An Early-Flowering Genotype of *Populus*

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Unlike herbaceous, annual crops, trees are not highly domesticated and, therefore, have wild relatives with which they are interfertile. They are also long-lived perennials that produce copious amounts of pollen and seed, which are often disseminated over considerable distances by the wind. Federal regulators have made it clear that before transgenic trees can be grown commercially in the U.S., it will be necessary to develop a strategy to mitigate the risk of transgene spread into the environment. One way to satisfy this requirement is to genetically engineer reproductive sterility. Because of its many useful attributes, poplar has become *the* model tree species for research community. However, because of its relatively long juvenile period, the development of a reliable sterility system for poplar is taking longer than expected. By having an early-flowering genotype of poplar, it will be possible to make much faster progress in our efforts to develop a reliable transgene-confinement system. We have identified a genotype of *Populus alba* that can be induced to flower within nine months of being regenerated.

Keywords: genetic engineering, *Populus*, precocious flowering, sterility

Genetic engineering has been used to introduce commercially useful traits, such as insect resistance, altered seed composition, and herbicide tolerance, into a wide variety of herbaceous agronomic crops (http://www.aphis.usda.gov/biotech/). The potential benefit of these introduced traits has also been demonstrated in transgenic trees (Meilan et al., 2002; Tzfira et al., 1998). A major concern over the use of transgenic trees, however, is the potential for transgene dispersal through pollen and seeds (Ellstrand, 2001; Kuvshinov et al., 2001; Meilan et al., 2001). Most trees are cultivated in close proximity to wild or feral relatives, increasing the probability of transgene spread (Skinner et al., 2000; Strauss et al., 1995). A major effort is underway to engineer reproductive sterility, which could be used to mitigate the risk of transgene migration (Meilan et al., 2004).

Poplar is an important model tree species because of its: 1) small genome, only 550 Mbp (similar to rice, only 4X larger than *Arabidopsis*, and 40X smaller than pine); 2) rapid juvenile growth, allowing rapid phenotypic assessments; 3) ease of transformation/regeneration; and 4) facile clonal propagation (Brunner et al., 2003; Han et al., 2000; Kim et al., 1997). Moreover, a 6.8X draft of its entire genome sequence has recently

been made available to the public (http://genome.jgi-psf.org/poplar0/poplar0.home.html). Although poplar has a relatively short juvenile phase, in comparison with other tree species, it still requires between five and seven years to become sexually mature (Braatne et al., 1996). This delay poses a significant impediment to more rapid progress in engineering flowering control.

Techniques used to accelerate flowering in gymnosperms are generally not successful when used with woody angiosperms (Meilan, 1997). Attempts to stimulate poplar to flower early using conventional means have not been successful (Strauss et al., 1998). Strauss et al. (1999) have also ectopically expressed in variety of poplar genotypes several genes (*AGL20*, *CONSTANS*, *OsMADS1*, and *APETELA1*, all under control of the 35S promoter) known to accelerate flowering in heterologous species. None of the independent poplar lines produced resulted in an early-flowering phenotype (51 lines produced with *AGL20* in four poplar genotypes, 71 lines with *CONSTANS* in five genotypes, 28 lines with *OsMADS1* in two genotypes, and 46 lines with *APETELA1* in three genotypes).

Constitutive expression of the *Arabidopsis thaliana* *LEAFY* gene in hybrid aspen led to the production of precocious flowers in hybrid aspen (Weigel and Nilsson, 1995). Unfortunately, ectopitc *LEAFY* expression caused all vegetative meristems to be converted to reproductive meristems which ultimately were lethal. In similar work

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by Strauss et al. (1997), male hybrid aspen (INRA 353-38; *Populus tremula* x *P. tremuloides*) flowered repeatedly in the greenhouse (GH) when transformed with 35S::*LEAFY* but it was not possible to regenerate primary transformants *in vitro*. Yuceer et al. (2003) recently reported that paclobutrazol (a gibberellic acid biosynthesis inhibitor) treatment combined with root pruning stimiluated flowering in three-month-old rooted cuttings of *P. deltoides*.

Lavendar and Zaerr (1985) reported that maintaining soil temperatures at about 3°C for six months stimulated the production of both male and female floral buds on Douglas-fir (*Pseudotsuga menziesii*) scions that were grafted onto four-year-old seedling rootstocks. In this report, we describe a genotype of *P. abla* that can be induced to flower within nine months of being propagated vegetatively through the use of the same root chilling technique.

MATERIALS AND METHODS

Germplasm Resources

During 1988 and 1989 seeds were collected from *P. alba* growing in different provenances along the Italian peninsula, spanning latitudes from 40° to 46°N. Seeds were sown immediately after collection and the resulting seedlings were transplanted into a nursery to serve as a germplasm source for future studies. In 1991, a *P. alba* common garden study was established in Northern Italy (Cavallermaggiore, Cuneo province) using cuttings taken from 706 genotypes belonging to 50 half-sib families and 10 provenances. At the end of February, one-year-old stems were harvested from stool beds to produce 30-cm hardwood cuttings. This material was stored at 4°C in sealed plastic bags; several days before planting the cuttings were rehydrated. In mid-March, the cuttings were planted in a sandy loam soil on a 3 x 1 m grid, with no more than 2-3 cm of the cutting exposed. Plants were cultivated following the normal practice used in the nursery but no irrigation was provided during the growing season. The early flowering clone, designated 6K10, was the only plant among all the planted genotypes of the common garden study to bear flowal buds at the end of the first year of vegetative growth (Fig. 1). This clone (genotype number 10) was obtained from an open-pollinated seedlot collected in 1989 on a wild mother plant (number 6) along the Bormida River (provenance K) near Carcare (Savona province, Italy; 44° 21'N latitude, 8° 17'E longitude). When hardwood cuttings taken from 6K10 were rooted and grown in the greenhouse, the resulting plants flowered. These plants were maintained at the Uni-
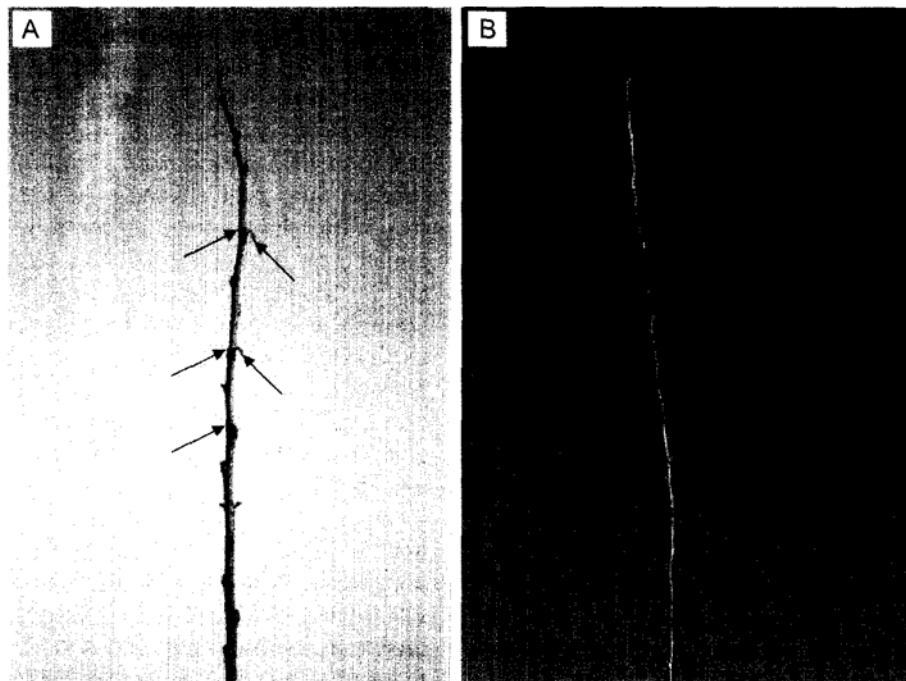
Figure 1. One-year-old *Populus alba* growing in the field in northern Italy in the spring. A) Clone 6K10 (arrows indicate catkins). B) Clone 6K6t, a normal (non-flowering) member of the same half-sib family as the flowering genotype.
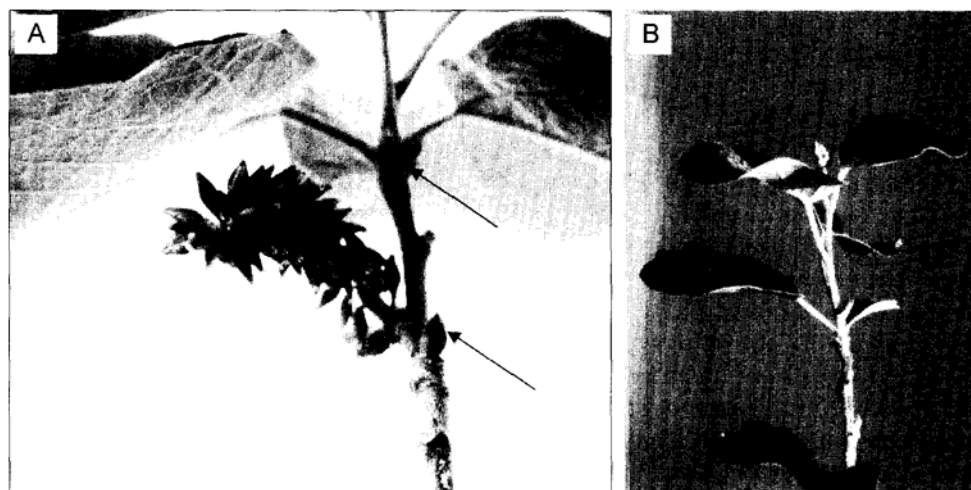
Figure 2. Nine-month-old *P. alba* clone 6K10 growing in the greenhouse. **A)** Catkin on a ramet that received inductive treatments. Conditions included six months of root chilling (4°C), eight weeks in a growth room set for short days (8-h photoperiod) and constantly cool temperatures (13°C), and ten weeks in a walk-in cooler (4°C), before plants were moved to a greenhouse to induce bud-flush. Three out of the four plants receiving this combination of treatments produced between one and three buds each. Arrows indicate floral buds that have not yet flushed. **B)** A ramet of the same age that did not receive inductive treatments.

versity of Tuscia experimental farm (Viterbo, Italy) and used to regenerate plants for export to the U.S.

Establishment In Vitro

Leaf explants were surface sterilized by exposure to 70% ethanol for 5 min, followed by a 1.05% solution of sodium hypochlorite containing 0.1% Triton X-100 for 25 min (treatments performed on an orbital shaker, 200 rpm). Explants were rinsed five times with sterile ddH$_2$0 before undergoing organogenic induction (Leple et al., 1992). The resulting in vitro grown plantlets were imported into the U.S. under USDA-APHIS Departmental Permit number #62673.

Propagation

Plants were propagated *in vitro* by excising the top 1 cm from each plant and inserting the cut surfaces into root-induction media (Han et al., 2000). When approximately 25 cm tall, 160 plants were transplanted into 2.5-inch Rosepots (OBC Northwest Inc., USA) containing a 1:1 Sunshine mix and perlite (McConkey Co., USA) potting soil for conditioning as described by Meilan et al. (2002). After one month, plants were transferred to long, cylindrical containers (approximately 7 cm in diameter and 22 cm long) that were sealed at their bases and filled with the same potting soil.

Induction Treatments

When it became obvious that the imported plants that were propagated *in vitro* had lost the ability to flower precociously, we propagated 50 additional plantlets for a preliminary inductive experiment. Ten plants were randomly assigned to each of five groups. Inductive treatments included: fertilization (fertilized plants weekly with 200 ppm of 20:10:20 fertilizer), water stress (plants were watered only after they began to wilt), paclobutrazol (250 ml of 75 ppm, root drench), and root chilling (4°C for six months); the rest of the plants served as controls. Treatments were initiated on 2 March 2001. On 10 August 2001, five out of 10 plants from each treatment group, plus a control, were transferred to a GH where they were exposed to an 8-h photoperiod (with the aid of an aluminized curtain that was automatically drawn and retracted each day under the control of a 24-h clock) and constantly cool temperatures (10°C) until buds were set. The remaining plants were moved to another GH, where they were grown under supplementary light (sodium halide lamps, 16-h photoperiods). Day and night temperature settings in the latter, long-day GH were 27.5°C and 16.0°C, respectively. Once buds were set by the plants exposed to short days, they were transferred to a walk-in cold room (4°C) for 8 weeks to satisfy their chilling requirement. These plants were then transferred to the long-day GH.

In a subsequent experiment, we tested various concentrations of paclobutrazol (0, 5, 10, 25, 50 and 75 ppm, all root drenches). Five rooted plants each received 1,250 mL of a given treatment and were grown for two weeks in a GH before being transferred to a growth room (GR), where they were exposed to short days (8-h photoperiod) and constantly cool temperatures (13°C). After two months, all plants were moved to the long-day GH.

Root Chilling

Containers (7 cm x 22 cm) for 150 four-week-old plants were immersed in a water bath maintained at 4°C. The above-ground parts for the root-chilled plants and 10 control plants were exposed to normal GH conditions (16-h photoperiod, with supplemental lighting from high-pressure sodium lamps; 24/13°C day/night temperatures). At monthly intervals, for six months, 25 plants were removed from the water bath and transferred to the short-day GR (described above) to induce bud set. After eight weeks in the GR, five of the cohort of 25 plants were moved from the GR to the GH to flush buds immediately; at the same time, another five plants were transferred from the GR to a walk-in cooler (4°C) for 10 weeks, to satisfy their chilling requirement, before being moved to the GH to induce bud-flush. At monthly intervals, the remainder of the plants in the GR were transferred, in groups of five, to the long-day GH. Due to mortality, only four plants remained for the final transfer from the GR to the walk-in cooler.

RESULTS

Initial Experiments

Two plants that had received the root chilling and one plant that received the water stress treatment set reproductive buds after being exposed to dormancy-inducing conditions. Within one month of transfer to the long-day GH, catkins emerged (Fig. 2).

Root Chilling

Only those plants receiving six months of root chilling; eight weeks of short, cool days; and 10 weeks of 4°C storage formed floral buds. Three out of the four plants receiving this treatment combination produced between one and three buds each. All floral buds flushed shortly after plants were transferred to

the GH. The flowers that emerged appeared normal in every respect. We attempted to hand pollinate every catkin with *P. alba* pollen that had been desiccated and stored at −20°C, but no seeds formed.

DISCUSSION

It would appear that exposing the roots of clone 6K10 to some form of stress is critical to restorting the early-flowering phenotype, and that a minimum of six months of cold induction is required. We are now experimenting with various combinations of treatments in order to achieve more complete flowering.

Although the catkins were normal in appearance, we believe that pollination was attempted prematurely, and with pollen that may have had low viability. While optimizing conditions for floral induction, we will also use fresh pollen to try pollinating flowers at various times after anthesis to identify the window within which the stigmas are most receptive.

Unfortunately this poplar genotype is very difficult to transform and regenerate *in vitro*. In separate experiments, we are now attempting to improve the efficiency with which we are able to recover transgenic plants. Once floral induction, pollination and transformation/regeneration have been optimized, this genotype will become a valuable tool for molecular biologists who are trying to genetically engineer trees to be reproductively sterile, and to improve our understanding of the genes involved in the regulation of floral development in trees.

Finally, in the spring of 2003, a cross was made at the University of Tuscia (Viterbo, Italy) between the early-flowering female genotype described herein (6K10) and a male clone from the same half-sib family (6K). The offspring has been maintained in a nursery and will be used to propagate plants for a field experiment in which we determine the potential for early flowering in both male and female progenies. This activity also represents the first step in our efforts to use molecular markers to identify the putative mutation responsible for this early-flowering phenotype.

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