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Research paper

Low temperatures are required to induce the development of fertile flowers in transgenic male and female early flowering poplar (*Populus tremula* L.)

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Until now, artificial early flowering poplar systems have mostly led to the development of sterile flowers. In this study, several strategies aimed at inducting fertile flowers in pHSP::*AtFT* transgenic poplar were evaluated, in particular the influence of temperature and photoperiod. Our results provide evidence that temperature, and not photoperiod, is the key factor required for the development of fertile flowers in early flowering poplar. Fertile flowers were only obtained when a cold treatment phase of several weeks was used after the heat treatment phase. Heat treatments induced *AtFT* gene activity through activation of the heat-shock promoter (pHSP). Photoperiod did not show a similar influence on flower fertility as pollen grains were obtained under both long- and short-day conditions. Fertility was confirmed in flowers of both male and female plants. For the first time, crosses were successfully performed with transgenic female early flowering poplar. All mature flowers obtained after 8 weeks of inductive treatments were fertile. Gene expression studies also confirmed that cold temperatures influenced expression of poplar genes homologous to 'pollen development genes' from *Arabidopsis thaliana* (L.) Heynh. Homology and expression patterns suggested a role for *PtTDF1*, *PtBAM1*, *PtSERK1/2* and *PtMS1* on anther and pollen development in poplar flowers. The system developed in this study allows a fast and very reliable induction of fertile poplar flowers in a very short period of time. The non-reproductive phase, usually 7–10 years, can now be shortened to 6–10 months, and fertile flowers can be obtained independently of the season. This system is a reliable tool for breeding purposes (high-speed breeding technology), genomics and biosafety research.

Keywords: cold treatment, fertility, ovule development, pollen development, poplar breeding, sterility.

Introduction

Forest tree species reach reproductive maturity only after many years or even decades of juvenile growth (Meilan 1997). The induction of early flowering in transgenic poplar with the heat-inducible *FLOWERING LOCUS T* gene from *Arabidopsis thaliana* (L.) Heynh (pHSP::*AtFT*) allowed a few fertile flowers to be obtained. Shortening of the reproductive phase from the usual 7–10 years to about only 1 year was reported (Hoenicka et al. 2014). However, hitherto available early flowering systems for poplar almost exclusively promoted the development of sterile flowers (Weigel and Nilsson 1995, Rottmann et al. 2000, Böhlenius et al. 2006, Hsu et al. 2006, Tränkner et al. 2010, Zhang et al. 2010, Xiaoming et al. 2011, Shen et al. 2012, Hoenicka et al. 2014, Xiaoming and Huanling 2014). The heat stress used for the activation of 'flowering time genes' in some gene constructs and the lack of specific environmental signals have been proposed as the causes of these results (Hoenicka et al. 2014).

Plant morphogenesis relies on intricate interactions between environmental signals and genetically encoded developmental programs. The reproductive development in plants is also

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influenced by many environmental factors. Stress-induced alterations on the reproductive program, sporogenesis (micro- and megasporogenesis) and gametogenesis (micro- and megagametogenesis), have been studied in many plant species (Oshino et al. 2007, Zou et al. 2010, Wilson et al. 2011, McDowell et al. 2013, Su et al. 2013, De Storme and Geelen 2014, Sakata et al. 2014, Sharma and Nayyar 2014). Similar studies are only available in few tree species (Issarakraisila and Considine 1994, Webber et al. 2005, Huang et al. 2010).

Whereas reproductive development lasts only a few weeks in annual plants, in forest tree species, development requires several months (Jansson and Douglas 2007). Vernalization, which is the cold requirement for flower induction, and winter dormancy (Rohde and Bhalerao 2007) have allowed adaption of forest tree species to seasonal changes in the temperate regions. In poplars, floral initiation occurs between mid-May and mid-June (Böhlenius et al. 2006). The floral buds hibernate in winter and anthesis occurs in the next growing season (Böhlenius et al. 2006, Hanke et al. 2012). In eastern cottonwood *Populus deltoides* Bartram ex Marshall, a 3-year flowering cycle has been proposed (Yuceer et al. 2003).

The growth–dormancy cycle of trees growing in temperate zones is driven by environmental cues, such as photoperiod and temperature, and involves the regular division of meristem cells in the shoot and stem (Li et al. 2009, Olsen 2010). Effects of these environmental signals on the activity of meristems have been extensively explored (Oribe and Kubo 1997, Oribe et al. 2001, Gričar et al. 2006). The *CO*/*FT* regulatory module, which controls flowering time in annual plants in response to variations in day length, also controls the flowering, short-day-induced growth cessation and bud set occurring during the autumn in aspen trees (Böhlenius et al. 2006). However, the connective links between environmental signals and the initiation of sporogenesis and gametogenesis are still unknown in tree species.

Genetic analyses have demonstrated the function of some genes regulating pollen development (reviewed in Ma 2005, Wilson et al. 2011), e.g., *DEFECTIVE IN TAPETAL DEVELOPMENT AND FUNCTION1* (*TDF1*) (Zhu et al. 2008), *BARELY ANY MERISTEM1* (*BAM1*) and *BAM2* (Hord et al. 2006, Feng and Dickinson 2007), *SPOROCYTELESS* (*SPL*) (Yang et al. 1999), *SOMATIC EMBRYOGENESIS RECEPTORLIKE KINASES1* and *2* (*SERK1/2*) (Albrecht et al. 2005, Colcombet et al. 2005) and *MALE STERILITY1* (*MS1*) (Wilson et al. 2001). Microsporogenesis (Smith 1943, Boes and Strauss 1994, Zhang et al. 2008, Wang et al. 2009) and megasporogenesis (Xi et al. 2014) have been studied in different *Populus* species, but not at the molecular level. The influence of environmental factors on pollen development, its genetic regulation and the interactions with other related regulons, including flowering and dormancy, remain broadly unknown in poplar.

Genetic transformation of poplar using hitherto available early flowering-inducing systems revealed a recalcitrant initiation of pollen and ovule development in poplars compared with other tree species (Hoenicka et al. 2014). Expression of the *FT*-homologous gene of apple (*MdFT2*) induces fertile flowers in apple (*Malus* × *domestica* Borkh.) but not in poplar (*P. tremula* L.) (Tränkner et al. 2010). Genetic transformation of citrange (*Citrus sinensis* L. Osbeck × *Poncirus trifoliata* L. Raf.) (Peña et al. 2001) and poplar (Weigel and Nilsson 1995) with 35S::*AtLFY* promoted development of fertile flowers only in citrange. Interestingly, both the constitutive and heat-inducible expression of *PtFT1*, from *P. trichocarpa* Hook., allowed a very reliable induction of fertile flowers in other tree species such as plum (*Prunus domestica* L.) (Srinivasan et al. 2012) and apple (Wenzel et al. 2013), but again not in poplar (Zhang et al. 2010). The reasons behind these differences are unknown so far.

This study aimed to promote fertility of male flowers of pHSP::*AtFT* transgenic poplar. We evaluated the influence of temperature and photoperiod on the promotion of pollen development, and provide evidence that low temperatures are the key factor for the development of fertile flowers in male early flowering pHSP::*AtFT* poplar. Chilling also promoted fertility in female pHSP::*AtFT* poplar enabling the first crosses to be performed within few months. The flower induction system described in this study allows a fast and very reliable induction of fertile flowers in 6- to 10-month-old poplar plants that can be used for breeding, genomics and biosafety research.

Materials and methods

Plant material, culture and genetic transformation

Female and male early flowering poplars were obtained through genetic transformation of aspen (*P. tremula* L., clone W52, ♂) and hybrid aspen (*P. tremula* L. × *P. tremuloides* Michx., clone Esch5, ♀). Male early flowering pHSP::*AtFT* poplars used in this study have been described previously (Hoenicka et al. 2012). The plants were grown on solid McCown Woody Plant Medium (WPM, Duchefa M0220) (Lloyd and McCown 1980) containing 2% sucrose and 0.6% Agar (Agar Agar, Serva, 11396). Genetic transformations were carried out employing the *Agrobacterium*-mediated approach (Fladung et al. 1997) using *Agrobacterium tumefaciens* (Smith & Townsend 1907) Conn 1942, strain EHA105. For regeneration of transgenic plants, WPM was supplemented with 0.01% Pluronics F-68 (Sigma P-7061, Steinhein, Germany), thidiazuron (0.01 μM) and antibiotics cefotaxime (500 mg L^{-1}) for Agrobacteria elimination and kanamycin (50 mg L^{-1}) for selection of transgenic shoots. Plants were transferred to growth chambers (Weiss Technik, Reiskirchen, Germany) under the following culture conditions—light period: 16/8 h (day/night), light intensity: 300 μE m^{-2} s^{-1} (lamps: Phillips TLM 140W/33RS, Amsterdam, The Netherlands), relative humidity: 70% and temperature: 22/19 °C. After a culture period of 6–18 months in growth chambers, transgenic plants were transferred to a standard S1 greenhouse under natural daylight conditions. Studies were carried out in the Institute of Forest Genetics in Großhansdorf,

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Germany (Latitude: 53° 39′ 42.5952″ N, Longitude: 10° 15′ 12.7764″ E).

Induction of fertile flowers in male and female pHSP::AtFT transgenic poplar

One- to five-year-old greenhouse plants from four selected single-copy transgenic lines (Hoenicka et al. 2012) were subjected to different treatments and culture conditions aimed at producing fertile flowers. Randomization was used to assign the ramets (i.e., genetically identical plants) to each experimental group. Flowering induction was carried out in growth chambers (Weiss Technik). The following crossing experiments were performed: (i) pHSP::*AtFT* (♀) × pHSP::*AtFT* (♂), (ii) pHSP::AtFT (♀) × wild-type poplar (♂) and (iii) wild-type poplar (♀) × pHSP::*AtFT*.

Two culture phases were used for flowering induction in pHSP::*AtFT* poplars. During Phase 1 (P1; flower induction), heat treatments (40 °C, 90 min, 3–5 weeks) were applied daily until appearance of the first flowers. The poplar plants were then subjected to different culture conditions: (i) winter conditions (WC) (day/night: 10/6 °C, 10/14 h), (ii) summer conditions (SC) (day/night: 22/16 °C, 16/8 h) or (iii) modified summer conditions (MSC) (day/night: 22/16 °C, 10/14 h).

During Phase 2 (P2; fertility induction), no heat treatments were applied to plants. The poplar plants were cultivated under different conditions for 8 weeks: (i) WC as in P1, (ii) SC as in P1 or (iii) modified winter conditions (MWC) (day/night: 10/6 °C, 16/8 h). Plants were kept under the respective P2 growth conditions until the crosses were completed.

Evaluation of pollen viability

Anthers obtained from early flowering plants were observed under an optical fluorescence microscope (Olympus BH-2, Tokyo, Japan) to confirm viability of pollen grains. Microspore viability was estimated by staining with fluorescein diacetate (FDA tests) (Wildholm 1972).

Molecular analysis of transgenic lines

Molecular analyses of male pHSP::*AtFT* transgenic lines have been published previously (Hoenicka et al. 2012). Genomic DNA from female pHSP::*AtFT* transgenic lines was extracted from leaves. Polymerase chain reaction (PCR) analyses were carried out with specific primers using 60 °C annealing temperature as described before (Hoenicka et al. 2012, 2014). Southern blot analyses were carried out with 20 μg genomic DNA digested with the restriction enzyme BglII (Fermentas, Waltham, MA, USA), according to the supplier's instructions. DNA electrophoresis and transfer of DNA to Biodyne A membranes (Pall Europe Limited, Portsmouth, UK) were performed as described elsewhere (Fladung et al. 1996, 1997). Prehybridization and hybridization of Southern blots were performed with the non-radioactive DIG (digoxigenine) system using a DIG-dUTP PCR-labeled probe as described earlier (Fladung and Ahuja 1995, Fladung et al. 1996). The gels were stained with Roti-Safe (Roth, Karlsruhe, Germany) shortly before blotting to confirm similar loaded DNA amounts and uniform restriction patterns.

RNA extraction and reverse transcription

Selected flowers from early flowering poplar and wild-type flower buds were frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Fifty to 70 mg of liquid nitrogen frozen tissue was ground in Eppendorf tubes using metal balls and a Retsch mill (Retsch MM300, Haan, Germany). Total RNA was isolated (Chang et al. 1993) and purified with the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany). RNA was quantified using spectrophotometric OD_{260} measurements with a Nanodrop 1000 (Thermo Scientific, Wilmington, DE, USA) and the Qubit 2.0 Fluorometer (Life Technologies, Paisley, UK). RNA quality was assessed by OD_{260}/OD_{280} and OD_{260}/OD_{230} ratios (maintained between 1.8 and 2.1) and with the Agilent Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA, USA) (samples with RIN values higher than 7 were selected). Contaminating DNA was removed from RNA samples using the Ambion turbo DNA-free (Ambion, Austin, TX, USA) according to the manufacturer's protocol. Genomic DNA contamination was evaluated by performing qPCR using primers targeting gene *Act 7* (see Table S1 available as Supplementary Data at *Tree Physiology* Online) and genomic DNA as control. The cDNA was synthesized with 2.31 μg RNA using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and the reaction mix was diluted 1 : 10 for expression studies.

Gene expression studies with male pHSP::AtFT poplar flowers

Information available on the genetic regulation of pollen development in *A. thaliana* (Wilson et al. 2001, Albrecht et al. 2005, Colcombet et al. 2005, Hord et al. 2006, Zhu et al. 2008, Feng and Dickinson 2007, reviewed in: Ma 2005, Wilson et al. 2011) was used to clone homologous genes in poplar. Sequences of the respective *Arabidopsis* genes were blasted against the poplar genome sequence (Phytozome 10.2, http://phytozome.jgi.doe.gov). Expression of the most homologous poplar genes of *TDF1*, *BAM1*, *SERK1/2* and *MS1* (see Table S1 available as Supplementary Data at *Tree Physiology* Online) was evaluated by quantitative PCR (qPCR). Poplar genes showing the highest homology to *Act7*, *PP2a*, *UBQ* and *Tip4*-like (see Table S1 available as Supplementary Data at *Tree Physiology* Online) were evaluated for reference gene normalization. Primer design was carried out with QuantPrime (Arvidsson et al. 2008, http://www.quantprime.de/) or Primer3plus (Rozen and Skaletsky 2000, http://primer3plus.com/cgi-bin/dev/primer3plus.cgi) with 60 °C melting temperature (see Table S2 available as Supplementary Data at *Tree Physiology* Online). Primer specificity was evaluated *in silico* using Phytozome 10.2. Primers for *PtTIP4-like* gene were described before (Pettengill et al. 2012). The qPCR experiments were

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carried out following the MIQE guidelines (Bustin et al. 2009, 2010). RNase-free water was added to lyophilized primers to obtain a 100 μM stock concentration, stored at −20°C. Primers were diluted to 10 μM use concentration. Melting and calibration curves were performed for each primer pair. Amplicons were analyzed on 1.5% agarose gel electrophoresis followed by orange G loading dye staining. Melting curve analysis was carried out to confirm the specificity of the amplification by the candidate genes. Calibration curves using a dilution series of the cDNA pool were made to calculate the PCR amplification efficiencies ($E = 10 - 1/$slope) for each quantified candidate gene. The reverse transcription (RT)-qPCR amplifications were performed on Stratagene Mx3000P (Stratagene, La Jolla, CA, USA) in 96-well reaction plates (Eppendorf LoBind® twin.tec® PCR Plates, Eppendorf, Hamburg, Germany) using the following parameters: 10 min at 95 °C and 40 cycles of 95 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min. Quantitative PCRs were repeated twice and a melting curve was included. Each 20 μl reaction mixture comprised 5 μL of template cDNA (diluted 50-fold) (~3.5 ng of input RNA), 10 μL of Fast Plus EvaGreen Master Mix with low Rox (Biotium, Hayward, CA, USA), 4.1 μl of ultrapure water and 0.44 μl of each primer (0.225 μM). Three biological and three technical replicates were used in each run for RT-qPCR analysis, and no template controls were also included. The melting curves were analyzed at 65–95 °C for 20 min after 40 cycles. The baseline and quantification cycle (C_t) were determined using the MxPro™ qPCR Software (version 4.10).

Gene expression studies were carried out with flowers obtained from male pHSP::*AtFT* transgenic poplar, collected in the early P2 (controls) and after 4 weeks in the P2 (test samples). Flower buds from wild-type poplar were collected in November (control group) and January (test samples). The relative gene expression levels (based on the relative quantities after the ΔΔCq method) were calculated from C_t values using the REST software (Relative Expression Software Tool, version 2.0.13), which bases its performance on pairwise comparisons using randomization and bootstrapping techniques (Pairwise Fixed Reallocation Randomization Test) (Pfaffl et al. 2002). The gene expression was normalized by the expression levels of *PtAct7*, and results were confirmed by normalization with *UBQ*.

Results

Genetic transformation of female hybrid aspen

Transformation of male transgenic lines has been described previously (Hoenicka et al. 2012, 2014). Transgenic lines obtained after genetic transformation of female clones with the pHSP::*AtFT* gene construct were selected according to PCR and Southern blot results (see Figure S1 available as Supplementary Data at *Tree Physiology* Online). Preliminary flowering induction experiments, using heat treatments (40 °C, 90 min, 3–5 weeks), allowed selection of the single-copy transgenic lines N221-1 and N224-1, which showed reproducible flowering rates and were used for crossing experiments.

Influence of temperature on flower fertility in male pHSP::AtFT transgenic poplar

Flower development could be observed after 3 weeks of heat treatments (P1). Induction of flowering was successful in male plants growing under SC (day/night: 22/16 °C, 16/8 h) and MSC (day/night: 22/16 °C, 10/14 h). Heat shocks (HS) were

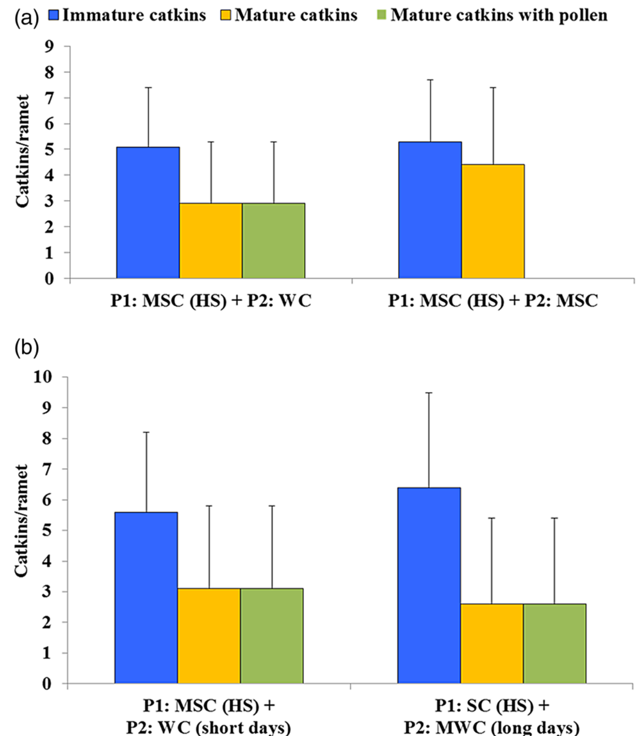

Figure 1. Influence of temperature (a) and photoperiod (b) treatments on induction of pollen development in flowers of male pHSP::*AtFT* poplar. (a) Phase 1 (flower induction phase): plants were kept under MSC (day/night: 22/16 °C, 10/14 h) and HS (40 °C, 90 min, 3–5 weeks) was applied daily for activation of the pHSP::*AtFT* gene construct. Phase 2 (fertility induction phase): plants were kept under WC (day/night: 10/6 °C, 10/14 h) or MSC (day/night: 22/16 °C, 10/14 h) with the same lighting regime but no HS. Pollen grains were only obtained when plants were subjected to low temperatures during P2. Cold temperatures are the key factor for the induction of pollen development in transgenic poplars. (b) Phase 1: plants were kept under SC (day/night: 22/16 °C, 16/8 h) or MSC (day/night: 22/16 °C, 10/14 h) and HS was applied as in (a). Phase 2: plants were kept under WC or MWC (day/night: 10/6 °C, 16/8 h) with no HS. Pollen grains were obtained under both short- and long-day conditions in catkins after 4 weeks in P2. Pollen grains were only obtained after 4 weeks in P2 for both treatments. This time threshold was established for this study, and sterile catkins developed before that time were discarded. Data collection took place after 8 weeks in P2. Catkins containing dark red anthers, instead of green ones, were classified as 'mature catkins'. These data show the number of catkins developed during the P2. Five ramets from two selected independent transgenic events were used for experiments. Number of catkins per ramet (mean values) and standard errors (bars) are shown. Experiments were repeated twice.

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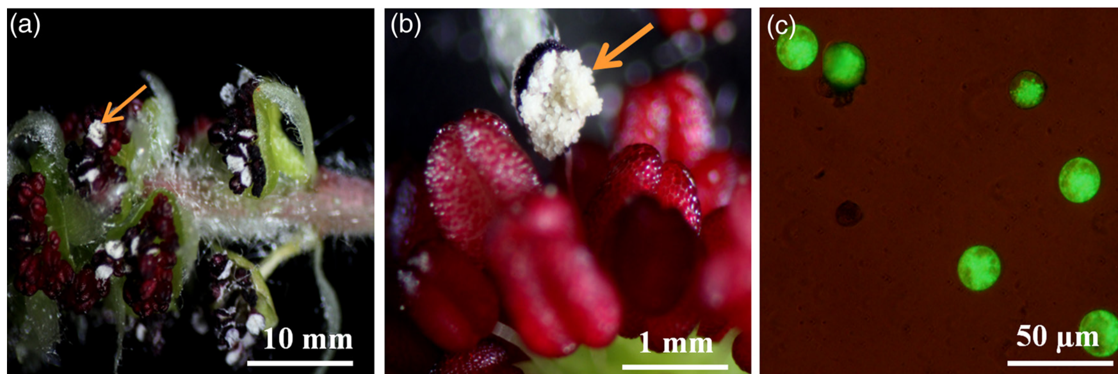

Figure 2. Artificial induction of fertile flowers in catkins of male early flowering HSP::*AtFT* poplars. (a) Catkin with fertile flowers*, (b) detail of mature anther (Stage 14) showing both normal dehiscence and pollen grains and (c) viable pollen (FDA test). Scale bars: 10 mm (a), 1 mm (b) and 50 µm (c). Arrows show pollen grains. *Fertility was confirmed through crossings.

detrimental to plants growing under WC (day/night: 10/6 °C, 10/14 h) and no flowers were obtained (data not shown). Plants kept under MSC during P1 were further cultivated in P2, either under WC or MSC (Figure 1a), to evaluate the influence of temperature on flower fertility under the same photoperiod regime. Flowers developed both under high and low temperatures. However, plants developed fertile catkins only after >4 weeks of exposure to low temperatures in P2 (Figure 1a). Sterile catkins developed before this time threshold were discarded. Anther dehiscence and the presence of pollen grains were clear indicators of potential flower fertility (Figure 2). Sterile flowers did not show anther dehiscence. FDA tests (Figure 2, 75–100% viability) and crossing experiments (percentage not calculated) confirmed pollen viability and fertility.

Influence of photoperiod on flower fertility in male pHSP::AtFT transgenic poplar

In a second approach, the influence of photoperiod on male fertility was studied. During P2, both groups were kept under chilling temperatures and two photoperiods were tested (long- or short-day conditions). Plants were maintained under either MSC (day/night: 22/16 °C, 10/14 h) or SC (day/night: 22/16 °C, 16/8 h) in P1, and WC (day/night: 10/6 °C, 10/14 h) or MWC (day/night: 10/6 °C, 16/8 h) during P2. Similar numbers of mature catkins containing pollen grains were obtained under both short- and long-day conditions (Figure 1b).

Induction of fertile flowers in female pHSP::AtFT transgenic poplar

The presence of pollen grains allowed the early identification of potentially fertile flowers in pHSP::*AtFT* transgenic male poplar. However, no fast and easy morphological criteria could be found to identify fertile female flowers. Therefore, seed production was used as an indicator of female flower fertility. The influence of temperature on the promotion of fertility in female flowers was confirmed using the same conditions described for male poplars (Figure 3).

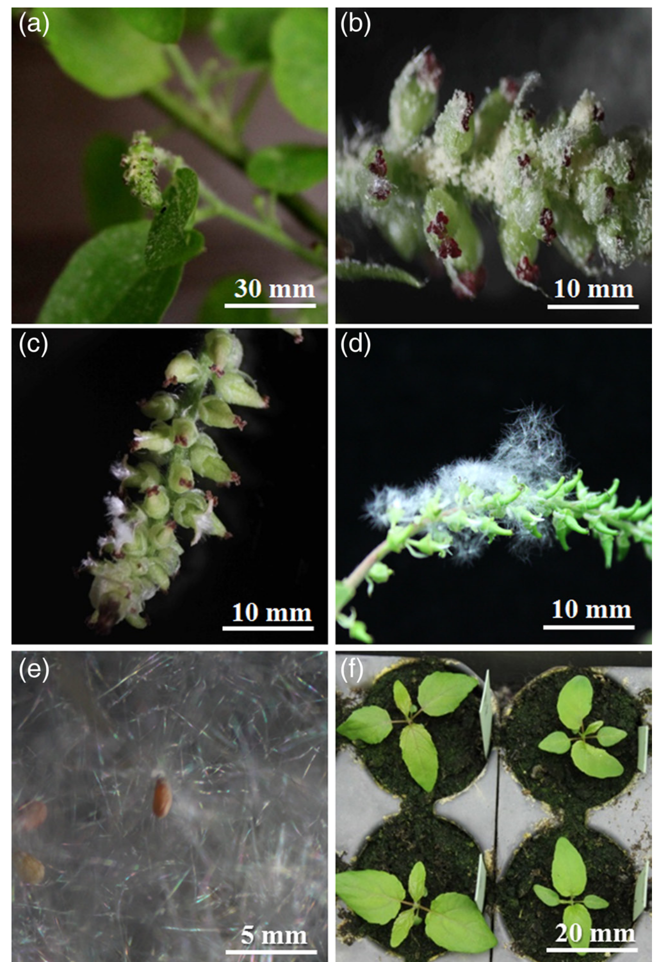

Figure 3. Crossings performed between pHSP::*AtFT* early flowering (♀) and wild-type poplar (♂). (a) Fertile flower, (b) pollinated flowers, (c) infructescence, (d and e) seeds and (f) seedlings. Scale bars: 30 mm (a), 10 mm (b–d), 5 mm (e) and 20 mm (f).

Development of crossing strategies based on pHSP::AtFT transgenic poplar

The use of a cold treatment after the heat treatment phase was found to induce fertile flowers in both male and female

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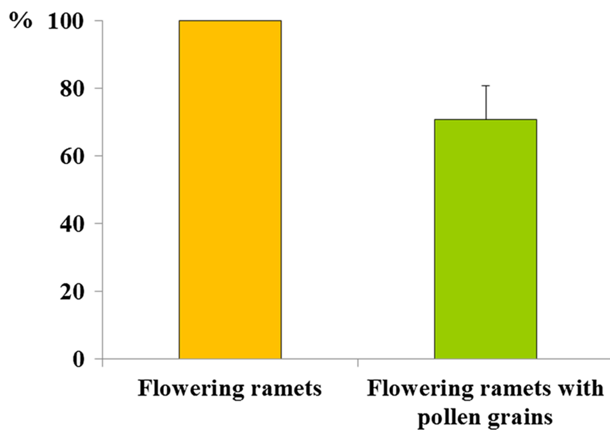

Figure 4. Induction of pollen development in male early flowering pHSP::*AtFT* poplar. Phase 1 (flower induction phase): plants were kept under MSC (day/night: 22/16 °C, 10/14 h) and HS (40 °C, 90 min, 3–4 weeks) were applied daily for the activation of the pHSP::*AtFT* gene construct. Phase 2 (fertility induction phase): plants were kept under WC (day/night: 10/6 °C, 10/14 h) and no HS were applied. Pollen grains were only obtained after 4 weeks in P2. This time threshold was established for our study, and sterile catkins developed before that time were discarded. Data collection took place after 8 weeks in P2. Catkins containing dark red anthers, instead of green ones, were classified as 'mature catkins'. Three to five ramets from two selected independent transgenic events were used for experiments. Percentage of flowering ramets (mean values) and standard errors (bars) are shown. Experiments were repeated twice.

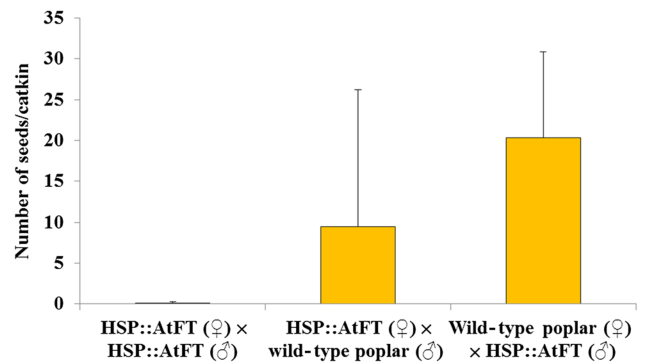

Figure 5. Number of seeds obtained per catkin in different crossing experiments with pHSP::*AtFT* and wild-type poplar (*P. tremula* L.). Phase 1 (flower induction phase): plants were kept under SC (day/night: 22/16 °C, 16/8 h) and HS (40 °C, 90 min, 3–4 weeks) were applied daily for the activation of the pHSP::*AtFT* gene construct. Phase 2 (fertility induction phase): plants were kept under winter (WC: day/night: 10/6 °C, 10/14 h) conditions. Number of seeds per catkin (mean values) and standard errors (bars) are shown. Results were obtained from crossings performed with 10–20 female flowers and the same number of male flowers. Experiments were repeated twice.

pHSP::*AtFT* transgenic poplars (Figures 1–5). Therefore, flower induction for crossing experiments was performed in two steps—P1 (flower induction phase): plants were kept under SC and HS (40 °C, 90 min, 3–4 weeks) were applied daily for the activation of the pHSP::*AtFT* gene construct. Phase 2 (fertility induction phase): plants were kept under WC. More than 95% of male ramets produced flowers, but only 70% of ramets had fertile flowers (Figure 4). A third of the male catkins developed fully (Figure 1). Mature catkins produced dark red anthers instead of green ones and contained pollen grains. Immature catkins showed arrested development, green anthers and some reversions, i.e., vegetative growth at the catkin tip, were observed. Dehiscent anthers and pollen grains clearly indicated potential male flowers fertility. Anthers reached Stage 14 (Sanders et al. 1999). Pollen grains showed a normal shape and high viability (75–100%) according to FDA tests (Figure 2). Pollen fertility was confirmed through crossings. Seeds obtained from crossings showed high viability (>80%). Fertile catkins could be obtained with this method independently of the season in growth chambers.

Crossings performed between either male or female transgenic pHSP::*AtFT* and their respective wild-type poplar allowed the production of seeds. These experiments indicated a clear influence of low temperature treatment on flower fertility. Chilling promoted development of pollen grains in >70% of male ramets (Figure 4). Crossing experiments with wild-type female flowers and pollen from pHSP::*AtFT* plants produced a mean of 20.3 seeds per catkin (Figure 5). An average of 9.5 seeds per catkin was obtained from crossings between flowers of pHSP::*AtFT* female transgenic and pollen from wild-type male poplar. Crossing experiments using both male and female pHSP::*AtFT* flowers produced seeds with a low performance as only a mean of 0.1 seeds per catkin was obtained.

Gene expression studies with male pHSP::AtFT transgenic poplar flowers

Different reference genes were evaluated for normalization of qPCR results (see Tables S1 and S2 available as Supplementary Data at *Tree Physiology* Online). The *PtAct7* gene showed the most stable gene expression for all samples, and was used for normalization. In 'no template controls' (NTC) no signals were obtained. Changes in gene expression were significant, according to C_t analyses carried out with the software REST 2009, for both pHSP::*AtFT* transgenic and wild-type poplar, remaining stable even without qPCR normalization (Figure 6). Fertile pHSP::*AtFT* flowers, collected in the early (controls) and late (test samples) P2, showed a strong increase in the expression of 'pollen development' *PtTDF1* and *PtMS1* genes (Figure 6a). Sterile pHSP::*AtFT* flowers (P2 under SC) showed no significant increase on expression of *PtTDF1* and *PtMS1* genes (Figure 6c).

A comparison of the gene expression profiles of fertile and sterile flowers showed that chilling promoted strong expression changes in the flowers of the pHSP::*AtFT* plants (Figure 6a), while warmer growth conditions did not result in any expression changes (Figure 6c). The *PtTDF1*, *PtBAM1*, *PtSERK1/2* and *PtMS1* genes showed 135-, 5-, 4- and 16-fold increases on gene expression, respectively (Figure 6a). Gene expression patterns in wild-type flower buds collected in November (controls) and January (test samples) were similar to those of fertile

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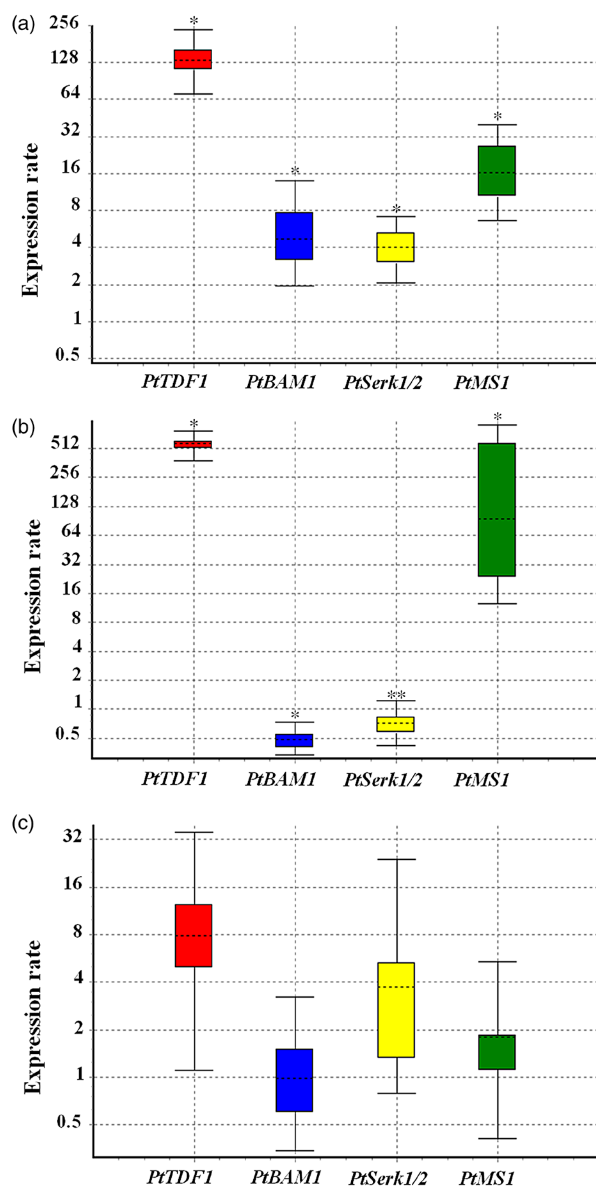

Figure 6. Gene expression studies of selected 'anther and pollen development genes' in male pHSP::*AtFT*, after heat (P1) and cold (P2) treatments, and wild-type poplar collected in autumn and winter. Expression changes were studied in poplar genes showing homology toward the anther and pollen development genes *TDF1*, *BAM1*, *SERK1/2* and *MS1* from *A. thaliana*. Transgenic pHSP::*AtFT* poplars were maintained under growth chamber conditions and wild-type poplar was collected in the field. Studies were carried out with (a) male pHSP::*AtFT* flowers collected in summer after the end of P1 (controls) and after 4 weeks under P2 (test samples), (b) male pHSP::*AtFT* flower buds from wild-type poplar collected in November (controls) and January (samples), and (c) male pHSP::*AtFT* poplar flowers collected in summer after the end of P1 (controls) and after 4 weeks growth under summer temperatures (test samples). Relative expression distribution (*Y*-axis) represented as a ratio of gene expression in controls vs test samples normalized against reference gene *PtAct7* and shown by box-and-whisker plots as medians (black dashed lines), interquartile ranges (boxes) and ranges (whiskers). Ratios >1 indicate higher gene expression of samples compared with controls, while ratios <1 indicate genes with a lower expression. Experiments were repeated twice using three biological replicates. Data analyses with program REST 2009 (Pfaffl et al. 2002). *Significant gene expression changes, $P < 0.001$ or $P = 0000$, **$P < 0.024$.

pHSP::*AtFT* flowers (Figure 6b). However, some differences were detected. Gene expression of *PtTDF1* and *PtMS1* was upregulated in wild-type flowers as in pHSP::*AtFT* flowers, but the increase was more pronounced (Figure 6b). Expression of *PtBAM1* and *PtSERK1/2* was significantly upregulated in pHSP::*AtFT* flowers but downregulated in wild-type poplar (Figure 6). Sterile pHSP::*AtFT* flowers showed weak expression of 'pollen development genes', and none of these genes showed significant expression changes (Figure 6c).

Discussion

Induction of fertile flowers in male and female pHSP::AtFT transgenic poplars

The genetic transformation of poplar with different genes associated with flowering control has long been known to induce flowering in poplar. However, flowers obtained after inductive treatment were hitherto sterile. A lack of appropriate environmental signals has been proposed to cause sterility in early flowering poplar systems (Hoenicka et al. 2014). In natural conditions, wild-type poplars develop flowers in completely different environmental conditions. Both 2- and 3-year flowering cycles have been observed in eastern cottonwood (Yuceer et al. 2003, Böhlenius et al. 2006, Hanke et al. 2012). Anthers are completely formed 1 year before flowering in the late summer. Archespore formation takes place in autumn (Boes and Strauss 1994). Pollen development continues during winter after the endodormancy phase. In contrast, flowers from pHSP::*AtFT* poplar develop in just a few weeks after heat induction. Since this flower induction is not natural, it is unlikely that naturally occurring endogenous signals are induced in this very short time period to activate regulons of sporogenesis and gametogenesis.

Our results show that chilling plays a key role in fertility induction in poplar flowers from both female and male plants. This modified system allowed anthers to reach full development, i.e., anthers reached development stage 14 (Sanders et al. 1999). An incomplete anther development (Stage 12) was previously reported for pHSP::*AtFT* poplar (Hoenicka et al. 2014). Crossings were only successful when low temperatures were applied during P2. This may explain the difficulties previously reported by many groups for the induction of fertile flowers in poplar with different gene constructs (Weigel and Nilsson 1995, Rottmann et al. 2000, Böhlenius et al. 2006, Hsu et al. 2006, Tränkner et al. 2010, Zhang et al. 2010, Xiaoming et al. 2011, Shen et al. 2012, Hoenicka et al. 2014, Xiaoming and Huanling 2014). The requirement for a chilling period has not been reported in any other transgenic early flowering tree species, including apple (Flachowsky et al. 2007, Tränkner et al. 2010, Wenzel et al. 2013), citrange (*C. sinensis* L. Osbeck 9 *Poncirus trifoliata* L. Raf.) (Peña et al. 2001), plum (*Prunus domestica*; Srinivasan et al. 2012) and pear (Freiman et al. 2012). This difference could be due to the different natural flowering time span of

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these species: winter to spring for poplars, and spring to summer for the fruit tree species mentioned.

The incidence of reverted catkins (which had resumed vegetative development) was very low. We confirm here the positive effect of a longer heat treatment period (Zhang et al. 2010) to prevent reversions in catkins from early flowering poplar to vegetative structures. However, development of many catkins remained incomplete. This was probably due to the decreasing amount of FT protein in the plants during the cold treatment phase. Our results show that chilling temperatures have a clear influence on promotion of flower fertility in both male and female pHSP::*AtFT* poplars. The photoperiod did not have a similar influence on pollen development, as pollen was obtained both under short- and long-day conditions. Photoperiod revealed no influence on flower fertility in wild-type rice (Lu et al. 2008). However, some mutants that show photoperiod-sensitive male sterility (PSMS) have been identified (Shi 1985, Ding et al. 2012). A major characteristic of the PSMS plants is that pollen fertility is regulated by day length: the pollen is completely sterile under long-day conditions, while fertility varies from partial to full under short-day conditions. Photoperiod-sensitive male sterility germplasms have been explored in several crop species (Smith et al. 2001, Virmani and Ilyas-Ahmed 2001, Guo et al. 2006). Stress factors, e.g., heat, cold and drought, are known to often disturb pollen and ovule development (Oshino et al. 2007, Zou et al. 2010, Wilson et al. 2011, McDowell et al. 2013, Su et al. 2013, De Storme and Geelen 2014, Sakata et al. 2014, Sharma and Nayyar 2014). Plant microsporogenesis can even be diverted from normal pollen development pathway toward an embryogenic route after exposure to stress pretreatments (reviewed in Islam and Tuteja 2012). However, most studies have focused on the impact of environmental stress on reproductive development of annual plants.

A heat treatment phase is required to induce *AtFT* expression in pHSP::*AtFT* transgenic plants. It is known that prolonged heat is detrimental to pollen development in wild-type poplar (Hoenicka et al. 2014) and many other plant species (Endo et al. 2009, Jain et al. 2010, Sakata et al. 2010, De Storme and Geelen 2014). Reproductive development is very sensitive to adverse environments and (a) biotic stresses (reviewed in De Storme and Geelen 2014). Our results reveal that at least 4 weeks of growth under chilling conditions is required to compensate for the detrimental effects of heat on flower fertility.

Low temperatures can also cause pollen sterility in rice and other plant species growing in sub-tropical regions of the world (Oliver et al. 2007, Sharma and Nayyar 2014). Disturbed ovule development was reported for various species when cultivated under low temperatures (Sun et al. 2005, McDowell et al. 2013), salt (Sun et al. 2004) and drought stress (Su et al. 2013). In poplars, however, pollen and ovules develop normally during the winter and spring, when temperatures are low. Therefore, it is not surprising that high rather than low temperatures have a detrimental effect on flower development in this species.

Gene expression studies in flowers of male pHSP::AtFT transgenic poplar

Our gene expression studies confirm that chilling influences the expression of several orthologous poplar genes associated with pollen development in *A. thaliana* (reviewed in Ma 2005, Wilson et al. 2011). Homology and expression patterns confirmed the potential role of *PtTDF1*, *PtBAM1*, *PtSERK1/2* and *PtMS1* on anther and pollen development in poplar flowers.

Anthers with viable pollen that developed in pHSP::*AtFT* transgenic poplars following the combination of HS and chilling treatments had still a lower developmental potential compared with wild-type flower buds. This explains the upregulation of genes associated with anther development, *PtBAM1* and *SERK1/2* (Wilson et al. 2011), in pHSP::*AtFT* transgenic and their downregulation in wild-type poplar (Figure 6). The upregulation of *PtTDF1* and *PtMS1* (Figure 6), both classified as 'pollen development genes', in flowers of wild-type and pHSP::*AtFT* transgenic poplar confirms their role in pollen development in poplar.

Development of crossing strategies based on male and female pHSP::AtFT transgenic poplars

The results presented in this study represent a major step toward development of strategies for faster breeding in poplars. Fertile flowers in female pHSP::*AtFT* poplar can reliably be obtained and crosses successfully performed. The number of seeds per catkin obtained is still low compared with those resulting from crosses with wild-type poplars; however, this is not a problem for breeding purposes. Between 200 and 400 seeds per catkin can be obtained from crosses between normal wild-type poplar plants (Koivuranta et al. 2012). Crosses between male and female pHSP::*AtFT* poplar produced particularly few seeds, with less than one seed obtained per catkin on average. Again, this is not a basic problem for breeding purposes. Combined crossings using early flowering male pHSP::*AtFT* and female wild-type poplar produced a mean of 20.3 seeds per catkin. These results indicate that our early flowering system still does not reach the fertility level of wild-type poplars.

Anther dehiscence and presence of pollen grains are reliable indicators of flower fertility in male pHSP::*AtFT*. However, no reliable indicators were found for flower fertility in female pHSP::*AtFT*. Lack of flower fertility indicators in female pHSP::*AtFT* poplar was detrimental to crossings involving these plants (Figure 5). The highest seed production achieved in crossings between female wild-type and male pHSP::*AtFT* poplar confirmed this statement. On the other hand, even these crossings still show a lower seed production than crossings involving wild-type poplar. A disturbed gametogenesis could be behind these results.

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The heat and cold treatments required for complete flower development still have a detrimental effect on flower fertility and seed development yields. Seed development in poplar is known to be influenced by temperature (Koivuranta et al. 2012). Flowers obtained directly after P1 (heat treatment phase) were sterile. Fertile flowers could only be obtained 4 weeks after heat treatments, during P2 (cold treatment phase). Low temperatures used during this phase (10/6 °C) may not be ideal for optimal seed development. In the wild, poplar seeds normally develop between 15.7 and 17.1 °C (Koivuranta et al. 2012). The introduction of a third warmer culture phase (Phase 3) in the artificial crossing procedure might improve the performances of this system.

Conclusions

The combination of HS and chilling treatments described in this study allows the reliable formation of male and female fertile flowers in pHSP::*AtFT* transgenic poplars, independently of the season. As this system is efficient even with very young plants (6–8 months), it can be employed in breeding programs as established for annual plants (high-speed breeding technology) (Flachowsky et al. 2011, Hoenicka et al. 2014) as well as for research purposes (genomic and biosafety research). For instance, the widespread use of transgenic early flowering systems for tree breeding would require the elimination of transgenes from plants before release. In accordance with Mendelian inheritance, we showed that transgene elimination can take place after crossings in 50% of the offspring (Hoenicka et al. 2014). Fast breeding under controlled conditions (biosafety level 1) and selection of transgene-free plants once the breeding process is concluded offer a very attractive alternative breeding strategy for trees even under very restrictive biosafety regulations.

Supplementary data

Supplementary data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared.

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