A female poplar clone selected for a specific trait would first be transformed with *FT*. The induced flowers are pollinated with pollen of any male 'plus'-tree (not early flowering). Seeds are harvested and germinated. As with other characteristics the trait 'early flowering' segregates following Mendelian inheritance. Those trees carrying the early flowering gene *FT* start to flower 6–12 months after germination, while the others, not carrying *FT* do not flower. Using molecular markers, the characteristics of both parental trees can easily be confirmed in the seedlings. Early flowering male and female seedlings carrying the desired parental traits, preferably using molecular markers linked to those traits to enable selection in the juvenile stage, are then selected for back crossing. This circle can be repeated several times until the desired parental traits are combined in a seedling population not carrying any of the early flowering genes. Thus, simple Mendel genetics combined with DNA markers can be used to (a) introgress new, desired genes and (b) eliminate the early flowering gene (transgene). Because of the occurrence of bisexual flowers with *LEAFY*, use of this early flowering gene may also enable self pollinations. Of course, for rapid breeding progress the trait must be highly heritable and expressed in the juvenile phase, or very tightly linked to molecular markers previously identified to allow marker-aided breeding. This would be most feasible for traits such as oligogenically-controlled disease resistance or simple aspects of fruit quality.

In contrast to poplar, where such a breeding programme is still a hypothesis, in fruit trees the proof of concept was recently provided in apple. Using transgenic apple plants overexpressing the *BpMADS4* gene of silver birch (Flachowsky et al. 2007), a preliminary small breeding programme was started in winter 2005/2006. The early flowering trait is being used to obtain several crossbred generations in a few years. Such a strategy appeared to be useful to introduce single traits from distant apple wild species into the genome of the cultivated apple and to subsequently eliminate unwanted negative traits by numerous pseudo-backcrosses with high quality cultivars. Several backcrosses might be manageable within a decade if large progenies are produced, phenotypic evaluations are done rapidly and precisely, and the traits have a simple, strongly inherited genetic basis. In later stages of the breeding programme the transgene can be out-crossed (Fig. 3) aiming for a genetically improved, but still, non-transgenic plant (Elo et al. 2007).

The *BpMADS4* expressing apple clone T1190 (described by Flachowsky et al. 2007) was selected as the most promising genotype for this programme. This line has a single integration of the transferred T-DNA and first flowers were obtained within few months. In a preliminary test, glasshouse-grown plants of line T1190 were pollinated by wild species to find out ideal culture conditions for fruit and seed production. It was shown that pollination of one to three flowers per plant is most effective; the remaining flowers were removed by hand to avoid fruit drop. In winter 2005/2006 several plants of line T1190 were pollinated by wild species *Malus fusca* as described above. Three fruits with a total of 11 seeds were harvested in fall 2006, and the seeds were stratified and sown in winter 2006/2007. A total of seven seedlings were obtained and tested for the presence of the foreign genes *npt*II and *BpMADS4* by PCR and Southern hybridization (data not presented). Four of seven seedlings were transgenic whereas three seedlings were non-transgenic. The transgenic seedlings flowered within a few weeks after they had reached about 40 cm in height (Fig. 4). No flowers could be found in the first season on the non-

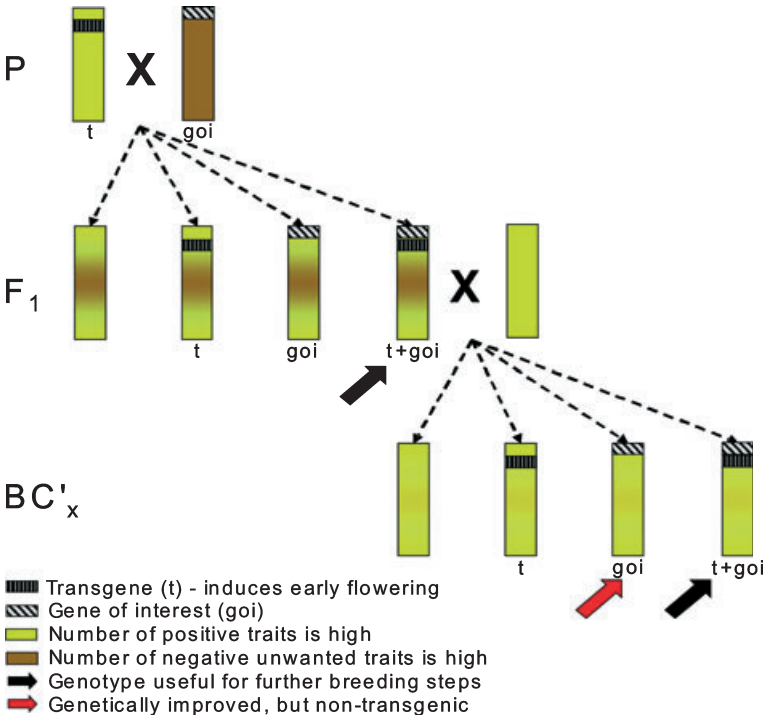

Fig. 3: Intended breeding programme using transgenic early flowering genotypes. Brown – wild species containing the gene of interest; green-high quality apple cultivar; P parental generation; F_1 first filial generation, seedlings contain 50% genome of each parent; BC'x generation after numerous pseudo-backcrosses 't + goi' genotypes by high quality cultivars, the percentage of wild species genome was reduced by convergence breeding. The arrows show genotypes we are looking for

Fig. 4: Transgenic F_1 apple seedlings of a cross between the transgenic line T1190 cv. 'Pinova' (Flachowsky et al., 2007) and the apple wild species *Malus fusca*. First flowers were obtained after the seedling has reached about 40 cm in height

transgenic seedlings. The transgenic seedlings were pollinated in spring 2007 with pollen of the apple cv. 'Topaz'. In October 2007 the resulting fruits were harvested and a total of 41 seeds were obtained. These seeds were stratified and sown in winter 2007/2008. It was shown that one crossbred generation per year is feasible. The actual stage of this experiment is summarized in Fig. 5.

Conclusions for Future Research

In this paper we propose that transgenic trees could be an important tool for speeding up breeding cycles for traits that can be rapidly determined in juvenile plants, or directly assessed via molecular markers. However, the use of plants constitutively overexpressing a flower inducing gene like *BpMADS4* is problematic. The transgenic plants are often malformed and the fruit yield and seed set is very low (Elo et al. 2007, Flachowsky et al. 2007). These problems could possibly be solved by using the transgenic pollen for pollination of flowers from non-transgenic trees, which are regularly developed. The use of an inducible promoter that can be turned on temporarily for flower production, as suggested by Flachowsky et al. (2007), may eventually help to solve this problem. Heat induction of *FT* genes have been successfully used in poplar to induce precocious flowering (Fig. 6; Strauss, unpublished data). Using the *FT* gene, whose derived protein product is mobile within plants (Corbesier et al. 2007), it should be possible to induce a flower inducing signal that moves from a transgenic rootstock to a non-transgenic scion thus avoiding any inheritance of transgenes, or risk of their release into the environment from the outdoor seed and breeding orchards that are common in tree breeding. Experiments are under-

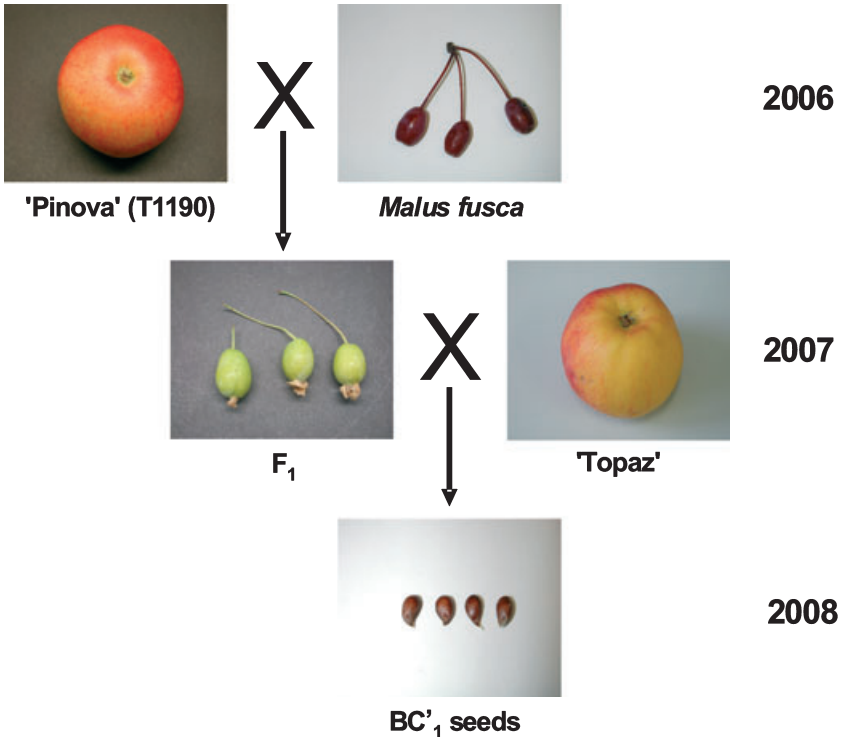

Fig. 5: Stages of experiments with transgenic apple trees in a first crossbred-breeding programme. Using the *BpMADS4* transgenic apple line T1190 cv. 'Pinova' (Flachowsky et a1., 2007) it was shown that one crossbred generation per year could be realized. The selection of the best seedlings for the next cross depends on the trait/s of interest and on the availability of usable selection methods (e.g. molecular markers)

Fig. 6: Image of a catkin produced from heat-shock induced expression of the Arabidopsis *FT* gene in a transgenic male hybrid aspen (*P. tremula* x *tremuloides*, INRA 353-53) growing in a greenhouse in Corvallis, Oregon, USA. Photo provided by Huanling Zhang, Oregon State University

way in our laboratories to test this system concept in apple and poplar.

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