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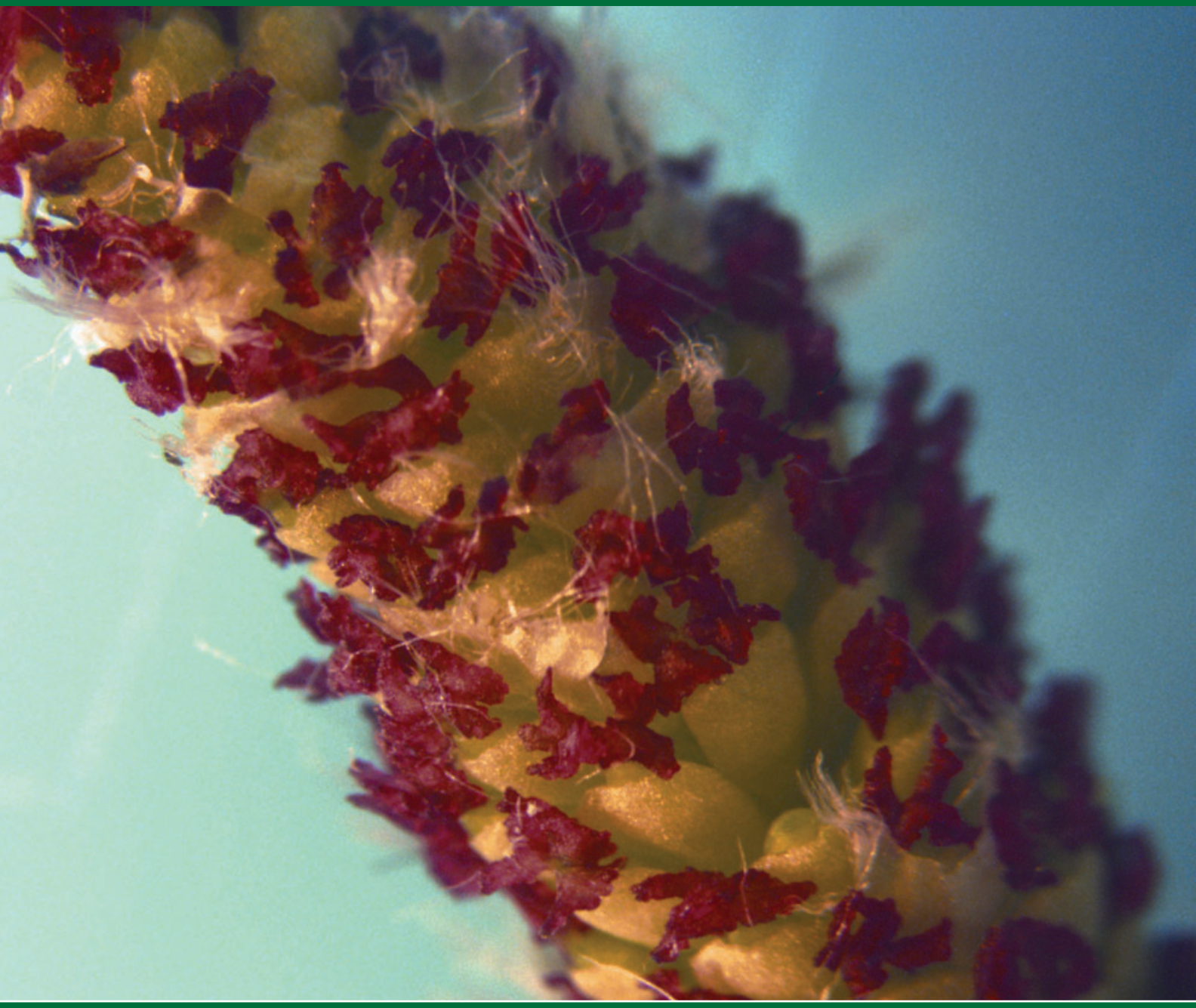

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Populus CEN/TFL1 regulates first onset of flowering, axillary meristem identity and dormancy release in *Populus*

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SUMMARY

Members of the *CENTRORADIALIS* (*CEN*)/*TERMINAL FLOWER 1* (*TFL1*) subfamily control shoot meristem identity, and loss-of-function mutations in both monopodial and sympodial herbaceous plants result in dramatic changes in plant architecture. We studied the degree of conservation between herbaceous and woody perennial plants in shoot system regulation by overexpression and RNA interference (RNAi)-mediated suppression of poplar orthologs of *CEN*, and the related gene *MOTHER OF FT AND TFL 1* (*MFT*). Field study of transgenic poplars (*Populus* spp.) for over 6 years showed that downregulation of *PopCEN1* and its close paralog, *PopCEN2*, accelerated the onset of mature tree characteristics, including age of first flowering, number of inflorescences and proportion of short shoots. Surprisingly, terminal vegetative meristems remained indeterminate in *PopCEN1*-RNAi trees, suggesting the possibility that florigen signals are transported to axillary mersitems rather than the shoot apex. However, the axillary inflorescences (catkins) of *PopCEN1*-RNAi trees contained fewer flowers than did wild-type catkins, suggesting a possible role in maintaining the indeterminacy of the inflorescence apex. Expression of *PopCEN1* was significantly correlated with delayed spring bud flush in multiple years, and in controlled environment experiments, *35S::PopCEN1* and RNAi transgenics required different chilling times to release dormancy. Considered together, these results indicate that *PopCEN1/PopCEN2* help to integrate shoot developmental transitions that recur during each seasonal cycle with the age-related changes that occur over years of growth.

Keywords: *TERMINAL FLOWER 1*, *Populus*, flowering, dormancy, branching, axillary meristem.

INTRODUCTION

Many features that distinguish tree growth habit from that of herbaceous plants are the result of the pattern of activity and identity of shoot meristems. Trees typically postpone flowering, producing only vegetative meristems for several years. In *Populus*, terminal shoot meristems remain indeterminate throughout a tree's life, but adult trees develop axillary inflorescence buds shortly after winter dormancy, and catkins complete development the following year (Boes and Strauss, 1994; Yuceer *et al.*, 2003). In contrast to annual plants with monopodial shoot growth that undergo a single vegetative to reproductive phase transition, both phases coexist within adult poplar trees (Brunner and Nilsson, 2004). Adult poplars contain branches with and without inflorescence buds, and individual branches produce both axillary vegetative and reproductive buds.

In temperate- and boreal-zone trees, shoot meristems seasonally cycle between growth and winter dormancy (Howe *et al.*, 2003; Welling and Palva, 2006; Rohde and Bhalerao, 2007). Decreases in day length and temperature during fall induce growth cessation, cold acclimation, endodormancy and, finally, maximal cold hardiness. Following an extended chilling period to release dormancy, the increasing temperatures and photoperiod in spring stimulate the resumption of growth. Genetic variation among

populations in the timing of these seasonal-related traits is associated with climatic and geographic gradients, reflecting the adaptive importance of maximizing growth while minimizing cold injury.

In addition to seasonal shoot-growth patterns, trees exhibit maturation-related changes in shoot architecture (e.g. branch frequency) that occur over years (Greenwood, 1995; Brunner *et al.*, 2003). The poplar crown is composed of shoot types ranging from extremely short shoots, which set buds very early in the growing season, to several meter-long shoots bearing both preformed and neoformed leaves, which continue growing until a critical day length for short day (SD)-induced bud set. There is considerable natural variation in the proportion of long shoots and a strong maturation trend in their frequency: over years, trees have proportionally fewer long shoots, and catkins tend to be most prevalent on short shoots in the upper crown (Critchfield, 1960; Dickmann *et al.*, 2001).

There is abundant evidence that the genes and pathways regulating shoot meristem activity are highly conserved among plants (McSteen and Leyser, 2005; Kobayashi and Weigel, 2007). However, there is also evidence that signaling modules for shoot meristem regulation have been adapted for control of seasonal growth in perennial plants such as poplar (Bohlenius *et al.*, 2006; Ruttink *et al.*, 2007). In Arabidopsis, *CONSTANS* (*CO*) and *FLOWERING LOCUS T* (*FT*) control long-day (LD)-induced flowering (Kardailsky *et al.*, 1999; Kobayashi *et al.*, 1999; Samach *et al.*, 2000). Poplar orthologs of *FT* also promote flowering in poplar, and, in addition, the *PtCO2/PtFT1* regulon mediates SD-induced growth cessation and bud set (Bohlenius *et al.*, 2006; Hsu *et al.*, 2006).

FT belongs to the phosphatidylethanolamine-binding protein (PEBP) superfamily. The plant PEBP genes group into three major subfamilies – *FT*-like, *TFL1*-like and *MFT*-like – with the *MFT*-like clade being ancestral to the other clades (Kobayashi *et al.*, 1999; Hedman *et al.*, 2009). *TFL1* acts in opposition to *FT*: *tfl1* mutants flower earlier, and the inflorescence meristem converts to a terminal flower (Shannon and Meeks-Wagner, 1991; Bradley *et al.*, 1997). Constitutive expression of *TFL1* results in a longer vegetative phase, a larger and highly branched inflorescence and delayed flower formation (Ratcliffe *et al.*, 1998). Study of *TFL1* homologs in various plant species have revealed similar but also distinctive functions, and diversification of paralogous genes has also been shown. Whereas *TFL1* is expressed in vegetative and inflorescence meristems, its paralog *Arabiodopsis thaliana CEN* (*ATC*) is expressed in the hypocotyl, and these expression differences have resulted in functional differences: *atc* mutants showed no effects on flowering, nor any obvious phenotype (Mimida *et al.* 2001). The snapdragon (*Antirrhinum* spp.) homolog, *CEN*, is only expressed in the inflorescence meristem, and *cen* mutants form terminal flowers, but flowering time is not altered (Bradley *et al.*, 1996). In pea (*Pisum sativum*), flowering time and meristem determinacy functions are subdivided between two *TFL1* homologs (Foucher *et al.*, 2003). *LATE FLOWERING* (*LF*) acts only as a repressor of flowering, whereas *DETERMINATE* (*DET*) maintains the indeterminate inflorescence meristem. In tomato, a recessive mutation in *SELF-PRUNING* (*SP*) causes premature termination of sympodial units into inflorescences (Pnueli *et al.*, 1998). In contrast to *FT* and *TFL1*, the function of *MFT* is unclear. No phenotypic changes were observed in the *mft-1* mutant, but overexpression of *MFT* slightly accelerated flowering in Arabidopsis, suggesting it might act redundantly to control flowering time (Yoo *et al.*, 2004).

To study the role of *PopCEN1* and *PopMFT* in the age/size-related developmental changes that occur in conjunction with recurrent seasonal transitions in temperate-zone trees, we overexpressed and downregulated these genes in poplar. The effects of misexpression were then studied during the onset of flowering over six growing seasons in the field. Whereas we did not identify a function for *PopMFT*, we show that *PopCEN1* and/or *PopCEN2* share functional commonalities with their homologs in herbaceous plants, in control of flowering and inflorescence architecture, but also find differences that reflect unique features of the tree growth habit, including a role in the control of shoot phenology.

RESULTS

The *Populus trichocarpa TFL1/FT* gene family

PopCEN1 and *PopMFT* were isolated from *P. trichocarpa* by homology-based cloning (Mohamed, 2006). Subsequent searches of the *P. trichocarpa* version 1.1 (POPTR v1.1) and recently released POPTR v2 genome assemblies (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html and http://www.phytozome.net/poplar) showed that poplar contains only one member of the *MFT* subfamily and three genes in the *TFL1*-like subfamily. *PopCEN2* is 91% identical in both nucleotide and amino acid sequence to *PopCEN1*. Within the *TFL1/CEN* group, eudicot members form a well-supported subgroup with *CEN*, or weakly group with *TFL1* (Figure S1). A number of eudicots, including Arabidopsis, grapevine (*Vitis vinifera*) and tomato (*Solanum lycopersicum*), contain members of both the *CEN* and *TFL1* groups, but the *Populus* lineage apparantly has lost the *TFL1*-like gene (also see Igasaki *et al.*, 2008). In addition, poplar has one ortholog of *BROTHER OF FT AND TFL1* (*BFT*), which groups separately from the *TFL1/CEN* group. Whereas five members of the *FT* subfamily were identified in POPTR v1.1, POPTR v2 only contains three, including a possible pseudogene that lacks 5′ exons (Table S1). Resolution of some *FT* group members as alleles from unassembled haplotypes may explain this difference. However, Igasaki *et al.* (2008) cloned five different *Populus nigra FT*-like genes, indicating that additonal

mapping is needed to resolve the number of *FT* homologs in *Populus.*

PopCEN1, *PopCEN2* and *PopMFT* have distinct expression patterns

PopMFT was expressed in both vegetative and inflorescence buds (Figure S2a). The seasonal pattern of *PopMFT* expression was similar in terminal and axillary vegetative buds, and also in juvenile and adult trees (Figure S2b,c). Expression decreased as buds approached the time of bud flush, remained low in actively elongating shoot tips and was upregulated in fall buds. *PopMFT* expression in reproductive shoots was also highest in fall (Figure S2d). *PopCEN1* and *PopCEN2* had very different tissue-type expression patterns (Figure 1a). *PopCEN1* was highly expressed in shoot tips and vegetative buds, whereas *PopCEN2* showed highest expression in the stem, leaf blade, petiole and immature inflorescence. In contrast to the seasonal pattern of *PopMFT* expression, *PopCEN1* was strongly upregulated in both terminal and axillary vegetative buds around the time of spring bud flush (Figures 1b–c and S3). *PopCEN1* expression decreased markedly a month later, when shoots were still elongating, and was lowest in late summer and fall buds. Although *PopCEN1* expression was relatively low during the summer, expression level was fourfold higher in actively growing long shoots compared with short shoots that had set bud, and threefold higher in newly formed axillary buds in the axils of neoformed leaves compared with older buds in the axils of preformed leaves (7/19 collection date; Figure 1b–c). The seasonal expression pattern of *PopCEN1* was similar for both terminal and axillary vegetative buds, and for both juvenile and adult trees (Figures 1b–c and S3b–e).

Functional analysis of *PopCEN1*/*PopCEN2* and *PopMFT* in transgenic poplars

We transformed poplar clone INRA 717-1B4 (*Populus tremula* × *Populus alba*) with *35S::PopMFT* and *35S::PopCEN1* transgenes. RNAi transgenes were introduced to downregulate endogenous *PopMFT* or *PopCEN1*. None of the transgenics exhibited flowering or other obvious phenotypic effects while in tissue culture, or during 2 months in the glasshouse. Four ramets per event and 10 non-transgenic control ramets were planted in the field in June 2003. We only studied events showing detectable transgene expression in the overexpression transgenics (15–18 events per construct), and events showing target endogene expression levels below that of non-transgenic controls for RNAi transgenics (9–10 events per construct). The selected events differed widely in transgene expression level or degree of target endogene downregulation (Figure S4). Because of their high sequence similarity, both *PopCEN1* and *PopCEN2* were suppressed in *PopCEN1*-RNAi transgenics (Figure S4d); thus, transgenic phenotypes are not specific to

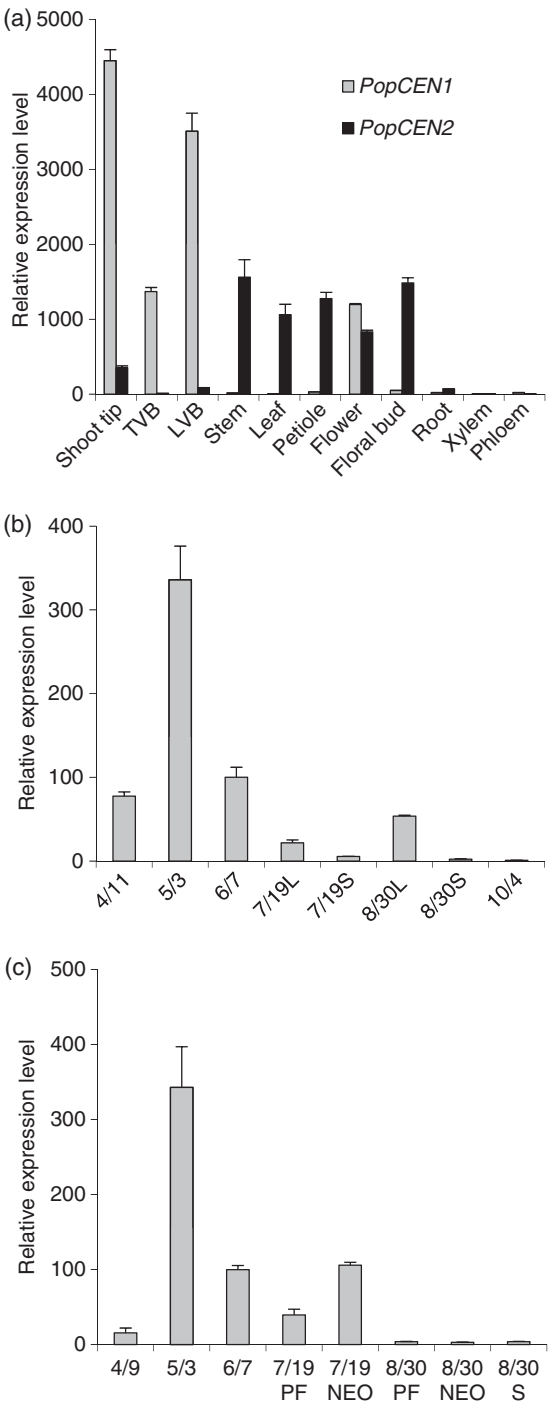

Figure 1. *PopCEN1* and *PopCEN2* are differentially expressed. Tissues were collected from *Populus alba* in Virginia, USA. (a) Expression in various tissues; LVB, axillary vegetative bud; TVB, terminal bud. Expression in terminal (b) and axillary (c) vegetative buds and shoot tips on the month/day indicated in 2006; L, shoot tip or bud from long shoot; NEO, bud from axil of neoformed leaf; PF, bud from axil of preformed leaf; S, bud from short shoot. Vegetative buds flushed shortly before the 3 May collection date. On 19 July, short shoots had set terminal buds, but long shoots were still elongating. On 30 August, terminal buds had set on long shoots. Error bars are standard deviations over three technical replicates. The *PopCEN1* expression pattern was similar in separate experiments with different *Populus* taxa (Figure S3).

PopCEN1, but indicate the combined function of *PopCEN1/PopCEN2.* We measured net growth for the 2004 growing season, when competition effects were still minor, and found no significant differences between the transgenic and control groups (Figure S4g). One event from the *35S::PopCEN1* trees grew slower than the non-transgenic trees ($P < 0.0009$, Dunnett's test), but no other transgenic events were significantly different from control trees (Dunnett's test, $\alpha = 0.05$). However, in later years, *35S::PopCEN1* trees were noticeably smaller, presumably because their delayed spring bud flush phenotype (described below) increasingly put them at a competitive disadvantage, as the closely surrounding trees that exhibited WT bud phenology grew larger.

PopCEN1/PopCEN2 expression regulates first onset of flowering and the identity of axillary buds

The first time that any tree initiated inflorescence buds was 2 years after planting (2005). Two ramets from each of two *PopCEN1*-RNAi events showed an unusual phenotype, in that some of the floral buds flushed in August and rapidly elongated into mature catkins, thus completing flower development in the same season in which they were initiated (Figure 2a,d). The inflorescence buds formed in the leaf axils of current-year stems, and, unlike normal pendulous inflorescences of poplar, the catkins were more branch-like: long and upright (Figure 2a). On one catkin of event 191, new vegetative shoots formed near the terminus of the catkin (Figure 2a, inset), indicating a reversion to vegetative identity.

Most of the floral buds initiated in 2005 overwintered as buds, flushed the following spring and produced pendulous catkins bearing female flowers with morphology indistinguishable from the wild type (Figure 2b–c). One of the 10 control trees produced a few catkins and two or more trees from each of the transgenic populations flowered, but the *PopCEN1*-RNAi population showed a much higher occurrence and intensity of flowering (measured as flowering index; Figure 3a). Moreover, the flowering index was inversely correlated with the degree of downregulation of the *PopCEN1* endogene (Figure 3b), indicating that *PopCEN1* regulates the first onset of flowering and the intensity of flowering. No correlations were found between flowering and expression level of the *PopMFT* endogene or the overexpression transgenes (data not shown).

The field trial was thinned in 2006, leaving all control trees and at least two ramets per transgenic event. The summer floral bud flush that occurred on a few trees in 2005 did not occur in subsequent years; all inflorescences elongated after winter dormancy. For control trees, there was little change in the number of flowering trees between 2006 and 2007, but by 2008, eight of the 10 control trees were flowering, although most were in the weakest flowering category (Figure 3c). Both the *35S::PopMFT* and *PopMFT*-RNAi transgenic populations showed an increase in the number of trees flowering between 2007 and 2008, although to a lesser degree than controls (Figure S5). Most *PopCEN1*-RNAi transgenics were flowering intensely by 2007. Whereas flowering showed an inverse linear correlation with *PopCEN1* endogene expression in 2006 (Figure 3b), by 2007 the *PopCEN1*-RNAi transgenics appeared to form two clusters, with all ramets from events showing more than a 50% reduction in *PopCEN1* endogene expression flowering heavily (Figure 3c,d and S4c). All ramets from *35::PopCEN1* transgenic events that had transgene expression levels in the top 50% remained non-flowering, except that one ramet from event 36 flowered in 2009 (Figure 3c,e).

By 2007, many of the catkins occurred on shorter, higher-order shoots in the intensely flowering *PopCEN1*-RNAi trees (Figure 2e), whereas control trees produced far fewer catkins (Figure 2f). Extremely short shoots with nearly all axillary buds giving rise to catkins became more frequent in the following years (compare Figure 4c with the catkin-bearing shoot of Figure 2b), a feature that is typically characteristic of older, larger trees. Terminal shoot apices always remained vegetative (Figure 4c), indicating that *PopCEN1/PopCEN2* regulates axillary meristem identity. Moreover, *PopCEN1/PopCEN2* downregulation affected the development of the axillary inflorescences, with RNAi transgenics having a significantly lower ($P < 0.0001$, Tukey–Kramer's test) average catkin length, weight and flower number per catkin, compared with controls (Figure 4a,b). In mid-April, when catkins had elongated and no new flowers were developing, *PopCEN1*-RNAi transgenic events had 26–51% fewer flowers/catkins than controls. On average, control catkins were 19% longer and 34% heavier (data not shown) than *PopCEN1*-RNAi transgenics.

PopCEN1/PopCEN2 affects shoot phenology and crown architecture

Because of the seasonal expression patterns of *PopCEN1* and *PopMFT*, we recorded the date of spring vegetative bud flush. In 2005, the *35S::PopCEN1* group flushed 9 days later than non-transgenic trees ($P < 0.0001$; Figure 5a). Seven out of 18 *35S::PopCEN1* events flushed late, ranging from 8 to 22 days later than control trees. The level of the *35S::PopCEN1* transgene expression was significantly correlated with the extent of delay in bud burst (Figure 5b). Although shoot phenology varies among years, the transgene expression level was again significantly correlated ($R = 0.838$; $P < 0.0001$) with delayed bud flush in the following year (Figure 5c–e). There was no evidence that bud flush of the two groups of RNAi trees differed from the non-transgenic trees (Tukey–Kramer's test, $\alpha = 0.05$). Four out of 19 *35S::PopMFT* events flushed significantly later than non-transgenic trees in 2005 ($P < 0.0009$, Dunnett's test), but bud burst date was not correlated with transgene expression level.

Figure 2. Flowering phenotypes in *PopCEN1*-RNAi transgenics. (a) Upright inflorescence shoots and (d) an axillary inflorescence bud flushing on 5 September 2005 in transgenic event 191. The inset in (a) is a close-up of inflorescence, indicated by the red arrow. Pendulous catkins (b) and female flowers (c) on transgenic event 178 that emerged after winter dormancy and had wild-type morphology (photos taken on 21 March 2006). Red stigmas are visible above the perianth cup in (c). (e) *PopCEN1*-RNAi transgenic event 178 bearing a large number of catkins on short shoots, and (f) non-transgenic control tree bearing a few catkins in 2007.

The time of bud flush depends on fulfilling both the chilling requirement to release dormancy and the subsequent heat sum required to promote the resumption of growth (Rohde and Bhalerao, 2007). To find out if *PopCEN1* affects dormancy release, we studied representative RNAi and overexpression transgenics in a growth chamber under a photoperiod and temperature regime previously shown to be effective for inducing, maintaining and releasing endodormancy in 717-1B4 (Figure 6a; Rohde *et al.*, 2007). In WT trees, *PopCEN1* expression in terminal buds increased during the chilling treatment phase (Figure 6b). All trees set bud during the SD treatment, with the *35S::PopCEN1* population completing bud set somewhat sooner than controls (Figure S6a). Ramets

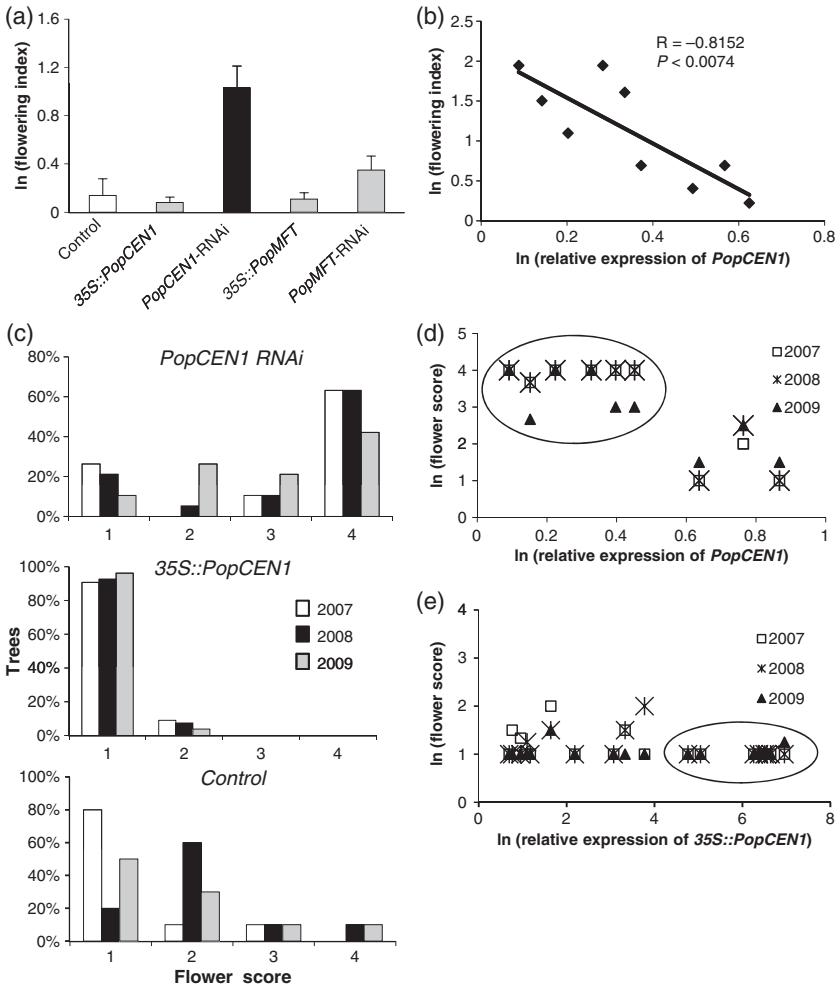

Figure 3. *PopCEN1/PopCEN2* regulates the first onset of flowering and flowering intensity. (a) Means with standard errors for flowering in 2006 for control and transgenic populations. Flowering index was defined as: (number of flowering ramets per event) × (mean flower score for each event), where the flower score was 0 = no catkins, 1 = 1–11 catkins, 2 = 11–30 catkins and 3 = more than 30 catkins (+1 was added to all indexes before ln transformation). The black bar indicates a mean that is significantly different from the control. (b) Negative correlation between increased flowering and expression level (mean per transgenic event) of endogenous *PopCEN1* in RNAi transgenics in 2006. (c) Flowering level of individual trees in 2007–2009. Flower scores were: 1 = no catkins, 2 = 1–50 catkins, 3 = 50–100 catkins and 4 = more than 100 catkins. (d) Relationship between 2007, 2008 and 2009 flowering intensity and expression level of endogenous *PopCEN1* in RNAi transgenics. Circled are events that flowered in 2006 and for which all ramets/events flowered profusely by 2007. (e) Relationship between 2007, 2008 and 2009 flowering and *35S::PopCEN1* transgene expression level. Circled are events for which all ramets, except one ramet from one event, have never flowered.

transferred to growth-promoting conditions on day 49 of the SD treatment were unable to resume growth (Figure 6d), indicating that dormancy was established in the controls and in both transgenic populations.

Monitoring dormancy release during chilling showed that the rate of release differed among the three groups (Figure 6c,d). Following 14 days of chilling, *PopCEN1*-RNAi transgenics resumed growth after an average of 37 days in LDs, but control and *35S::PopCEN1* terminal buds did not flush for 100 or more days (Figure 6c,d). After 28 days of chilling, RNAi and control plants flushed after an average of 18 and 21 days, respectively, but *35S::PopCEN1* trees required 94 days to flush. After 48 days of chilling, RNAi, control and *35S::PopCEN1* trees needed 10, 11 and 14 days, respectively, to resume growth. However, the flushed terminal shoots of *35S::PopCEN1* trees did not continue to elongate, and an axillary bud near the shoot apex grew out to become the dominant shoot (Figure 6d–f). *35S::PopCEN1*trees that had received various chilling times also had a lower percentage of axillary buds that flushed compared with controls (Figure S6b).

We measured shoot characteristics in the upper crown of representative events in June 2006, 3 years after planting. The numbers of primary branches were similar for *PopCEN1*-RNAi, *35S::PopCEN1* and control trees, but secondary branching occurred rarely or not at all in the *35S::PopCEN1* transgenic trees (Figure 7a–b). For both primary and higher-order shoots, *PopCEN1*-RNAi trees had the highest proportion of short shoots, whereas *35S::PopCEN1* trees had a very high percentage of long shoots (Figure 7c–f). The high proportion of short shoots in the *PopCEN1*-RNAi trees in 2006 correlates with the occurrence of many catkins from 2007 onwards on short, higher-order shoots in these heavily flowering trees (Figures 2e and 4c).

DISCUSSION

By studying *PopCEN1/PopCEN2* function over several years, we have shown that it has a major role in both age/size-related maturation and seasonal shoot development (Figure 8). *PopCEN1/PopCEN2* regulates the time of first flowering, and also the progression to increased proportion of short shoots and increased flowering intensity. Both these

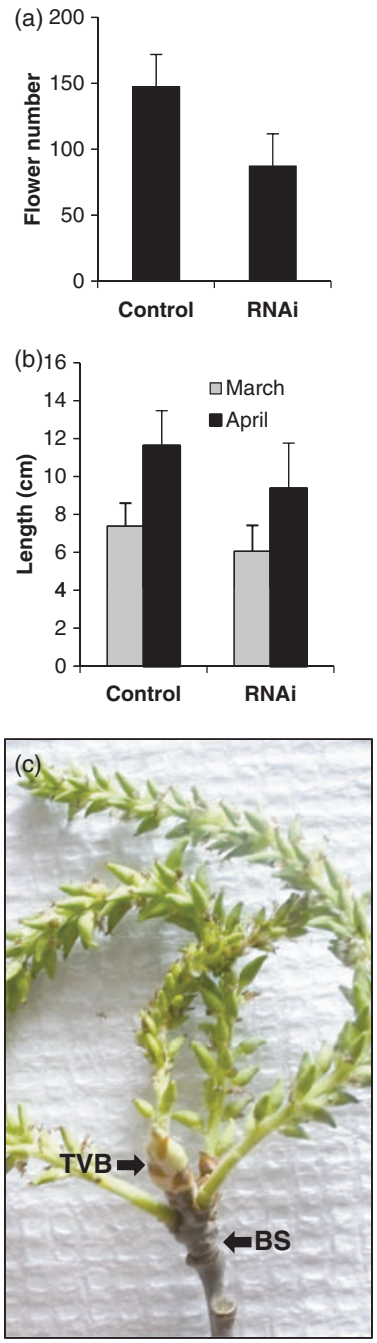

Figure 4. Inflorescence phenotypes in *PopCEN1*-RNAi transgenics. (a) Mean number of flowers per catkin in April 2009. (b) Mean catkin length. (c) Short, catkin-bearing shoot. Error bars are standard deviations for group means; BS, bud scale scar from previous year's terminal bud; TVB, terminal vegetative bud.

maturation trajectories typically continue for several years after first flowering. *PopCEN1* downregulation is important for bud dormancy release and its upregulation in spring, when growth resumes, correlates with the time that newly formed axillary meristem identity is established. In addition *PopCEN1/PopCEN2* appears to have a role in maintaining the indeterminacy of the terminal inflorescence meristem as floral meristems develop on the flanks.

PopCEN1/PopCEN2 regulates the first onset of flowering in poplar

Members of the *TFL1/CEN* subfamily vary with respect to their role in the regulation of flowering time. Downregulation of the apple ortholog, *MdTFL1*, and loss-of-function mutations in *TFL1* and *LF*, but not *CEN*, *SP* or *DET*, result in early flowering (Bradley *et al.*, 1996, 1997; Pnueli *et al.*, 1998; Foucher *et al.*, 2003; Kotoda *et al.*, 2006). Poplar transgenics showing strong downregulation of *PopCEN1* initiated floral buds after 2 years in the field, whereas most control trees first initiated floral buds at 4 years (Figure 3). In contrast, the eight *35S::PopCEN1* events showing the highest transgene expression levels never flowered, except for one ramet from event 36 that initiated floral buds during the sixth growing season (Figure 3). Because both *PopCEN1* and *PopCEN2* were suppressed in *PopCEN1*-RNAi transgenics (Figure S4d), it is possible that *PopCEN2* rather than *PopCEN1*, or both genes, regulate flowering time.

Overexpression of poplar *FT* homologs induced flowering within months following transformation (Bohlenius *et al.*, 2006; Hsu *et al.*, 2006). The complete absence of *PopCEN1/PopCEN2* activity might accelerate flowering to a similar degree as *FT* overexpression, but it is also possible that the more modest acceleration induced by *PopCEN1/PopCEN2* downregulation is because other genes have a predominant role in maintaining the long juvenile phase of poplar. In Arabidopsis, FLOWERING LOCUS C (FLC) and SHORT VEGETATIVE PHASE (SVP) directly repress the flowering pathway integrators *FT* and *SOC1* (Hepworth *et al.*, 2002; Michaels *et al.*, 2005; Helliwell *et al.*, 2006; Li *et al.*, 2008). In yeast two-hybrid studies, FT and TFL1 interact with the same proteins (Pnueli *et al.*, 2001; Wigge *et al.*, 2005), suggesting that TFL1 and FT might regulate flowering by competing for common interacting partners, and by having opposite effects on their partner's activity (Ahn *et al.*, 2006). In tomato, the local ratio of SP and the FT ortholog SINGLE FLOWERING TRUSS (SFT) regulates flowering as well as aspects of vegetative development (Shalit *et al.*, 2009). The expression level of two poplar *FT* orthologs gradually increases with age (Bohlenius *et al.*, 2006; Hsu *et al.*, 2006), suggesting the possibility that downregulation of *PopCEN1/PopCEN2* might have accelerated the time of first flowering by lowering the level of FT required to out-compete PopCEN1/PopCEN2.

Overexpression of *MFT* and *PopMFT* in Arabidopsis induces a modest acceleration of flowering (Yoo *et al.*, 2004; data not shown); however, overexpression of *PopMFT* did not detectably accelerate the year of flowering onset in poplar (Figures 3a and S5). *MFT* homologs in *P. nigra* and maize (*Zea mays*) were most strongly expressed in seeds,

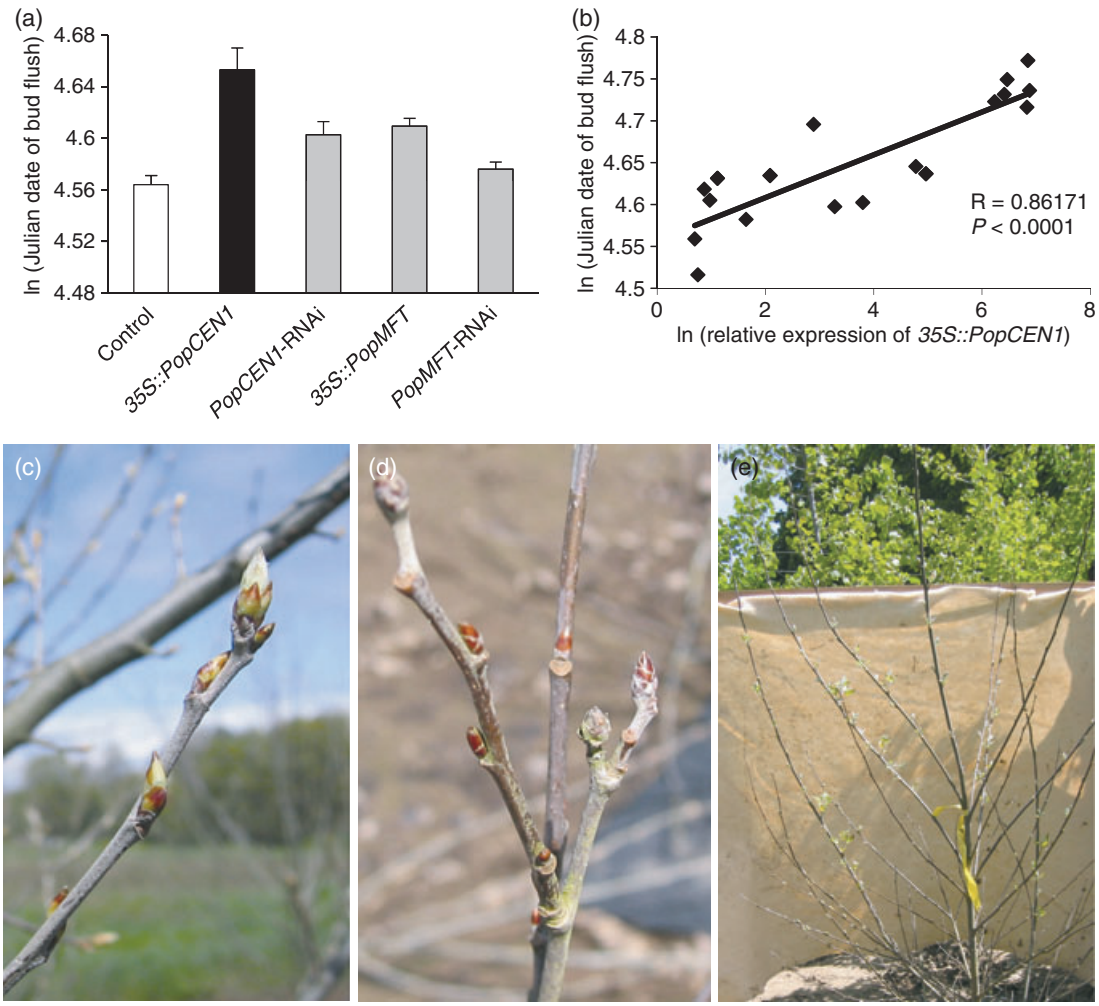

Figure 5. *35S::PopCEN1* poplar transgenics show delayed spring bud flush. (a) Means and standard errors for bud flush date for control and transgenic populations in 2005. The black bar indicates a population significantly different from controls. (b) Correlation between date of bud flush and expression level (mean per transgenic event) of the *35S::PopCEN1* transgene in 2005. (c) Flushing buds on control tree and (d) dormant buds on transgenic event 36 on 17 April 2006. (e) On 5 May 2006, buds on transgenic event 36 are in the process of flushing, whereas wild-type (WT) trees (in background, behind sheet) have expanded leaves.

suggesting *MFT* may have a role in seed development (Igasaki *et al.*, 2008; Danilevskaya *et al.*, 2008). *PopMFT* expression in buds is also seasonally regulated, with the highest expression in fall buds (Figure S2). Both SD-induced bud set and seed maturation are regulated by *ABSCISIC ACID-INSENSITIVE* (*ABI3*), and *PopMFT* is upregulated in poplars overexpressing *ABI3* just after the onset of SD (Rohde *et al.*, 2002; Gutierrez *et al.*, 2007; Ruttink *et al.*, 2007). Although *PopMFT* poplar transgenics formed buds and became dormant in the field, additional study under different, controlled conditions could perhaps reveal a functional role in bud set.

PopCEN1/PopCEN2 regulates axillary meristem identity

The most striking phenotype induced by the strong downregulation of *PopCEN1/PopCEN2* was an increase in inflorescence number. All 13 trees showing 50% or more downregulation had more than 100 catkins by 2007, whereas only one of 10 control trees reached this level of flowering, but not until 2008 (Figure 3). Moreover, *PopCEN1/PopCEN2* downregulation did not alter terminal vegetative meristem identity: inflorescences only developed from axillary meristems (Figure 4c). In monopodial annual plants such as Arabidopsis, flowering signals are translocated to the shoot apex, which transitions to an inflorescence meristem that only then gives rise to axillary flowers and secondary inflorescence shoots. Inflorescence meristems are converted to terminal flowers in *tfl1* mutants and growth ceases (Shannon and Meeks-Wagner, 1991). Thus, as discussed below, the *PopCEN1*-RNAi results shed light on the question of where florigen signals are translocated to and active in poplar (Hsu *et al.*, 2006).

There is a seasonal window after growth resumes in spring when an adult poplar tree is able to specify

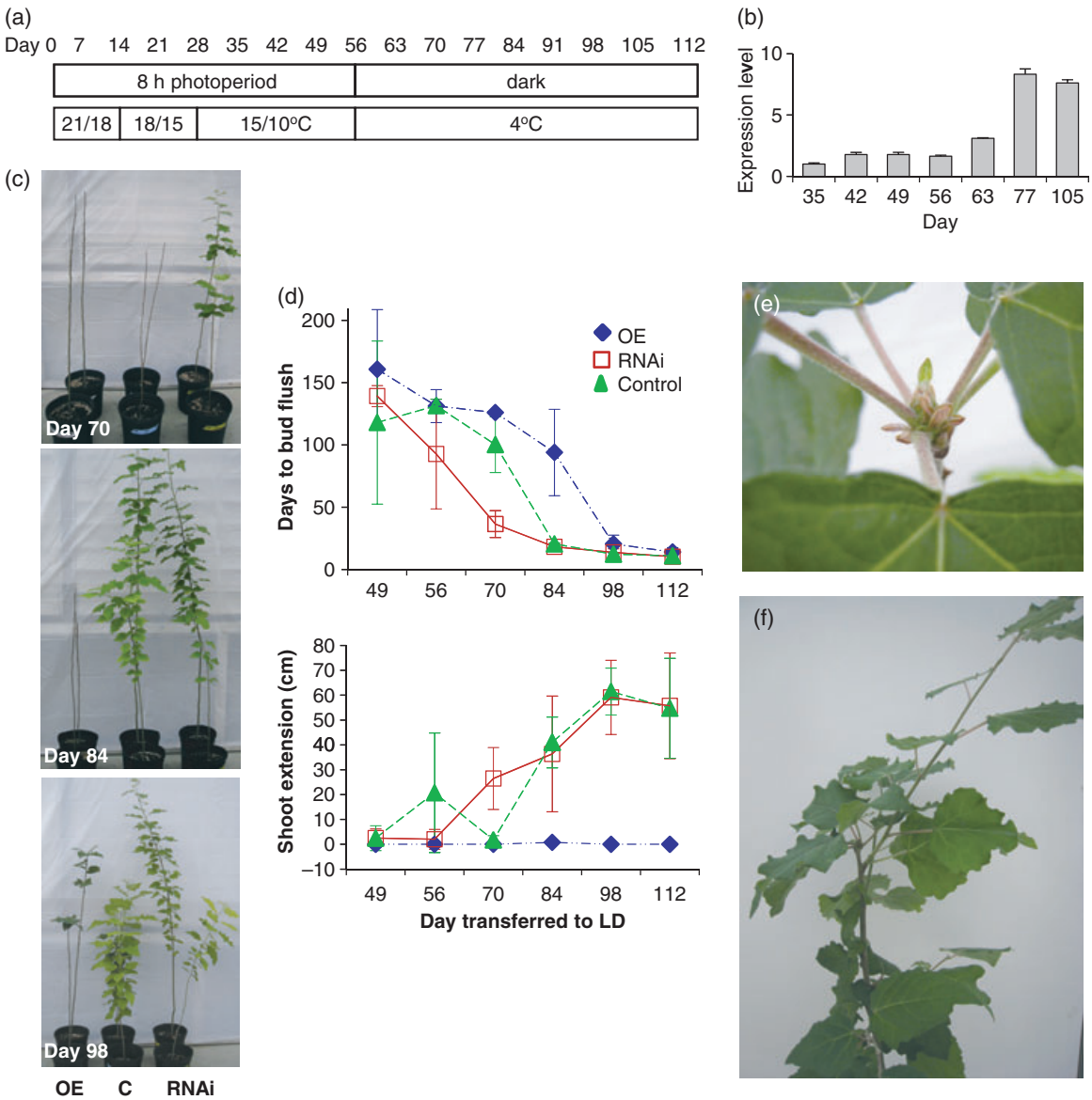

Figure 6. *PopCEN1* affects dormancy release. (a) Dormancy treatment (Rohde *et al.*, 2007). Day/night temperatures are shown for the short-day (SD) phase. At various times during the treatment, ramets were transferred to a long-day (LD) glasshouse. (b) Relative expression of *PopCEN1* in clone 717-1B4 terminal buds at different points during the treatment. Error bars indicate standard deviations of three technical replicates of samples pooled from four biological replicates. (c) Representative ramets of control (C), *PopCEN1*-RNAi and *35S::PopCEN1* (OE) trees transferred to LDs on the day indicated, and photographed 112 days after the start of the experiment. (d) Days to terminal bud flush and terminal shoot extension after transfer to LDs. Shoot extension was measured 231 days after the start of the treatment. Values are means and standard deviations of four biological replicates. (e) *35S::PopCEN1* transgenic showing a flushed terminal bud that produced four leaves, but the shoot did not elongate, and (f) extension of axillary shoot to become the dominant leader.

inflorescence identity to some of the axillary meristems, and then initiation and development of flowers continue within the inflorescence bud during the growing season (Boes and Strauss, 1994; Yuceer *et al.*, 2003). Overexpression of *FT2* and *LEAFY* (*LFY*) induced terminal flowers in poplar, indicating that terminal shoot apices are competent to respond to flower-promoting signals (Weigel and Nilsson, 1995; Rottmann *et al.*, 2000; Hsu *et al.*, 2006). One possibility is that flowering signals are translocated to the poplar shoot apex, allowing the initiation of axillary inflorescence buds, but signals are not maintained and the shoot meristem reverts to producing vegetative buds. If this is the case, our results indicate that factors other than PopCEN1/PopCEN2 maintain the indeterminacy of the terminal shoot meristem, or that greatly reduced levels of PopCEN1/PopCEN2 are sufficient to maintain terminal but not axillary meristems.

In Arabidopsis, axillary meristems arise acropetally during the vegetative phase, but arise basipetally after the

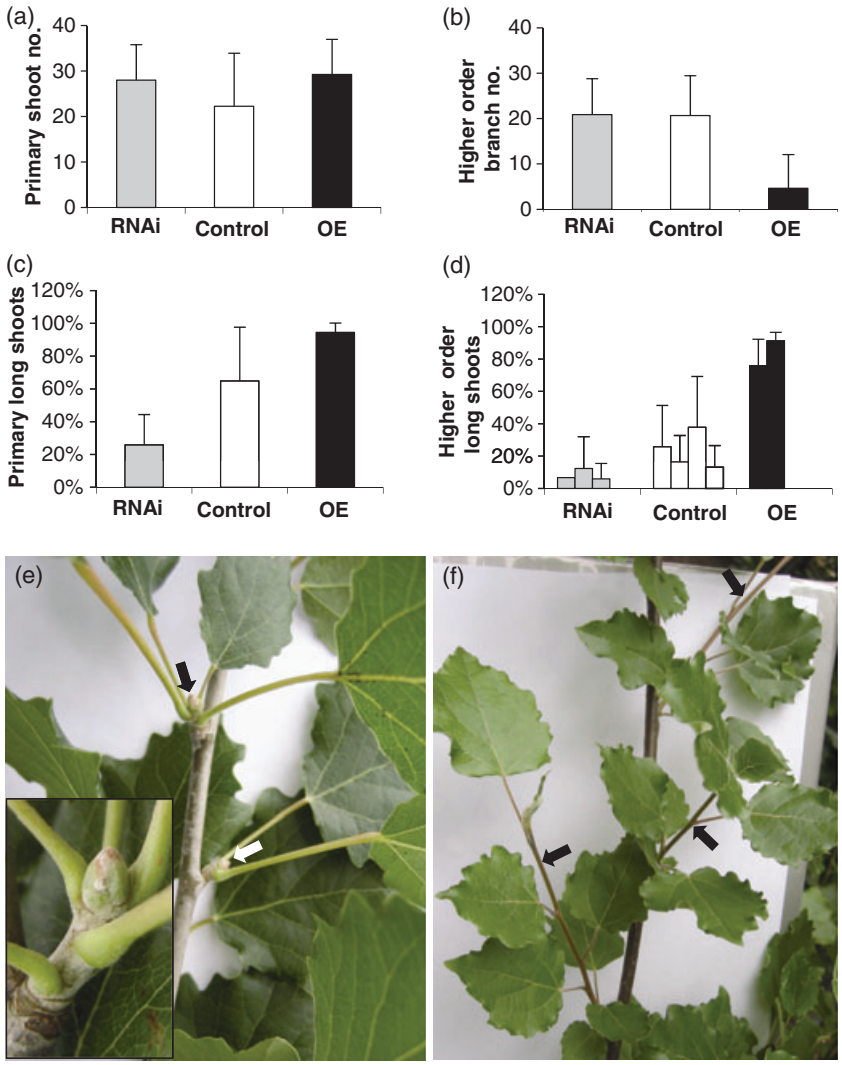

Figure 7. Branching phenotypes in *PopCEN1* transgenics. (a) Mean number of primary branches in the upper third of the crown. (b) Mean number of higher-order branches per primary/secondary branch. (c) Percentage of primary long shoots. (d) Percentage of higher-order long shoots for individual ramets. (a), (b) and (c) show means and standard deviations of three or four biological replicates; (d) shows mean percentage of secondary/tertiary branches on five primary/secondary long branches for each tree. For *35S::PopCEN1*, only between two and four primary branches had secondary shoots in two trees; the other two trees are not shown because they had no secondary branches in the upper crown. (e) Short shoots on the RNAi transgenic with terminal buds set, and (f) actively elongating long shoots of the *35S::PopCEN1* transgenic on 16 June 2006.

transition to flowering; thus, developing axillary meristems are subtended by leaves with well-developed vasculature only during the vegetative phase (Grbic and Bleecker, 2000; Long and Barton, 2000). *TFL1* is not upregulated in the shoot apex until the inflorescence phase, but is strongly expressed in axillary meristems during the vegetative phase, perhaps to provide protection from florigen signals originating from their subtending leaves (Conti and Bradley, 2007). In contrast, axillary meristems arise acropetally during all phases of poplar development. If florigen signals are translocated to axillary meristems in poplar, the transition of an individual axillary meristem to an inflorescence meristem would be analogous to the reproductive transition of an entire Arabidopsis plant. In this case, *PopCEN1* might not be needed to maintain terminal vegetative meristems, but by analogy, might be expected to maintain the identity of inflorescence apices.

Poplar inflorescences are thought to be indeterminate, but, to our knowledge, detailed SEM studies to conclusively show the absence of a terminal flower, such as those carried out for pea (Singer *et al.*, 1999), have not yet been reported. Nonetheless, catkins on *PopCEN1*-RNAi trees had significantly fewer flowers than those on control trees (Figure 4), suggesting a possible role in maintaining the inflorescence meristem. In Arabidopsis, *LFY* and *APETALA1* (*AP1*) repress *TFL1* expression in floral meristems, and *TFL1* represses *LFY*

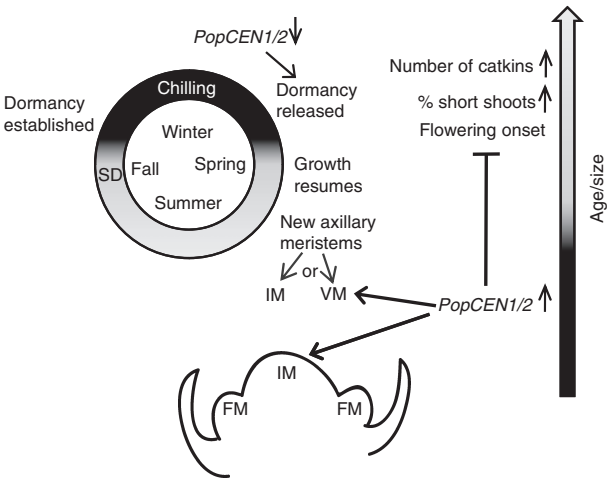

Figure 8. Model for *PopCEN1/PopCEN2* function in maturation and seasonal development in *Populus*. Arrows indicate promotion and bars indicate repression. Upward arrows next to the text indicate upregulation or increase, and downward arrows indicate downregulation; FM, floral meristem; IM, inflorescence meristem; VM, vegetative meristem. See main text for additional description.

and *AP1* in the inflorescence meristem (Liljegren *et al.*, 1999; Ratcliffe *et al.*, 1999). Consistent with the conservation of these regulatory interactions in poplar, poplar *LFY* (*PTLF*) and poplar *AP1* were strongly expressed in initiating axillary floral meristems, but were not detected in the apical inflorescence meristem (Rottmann *et al.*, 2000; Brunner *et al.*, unpublished data). Although we could not differentiate the functions of *PopCEN1* and *PopCEN2*, their different expression patterns (Figure 1a) suggest functional diversification. The higher expression of *PopCEN2* in developing inflorescences in summer suggests that it might maintain inflorescence meristem identity.

Vegetative bud and shoot tip samples included embryonic and immature leaves, and thus, initiating axillary meristems. The expression of *PopCEN1* in these samples (Figures 1 and S4) is, therefore, consistent with a role in regulating whether or not an axillary meristem transitions to an inflorescence meristem. Moreover, the striking upregulation of *PopCEN1* as growth resumes in spring corresponds to the seasonal time when new axillary meristems are developing, and some are transitioning to inflorescences (Boes and Strauss, 1994; Yuceer *et al.*, 2003). Thus, both expression and transgenic phenotypes indicate that a major role of *PopCEN1* is to promote the vegetative identity of axillary meristems, and that in adult trees, signals promoting flowering are able to switch some axillary meristems to inflorescence meristems. In addition to age-related increases in expression, poplar *FT1* and *FT2* were markedly upregulated in leaves in late spring, and *FT2* was also upregulated in axillary buds that subsequently formed inflorescences (Bohlenius *et al.*, 2006; Hsu *et al.*, 2006). Considered together, these results suggest that the local balance of PopCEN1/PopCEN2 and FT1/FT2 might regulate the seasonal determination of vegetative versus inflorescence identity of axillary meristems, as well as inflorescence versus floral meristem identity in the developing inflorescence.

Role of *PopCEN1/PopCEN2* in vegetative growth

Plant growth and size was markedly reduced in *tfl1*, *cen*, *det* and *sp* mutants, and by the downregulation of *MdTFL1* (Bradley *et al.*, 1996, 1997; Pnueli *et al.*, 1998; Foucher *et al.*, 2003; Kotoda *et al.*, 2006), but this phenotype was not induced by *PopCEN1* downregulation, because terminal vegetative meristems remained indeterminate. However, *PopCEN1*-RNAi and *35S::PopCEN1* transgenics exhibited opposite trends in branching, with RNAi transgenics having a high proportion of short shoots 3 years after planting (Figure 7). Over the years, higher-order branching and the proportion of short shoots increase in poplars (Critchfield, 1960; Dickmann *et al.*, 2001). Catkins occur on any length or order of shoot, but most commonly appear on higher-order short shoots. Hence, higher-order short shoots are not a prerequisite for flowering, but their occurrences might be coordinated; however, further studies are needed to determine if the onsets of heavy flowering and a skewed ratio of short/long shoots are significantly correlated. By reducing leaf display costs, the high leaf-to-stem mass of short shoots may be beneficial to mature trees that accumulate more non-photosynthetic organs (Suzuki, 2003). Perhaps coordination of these processes by *PopCEN1/PopCEN2* is an important feature of the poplar growth habit, allowing a large, self-supporting tree to thrive while displaying high levels of reproductive effort over many decades.

Poplars constitutively expressing oat *PHYA* do not set bud under SDs, and, unlike WT trees, *PopCEN1* expression was not downregulated in response to SDs (Ruonala *et al.*, 2008). Our studies did not reveal a role for *PopCEN1/PopCEN2* in dormancy induction, but indicate that downregulation of *PopCEN1/PopCEN2* is important for dormancy (Figures 6 and S6). Spring bud flush was delayed in *35S::PopCEN1* transgenics, yet *PopCEN1* transcripts increased during chilling, and showed a marked upregulation around the time of bud flush (Figures 1, 6 and S3). Under controlled conditions, *PopCEN1*-RNAi and *35S::PopCEN1* transgenics had different requirements for dormancy release, with *35S::PopCEN1* needing longer chilling than the WT. Thus, *PopCEN1* downregulation might be needed to enable dormancy release, and then later, as dormancy is released and growth resumes, upregulation of *PopCEN1* promotes meristem indeterminacy.

A chilling period sufficient to reduce the time to terminal bud flush for *35S::PopCEN1* transgenics was not able to sustain shoot elongation, and an axillary shoot became dominant (Figure 6). This suggests that resumption of shoot growth could have separable phases that require different

signals or different levels of a signal, and that at least some axillary buds require less chilling than the terminal bud. In beech (*Fagus* spp.), chilling had a strong effect on time of bud flush for terminal but not axillary buds; however, chilling accelerated the progression of bud flush in both types of bud, although the effect was more pronounced for terminal buds (Falusi and Calamassi, 1990).

During dormancy induction, plasmodesmata are blocked, and chilling restores symplastic circuitry (Rinne *et al.*, 2001). Moreover, storage vacuoles are produced during dormancy induction, and are distributed throughout the cytoplasm, but during chilling, they align along the plasma membrane. Delineating relationships between these cellular changes and *PopCEN1* function are important because TFL1 moves among cells to become evenly distributed across the shoot meristem (Conti and Bradley, 2007), and trafficking of proteins to storage vacuoles is impaired in *tfl1* mutants (Sohn *et al.*, 2007). Ruonala *et al.* (2008) localized *PopCEN1* expression to the rib meristiem (RM), suggesting that the dormancy-associated changes in symplastic circuity are relevant to *PopCEN1* function. For example, when *PopCEN1* is upregulated as growth resumes in spring, plasmodesmata are open, making it possible for the PopCEN1 protein to move from the RM into the SAM. Also important to determine is whether the functions of *PopCEN1* in dormancy and flowering are distinct or part of a shared regulatory pathway. The effect of *PopCEN1* expression level on dormancy release and its expression pattern suggest that it could have a role in measuring chilling accumulation, a process that might be shared between dormancy and vernalization (Horvath *et al.*, 2003; Sung and Amasino, 2005; Rohde and Bhalerao, 2007). Although there is no conclusive evidence linking vernalization with flowering in poplar, the seasonal timing of the floral transition and expression patterns of *FT1* and *FT2* (Bohlenius *et al.*, 2006; Hsu *et al.*, 2006) suggest that this is possible.

FT, *TFL1* and other meristem identity genes have been of interest as tools for modifying tree flowering (Brunner *et al.*, 2007). They may be useful for the promotion of flowering, and for its prevention as a means of reducing unwanted gene flow. Our data suggests that, with continued technological development, *PopCEN1* holds promise for both purposes. Its suppression caused large numbers of fertile flowers to form at least 2–3 years earlier and lower in the crown than would be expected in most poplars. In contrast, the more dramatic acceleration of the onset of flowering via overexpression of *LFY* or *FT* does not appear to give rise to normal catkins that are capable of completing the reproductive cycle to form viable seeds (Rottmann *et al.*, 2000; Strauss *et al.*, unpublished data). Overexpression of *PopCEN1* caused a nearly complete absence of flowering, but also caused severely disturbed shoot phenology and crown architecture. However, our results suggest that it might be productive to study the use of *PopCEN1* to prevent the transition to flowering via floral or inflorescence meristem-predominant promoters.

EXPERIMENTAL PROCEDURES

Sequence analysis

TFL1/*FT* family members were identified by BLASTP searches of predicted proteins in the Arabidopsis (TAIR, http://www.arabidopsis.org), rice (http://rice.plantbiology.msu.edu/index.shtml) and poplar (JGI, http://genome.jgi-psf.org) genome databases, and in the NCBI protein database (http://www.ncbi.nlm.nih.gov) (Table S1). Proteins were aligned using MUSCLE (Edgar, 2004), and phylogenetic analysis was performed using the neighbor-joining method in MEGA 3.1 (Kumar *et al.*, 2004).

Plant materials

Populus trichocarpa tissues were collected from trees near Corvallis, OR, USA, as previously described (Kalluri *et al.*, 2007). Buds and shoot tips were collected from juvenile and adult female hybrid poplar trees (*P. trichocarpa* × *P. deltoides*, clone 15–29) growing in plantations near Clatskanie, OR, USA, in 2001. *Populus alba* tissues were collected from a clonal group in Blacksburg, VA, USA, at different seasonal times. For Figure 1a, shoot tip, terminal and axillary vegetative buds, stems (primary/transitional growth region), leaves and petioles were collected from the upper crown of an adult tree on 19 July 2006. Flowers and inflorescence buds were collected from the same tree on 11 April 2006 and 30 August 2006, respectively. Roots were collected from a 1-year-old tree on 19 April 2007. Secondary xylem and phloem/cambium were collected from a 5-year-old tree on 9 May 2007.

Gene expression

Total RNA was extracted and treated with DNAse, as described previously (Brunner *et al.*, 2004). cDNA was synthesized from 1 μg RNA using SuperScript™ II (Invitrogen, http://www.invitrogen.com), according to the manufacturer's protocol.

Quantitative PCR was performed in a 25-μl final volume containing 12.5 μl of Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen), 0.4 μM each of forward and reverse primers, and 1 μl of a 1:5 dilution of the cDNA reaction mixture as template. Reactions were performed on an MX3000P™ Real-time PCR System (Stratagene, http://www.stratagene.com). The relative quantities were determined according to Pfaffl (2001), and were normalized to levels of *UBQ*. Normalization, calibration and standard deviation calculations were performed using qBASE v1.2.2 (Hellemans *et al.*, 2007). Experiments shown in Figures 1 and 6b were performed as described above, with the following exceptions: cDNA was synthesized from 2 μg RNA using the High Capacity cDNA kit (Applied Biosystems, http://www.appliedbiosystems.com), and qPCR was performed on an ABI PRISM™ 7500, according to the manufacturer's protocol, with relative quantities determined according to Livak and Schmittgen (2001). Primer sequences are shown in Table S2. PCR efficiencies were at least 95%, and poplar *UBQ* was validated as an internal control for the various tissues analyzed (Mohamed, 2006; Wang *et al.*, unpublished data), as previously described (Brunner *et al.*, 2004; Gutierrez *et al.*, 2008).

Vector construction and transgenic plant production

All sequences were amplified with primers introducing restriction sites suitable for subcloning (Table S2). For the *PopCEN1*-RNAi construct, a 147-bp fragment was amplified and inserted into pHANNIBAL to create an inverted repeat transgene (Wesley *et al.*,

2001). The transgene was then excised with *Not*I and ligated into pART27 (Gleave, 1992). A 239-bp fragment was amplified to create the *PopMFT*-RNAi construct. Full-length coding regions of *PopCEN1* and *PopMFT* were amplified and inserted into the 35S cassette (Hellens *et al.*, 2000), and the fusions were excised with *EcoR*V and ligated into the filled-recessed termini of the *Sst*I site of pART27.

INRA clone 717-1B4 (*P. tremula* × *P. alba*) was transformed, and DNA was isolated as described by Filichkin *et al.* (2006). The presence of the transgene was verified by PCR (see Table S2 for primers).

Phenotypic assessment of poplar transgenics

Plants were grown in the glasshouse for 2 months before being planted at a field site near Corvallis, Oregon, USA, in June 2003. Ten ramets of non-transgenic control and four ramets from independent transgenic events were transplanted in two pairs, with each pair being placed randomly on the site.

Height and diameter were measured in early spring 2004, and again in fall 2004, and the tree volume index (*VI* = height × diameter^2) was calculated. Net growth was defined as the difference between ln(*VI*) at the end and beginning of the measurement period. The vegetative bud flush date was recorded in spring 2005 when any buds along the main stem began to open. Flowering index was measured in early spring 2006 as: (number of flowering ramets per event) × (mean catkin number for each event). Growth, bud flush and flowering data were analyzed in SAS v9.1 (SAS Institute Inc., 2002–2003), using the MIXED procedures model to test the effects of constructs and the events within constructs. The response (Y-data) was the average of the two ramets in a pair, resulting in two independent data points for each transgenic event, and five data points for the control trees. To estimate and test differences between means, we used the LSMEANS protocol: Tukey–Kramer's adjustment was used for all possible pairwise comparisons between transgenic group means; Dunnett's adjustment was employed for comparisons between transgenic events and non-transgenic controls.

We assessed branching phenotypes in the upper third of the tree crown for representative events in June 2006. To measure higher-order branching, five primary or secondary long shoots with branches were randomly selected, and the percentage of higher-order long shoots was determined. We measured inflorescence number in 2007–2009 on all remaining trees. Between 10 and 27 catkins per *PopCEN1*-RNAi transgenic event for six events, or per four control ramets, were collected on 31 March 2009 and 17 April 2009, to measure average catkin length, weight and flower number. Data were analyzed using the MIXED procedures in SAS v9.1: LSMEANS and the Tukey–Kramer's test were used to estimate and detect differences between *PopCEN1*-RNAi and control groups.

Selected transgenic events and controls were propagated, grown for 10 weeks at 22°C with a 16-h photoperiod, and then subjected to a dormancy cycle regime (Rohde *et al.*, 2007) in a growth chamber. At various time points during the treatment, four ramets per transgenic event and WT were transferred to a glasshouse (22°C day/20°C night, with an extension of the photoperiod to 16 h), and time of bud flush (scored when the tips of the first leaves had emerged) and extent of regrowth was measured.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Phylogenetic analysis of the *TFL1/FT* family.

Figure S2. *PopMFT* expression in different tissues, ages and seasonal times.

Figure S3. *PopCEN1* expression in juvenile and adult trees at different seasonal times.

Figure S4. Growth of transgenic poplars and expression levels of the transgene and target endogene.

Figure S5. Flowering in *PopMFT* poplar transgenics.

Figure S6. Short-day-induced bud set and axillary bud flush in *PopCEN1* poplar transgenics.

Table S1. Sequences used for phylogenetic analysis of the *TFL1/FT* family.

Table S2. Primer sequences.

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