Overexpression of a SOC1-Related Gene Promotes Bud Break in Ecodormant Poplars

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Perennial species in the boreal and temperate regions are subject to extreme annual variations in light and temperature. They precisely adapt to seasonal changes by synchronizing cycles of growth and dormancy with external cues. Annual dormancy–growth transitions and flowering involve factors that integrate environmental and endogenous signals. MADS-box transcription factors have been extensively described in the regulation of Arabidopsis flowering. However, their participation in annual dormancy–growth transitions in trees is minimal. In this study, we investigate the function of MADS12, a Populus tremula × alba SUPPRESSOR OF CONSTANS OVEREXPRESSION 1 (SOC1)-related gene. Our gene expression analysis reveals that MADS12 displays lower mRNA levels during the winter than during early spring and mid-spring. Moreover, MADS12 activation depends on the fulfillment of the chilling requirement. Hybrid poplars overexpressing MADS12 show no differences in growth cessation and bud set, while ecodormant plants display an early bud break, indicating that MADS12 overexpression promotes bud growth reactivation. Comparative expression analysis of available bud break-promoting genes reveals that MADS12 overexpression downregulates the GIBBERELLINS 2 OXIDASE 4 (GA2ox4), a gene involved in gibberellin catabolism. Moreover, the mid-winter to mid-spring RNAseq profiling indicates that MADS12 and GA2ox4 show antagonistic expression during bud dormancy release. Our results support MADS12 participation in the reactivation of shoot meristem growth during ecodormancy and link MADS12 activation and GA2ox4 downregulation within the temporal events that lead to poplar bud break.

Keywords: poplar, MADS-box family transcription factors, dormancy, gibberellins, growth reactivation, SOC1, ecodormancy, bud break

INTRODUCTION

Woody perennial plants have acquired multiple adaptive mechanisms to coordinate their vegetative and reproductive growth with the seasonal weather changes (Cooke et al., 2012; Brunner et al., 2014). In temperate latitudes, trees switch between growth and dormancy at their shoot apical meristems to survive dehydration and freezing stress during winter months (Yordanov et al., 2014). Deciduous woody plants cease meristem activity to establish a dormant state before winter;
this is called endodormancy. The meristem is rendered insensitive to growth-promoting signals until the chilling requirement fulfillment (Rohde et al., 2007). The inability to initiate growth clearly distinguishes between endodormancy and the subsequent stage, called ecodormancy, when tree buds recover growth capacity in late winter without showing changes in morphology, maintaining plants cold protected (Groover and Cronk, 2017; Maurya and Bhalerao, 2017). During ecodormancy, the tree has the capacity to resume growth and bud break. Cell elongation of preformed leaves inside the buds precedes new cell divisions (Rohde et al., 2007). Photoperiod and temperature are the main regulatory signals of *Populus* dormancy establishment and release (Wareing, 1956; Weiser, 1970; Ding and Nilsson, 2016; Singh et al., 2017). Changes in photoperiod are constant every year in a given location, while the temperature is more variable among the years. This variation in the temperature has been exacerbated by global warming, causing significant ecological and economic detriment. Warmer springs substantially advance leaf unfolding and flowering time in perennials. On the contrary, warmer winters compromise the chilling fulfillment, which results in a delay of bud break (Wang H. et al., 2020). A better understanding of the *spring* phenology’s molecular control in perennials is crucial to avoid global warming damages and help perennials resist future climate.

The photoperiodic pathway drives *Arabidopsis* flowering and poplar shoot growth (Shim et al., 2017; Singh et al., 2017; Triozzi et al., 2018). The CONSTANS/FLOWERING LOCUS T (CO/FT) module conserves the day length measurement function of *Arabidopsis* and poplar (Yanovsky and Kay, 2002; Bohlenius, 2006). Shoot growth resumption after winter correlates with activation of *FT1* and gibberellin (GA) biosynthetic genes (Rinne et al., 2011; Pin and Nilsson, 2012). More specifically, they encode members of the *GA3* and *GA20 oxidases*. GAs correlate with the activation of glucanase GH17s gene to reopen the plasmodesmata channels. They reinitiate the symplastic growth-promoting cell-to-cell signaling within the SAM (Rinne et al., 2011; Tylewicz et al., 2018). In poplar, GAs work parallel to the FT pathway controlling shoot elongation since high levels of bioactive GA during short-day (SD) conditions were sufficient to sustain shoot elongation growth (Eriksson et al., 2015; Singh et al., 2018). Furthermore, inactivation of GAs in GA 2-OXIDASES (GA2ox) overexpressing (OE) poplars produced extreme tree dwarfism (Zawaski et al., 2011). Additionally, the timing of bud break in poplar requires APETAL2/ethylene-like responsive factor, *Early Bud-Break 1* (*EBB1*), which is a positive regulator of bud break (Yordanov et al., 2014). *EBB1* overexpression shows early bud break, and downregulation delayed bud break relative to wild-type (WT) plants (Yordanov et al., 2014). Finally, it is essential to highlight those dynamics in genomic DNA methylation level involved in regulating the dormancy–growth cycle in trees (Santamaria et al., 2009; Conde et al., 2013, 2017a,b). A progressive reduction of genomic DNA methylation in the apex precedes growth in the apical shoot during the bud break. The induction in the apex of a chilling-dependent poplar *DEMETERY-LIKE 10* (*DML10*) DNA demethylase causes a global reduction of DNA methylation levels before bud break (Conde et al., 2017a).

Multiple developmental pathways and stress responses in plants include MADS-box transcription factors (TFs), recently reviewed in Castelán-Muñoz et al. (2019). In trees, MADS-box has emerged as a crucial regulator of dormancy–growth transition during vegetative and reproductive development (Rodríguez-A et al., 1994; Hoenicka et al., 2008; Horvath et al., 2010; Leida et al., 2010; Kayal et al., 2011; Klocko et al., 2018; Singh et al., 2018; Falavigna et al., 2019; Wang J. et al., 2020). Genomic study of *Prunus persica* evergreen (ev) mutant revealed a deletion of six *DORMANCY ASSOCIATED MADS-box* (DAM) genes essential for endodormancy maintenance (Rodríguez-A et al., 1994). Consistent with this role of DAM, overexpression of *Malus domestica* MdDAMb gene showed delayed bud break (Wu et al., 2017). Endodormancy establishment and maintenance in *Populus* and *M. domestica* require a gene phylogenetically related to *Arabidopsis SHORT VEGETATIVE PHASE* (SVP) (Wu et al., 2017; Singh et al., 2018). Opposite to DAM and SVP, heterologous overexpression in poplar of a silver birch FRUITFUL homolog, *BpMADS4*, prevented bud dormancy (Hoenicka et al., 2008). Seasonal expression studies identified MADS-box homologs *Arabidopsis SUPPRESSOR OF OVEREXPRESSION OF CO1* (SOC1) during winter dormancy (Voogd et al., 2015; Kitamura et al., 2016; Wang J. et al., 2020). *Arabidopsis SOC1* integrates vernalization, photoperiodic, aging, GAs, and flowering signals and promotes meristematic activity-initiated floral meristems (Moon et al., 2003; Andrés and Coupland, 2012; Immink et al., 2012). *SOC1*-like homologs display a seasonal expression pattern and form a heterocomplex with SVP and DAM homologs of *Arabidopsis*, kiwifruit trees, Japanese apricot, and sweet cherry (de Folter et al., 2005; Voogd et al., 2015; Kitamura et al., 2016; Wang J. et al., 2020). Functional analysis of tree *SOC1* homologs during growth dormancy transitions is minimal. Nevertheless, Voogd et al. (2015) studied the phenology of kiwifruit *AcSOC1*-*, AcSOC1e*, and *AcSOC1f* OE lines, finding that only *AcSOC1f* presented earlier bud break than control plants. Even though *SOC1*, SVP, and DAM homologs could coexpress and interact, some members might have unique function during dormancy.

In this study, we investigate the role of the poplar *MADS12* gene in the dormancy–growth transition. Our phylogenetic analysis and protein sequence comparison indicate that *MADS12* belongs to the SOC1 clade. Our gene expression analysis demonstrates *MADS12* induction once chilling is fulfilled, showing a peak of expression before bud break. Moreover, we study the performance of *MADS12* OE lines (OE3 and OE5) and WT plants, under phenological assays. Our findings show that hybrid poplar OE *MADS12* downregulates *GA2ox4* gene and promotes early bud break during ecodormancy.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

The hybrid poplar *Populus tremula × alba* INRA clone 717 1B4 was used as the experimental model. Poplar plantlets were grown *in vitro* in Murashige and Skoog (MS) medium 1B (pH 5.7) supplemented with 2% sucrose and with indole acetic and indole
butyric acids (0.5 mg/L) containing 0.7% (w/v) plant agar under long-day (LD) 16-h light/8-h dark and 22°C conditions.

For the phenological assays, in vitro-cultivated poplars of WT and four selected independent MADS12 OE lines, OE1, OE3, OE5, and OE7, were transferred to pots containing blond peat, pH 4.5, and grown under LD and 22°C conditions for 3 weeks. The plants were fertilized once every 2 weeks with a solution of 1 g/L of Peters Professional 20-20-20 in LD conditions and 20-10-20 in SD conditions (Comercial Química Massó, Barcelona, Spain). Growth cessation and bud set were induced by exposing plants to SD conditions at 22°C (8-h light/16-h dark) during 8 or 10 weeks. Bud set progression was graded by scoring from stage 3 (fully growing apex) to stage 0 (fully formed apical bud) according to Rohde et al. (2010). Winter conditions were emulated by treating plant under SD 4°C for 4 or 6 weeks to fulfill the chilling requirement. Finally, plants were transferred back to LD 22°C to monitor bud break. The regrowth was scored according to the six developmental stages of bud break (stages 0 to 5) according to Johansson et al. (2014). Phenological assays to evaluate bud break of ecodormant plants were performed twice. In the first round, we assayed MADS12 OE lines OE1, OE5, and OE7 lines and WT. We observed that the highest level of MADS12 expression correlates with the earliest bud break. In the second round, we assayed only the MADS12 OE3 and OE5 lines showing the highest MADS12 expression levels and WT to increase the number of plantlets to make two biological replicates.

Generation of T1 MADS12 Overexpressing Lines

The MADS12 coding region (CDS) was amplified from hybrid poplar using gene-specific primers with attB sites (Supplementary Table 1). For polymerase chain reaction (PCR), Phusion DNA Polymerase (Thermo Fisher Scientific, Massachusetts, USA) was used, and the PCR products were purified and inserted into pDONR207 (Life Technologies, Massachusetts, USA) was used, and the PCR products were sequence verified. The Gateway cassettes carrying MADS12 OE lines OE1, OE5, and OE7 lines and WT. We observed that the highest level of MADS12 expression correlates with the earliest bud break. In the second round, we assayed only the MADS12 OE3 and OE5 lines showing the highest MADS12 expression levels and WT to increase the number of plantlets to make two biological replicates.

For comparative gene expression analysis among hybrid poplar MADS12 OE lines, OE3 and OE5, and WT, RNA was obtained from a pool of six ecodormant apical buds grown for 5 days under LD at 22°C, for each biological sample.

Mid-winter to mid-spring transcriptional profiles, used for making heat maps, were collected from hybrid poplar apical bud samples grown in natural conditions in Madrid (Spain). Normalized expression data are available in the gene atlas from Phytozone https://phytozone.jgi.doe.gov/pz/portal.html (Conde et al., 2019).

RNA Extraction, cDNA Synthesis, and qRT-PCR Analysis

RNA extraction was performed adding to frozen bud cetyltrimethylammonium bromide (CTAB) extraction buffer with 2% β-mercaptoethanol at 65°C for 5–8 min, following by three washes with chloroform. After that, 7.5 M LiCl2:50 mM EDTA was added, and samples were precipitated overnight at 4°C. The next day, RNA was collected by centrifuging at 10,000 RPM for 20 min, the supernatant was discarded, and the pellet was resuspended using NR buffer, and RNA was purified according to the manufacturer’s instructions of NZY RNA purification kit (NZYTech, Lisboa, Portugal). A total of 500 ng of RNA was retrotranscribed to cDNA using the Maxima First Strand cDNA Synthesis kit from Thermo Scientific (Thermo Fisher Scientific, Massachusetts, USA), and qRT-PCR was performed as described earlier (Ramos-Sánchez et al., 2019). The gene-specific primers used in these studies are listed in Supplementary Table 1.

Phylogenetic Analysis and Sequence Alignment

Arabidopsis protein sequences were obtained from TAIR10 website (www.arabidopsis.org), and Populus trichocarpa protein sequences from (Phytozone v10.3, www.phytozone.net). To
construct the phylogenetic tree, protein sequences were aligned with MAFFT E-INS-I algorithm; ProtTest 2.4 was used to select the best evolutionary model (JTT+I+G+F; 5 categories rate; alpha = 1.213); the phylogeny was inferred with RAxML algorithm (perform bootstrap 1000). Tree representation was made using iTol (https://itol.embl.de/) and selecting a bootstrap < 600, and clade coloring was performed according to phylogenetic relationships with Arabidopsis MADS-box genes. Multiple sequence alignment was inferred using ClustalW. MADS-box protein domains were annotated as described in Trainin et al. (2013).

In Silico Analysis of GA2ox4 Promoter
Promoter sequence (2 kb) of GA2ox4 was obtained from ASPENDB (http://aspendb.uga.edu/) and analyzed using the Plant promoter analysis navigator 3.0 (http://plantpan.itps.ncku.edu.tw/). MADS-box TF binding sites were selected and examined to search putative TFs that were identified using phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html).

MADS12 Coexpression Analysis
Genes coexpressed with MADS12 were identified using the Pearson Algorithm on bud mid-winter to mid-spring RNAseq dataset applying correlation coefficient higher than 0.9. We identified 258 genes in apical bud dataset coexpressed with MADS12. Gene expression values for each gene were normalized being the maximum of gene expression 1. From a total of 258 MADS12 coexpressed genes, a total of 242 unique homologs to Arabidopsis genome were identified.

RESULTS
Poplar SOC1 Clade Displays Seven Members
MADS-box TF gene family displays a large number of uncharacterized members in poplar. We investigated the phylogenetic relationship of MADS-box genes in Arabidopsis thaliana and Populus trichocarpa by generating a maximum likelihood phylogenetic tree using all annotated poplar and Arabidopsis full-length MADS-box protein sequences. Thirteen major lineages within the MIKC-types MADS-box have been resolved and named according to the Arabidopsis gene terminology: FLC/MAF, AP1/FUL, SEP, AGL6, SOC1, AG, AGL12, SVP, AGL15, AGL17, AP3, PI, and TT16. Also, we found two clusters that belong to MIKC-type proteins without any Arabidopsis orthologous (Figure 1A). Even though several SOCI-like genes were associated with dormancy release in trees (Voogd et al., 2015; Kitamura et al., 2016; Wang J. et al., 2020), the poplar SOCI-like members remain uncharacterized during dormancy phenology. We performed protein comparison of identified SOCI-like members of Arabidopsis, poplar, rice, and maize, revealing a high sequence similarity among MADS-box (M) domain and less conserved intervening (I) and keratin-like (K) domains (Supplementary Figure 1). Interestingly, most SOCI-like proteins display conserved C-terminal motif; however, there are members that lack this C-terminal sequence in monocots and dicots (Figure 1B). Together, these analyses indicate that SOCI members might have originated from a common MIKC-type MADS-box ancestor; nevertheless, they have evolved to a novel C-terminal sequence. Since all Arabidopsis SOCI-like genes promote flowering transition, the SOCI C-terminal motif is not critical for flowering (Schonrock, 2006; Lee and Lee, 2010; Dorca-Fornell et al., 2011; Pérez-Ruiz et al., 2015).

MADS12 Activation Occurs Once the Chilling Requirement Has Been Fulfilled
To understand the expression pattern of poplar SOCI-like genes during dormancy to growth shift, we studied their temporal expression in the Phytozome public repository, which deposited RNaseq-based gene expression analyses of hybrid poplar apical buds grown under natural conditions (Conde et al., 2019). All these genes display a particular mid-winter to mid-spring pattern of gene expression in shoot apical buds, showing a temporal specialization (Figure 2A). We focused on Potri.012G100200 gene, called MADS12, which shows a low expression level during the winter followed by a transitory expression peak with its maximum at Early Spring (Figure 2A). MADS12 is a spring gene activated during the shoot growth resumption period (Figure 2B). The repression of MADS12 observed during the winter suggested that its activation depends on fulfilling the chilling requirement (Figure 2B). We investigated MADS12 expression in dormant buds of pre-chilling and post-chilling cuttings, after moving them to growth-promoting conditions during 0, 6, and 12 days. Our qRT-PCR analysis confirmed that MADS12 is induced once the chilling requirement has been fulfilled (Figure 2C). Moreover, MADS12 shows its maximal expression at 6 days (83-fold) and decreasing ~50% at 12 days (Figure 2C). However, the expression of CYCLIN C6 (CYC6) gene, used as marker of cell proliferation (Conde et al., 2017a), peaks at 12 days (Figure 2D), suggesting that MADS12 induction precedes the cell proliferative stage. These results suggest that MADS12 could be involved in the resumption of growth after chilling period.

MADS12 Overexpression Promotes Shoot Growth Reactivation of Ecodormant Plants
To assess the functional role of MADS12 during dormancy-growth cycles, we generated 10 independent OE hybrid poplar lines, characterizing their MADS12 expression levels by qRT-PCR (Supplementary Figure 2). Because of the highest MADS12 expression level, we selected OE3 and OE5 lines to examine the growth and apical bud phenology in growth chambers under photoperiodic and temperature conditions that mimic seasonal transitions autumn–winter–spring (Rohde et al., 2010). Phenology of WT and MADS12 OE3 and OE5 genotypes did not show any differences in growth cessation and bud set, indicating that MADS12 overexpression does not impair these transitions (Supplementary Figures 3A, B).

To evaluate if MADS12 OE plants show changes in endodormancy release, we subjected those plants to 8 weeks of SDs at 22°C followed by 4 or 6 weeks of SD at 4°C.
FIGURE 1 | Identification of poplar SOC1-like proteins. (A) Phylogenetic tree analyses of MADS-box proteins from Arabidopsis and poplar. Maximum likelihood tree obtained using the RAxML tool with 1,000 bootstrap replicates. The right panel indicates the color code of the clades and bootstrap values. Clades are named FLOWERING LOCUS C (FLC), APETALA1/FRUITFUL (AP1/FUL), SEPALLATA (SEP), AGAMOUS-LIKE 6 (AGL6), SOC1, AG, AGL12, SVP, AGL15, AGL17, AP3, PISTILLATA (PI), and TRANSPARENT TESTA16 (TT16). (B) MAFF alignment of Populus, Arabidopsis, rice, and maize SOC1-like proteins. The alignment shows part of the K domain (green ink) and the SOC1 motif (red ink). Black boxes indicate identical amino acids. Gray boxes indicate conservative amino acid substitutions.
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Subsequently, we shifted them to LDs at 22°C for monitoring their bud break (Conde et al., 2017a). Noticeably, all genotypes tested remained dormant under growth-promoting conditions after 4 weeks of SD at 4°C, although they resumed growth after 6 weeks of SD at 4°C (Supplementary Figures 3A,C). We observed no differences in bud break scores among genotypes, suggesting that chilling requirement fulfillment caused an equal reactivation of shoot growth (Figures 3A,B). Once trees entered in endodormancy, WT and OE plants have same chilling requirement, and MADS12 overexpression does not induce endodormancy release.

Afterward, we tested whether MADS12 OE could restore growth on plants that had already ceased growing and set buds without any chilling treatment. We arranged a new set of WT, MADS12 OE3, and OE5 plants in LD at 22°C for 4 weeks and placed them under SD at 22°C for 8 or 10 weeks. Then, plants were subjected again to LD at 22°C, and bud break was monitored (Figure 3C and Supplementary Figure 3D). After 10 weeks in SD at 22°C, WT and most MADS12 OE3 and OE5 plants entered endodormancy; therefore, they could not resume growth under favorable environmental conditions without chilling (Supplementary Figure 3D). This observation indicates that MADS12 overexpression does not restrict the plants from reaching the endodormant state and that they could not break endodormancy without chilling. Finally, plants subjected to SD at 22°C conditions for 8 weeks ceased growth and set buds; however, they could not reach the endodormant state since plants resumed total shoot growth under LD at 22°C without chilling (Figure 3C). Significantly, the apical shoot of MADS12 OE3 and OE5 lines restored full growth ~10 days earlier than WT plants (Figures 3C,D). Furthermore, a phenological assay of additional lines confirmed that the lower the MADS12 overexpression level, the lower the differences in bud break observed (Supplementary Figure 4). Collectively, these results indicate that MADS12 overexpression promotes early bud break of ecodormant plants under favorable environmental conditions.

**GA2ox4 Is Downregulated in Ecodormant MADS12 Overexpressing Plants**

Our phenological assays revealed an accelerated shoot growth resumption of MADS12 OE lines with respect to WT, suggesting an improved growth-stimulating capacity. We hypothesize that MADS12 OE3 and OE5 might differentially express shoot growth-promoting genes with respect to WT. To test this, we collected apical buds of MADS12 OE3, MADS12 OE5, and WT plants treated with 8 weeks of SD at 22°C and exposed during 5 days to LD at 22°C conditions, before the observed phenotypic differences (Figure 3C). In poplar, one of the main factors involved in shoot growth is FT (Bohlenius, 2006; Hsu et al., 2011). qRT-PCR analysis did not show differences in FT1 expression in MADS12 OE3 and MADS12 OE5 lines with respect to WT (Figure 4A). We did not detect FT2 in these bud tissues (data not shown). This result indicates that an FT-independent pathway controls the activation of shoot growth. Bioactive GAs act as an FT parallel pathway to control poplar shoot growth (Eriksson et al., 2015). GA2ox genes cause the inactivation of bioactive GAs (Rieu et al., 2008). Only GA2ox3, GA2ox4, and GA2ox5 are expressed in the apical shoot, and RNAi downregulation of GA2ox4 and GA2ox5 promotes poplar aerial shoot growth (Gou et al., 2011). Our qRT-PCR analysis showed that GA2ox4 and GA2ox5 are downregulated in MADS12 OE3 and OE5 with

![Image](54x286 to 281x710)

**FIGURE 2** | Chilling requirement fulfillment is a prerequisite for MADS12 activation. (A) Heatmap showing normalized expression of hybrid poplar SOC1-like genes from mid-winter to mid-spring in apical buds. Groups are named “early,” “mid,” or “late” when sampling dates were within the first, second, or third month of each season, respectively, following the Northern Meteorological Seasons dates. “1” or “2” mean fortnight 1 and fortnight 2 within the month (Conde et al., 2019). (B) MADS12 annual expression pattern measured by qRT-PCR in 2-year-old poplar branches over 1 year. Ubiquitin7 is used as the housekeeping gene. Plotted values and error bars are fold-change means ± s.d. of two biological replicates. Note: MADS12 shows the highest mRNA accumulation during mid-winter–spring period. (C,D) qRT-PCR analysis of MADS12 (C) and CYCD6 (D) genes in dormant shoot apex of pre-chilling and post-chilling cuttings grown under LD and 22°C conditions. Ubiquitin7 is used as the housekeeping gene. Plotted values and error bars are fold-change means ± s.d. of two biological replicates. The x-axis scale is indicated in days. Pre-chilling and post-chilling data are shown in white and green backgrounds, respectively.

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FIGURE 3 | MADS12 overexpression promotes early bud break of ecodormant poplars. Bud break scoring of hybrid poplar MADS12 overexpressing OE3 and OE5 lines and wild type after transferring to LD and 22°C conditions (A,B) with chilling treatment and (C,D) without chilling treatment. (B,D) Representative picture of bud break differences in (B) plants after 15 days of LD exposure and (D) plants after 10 days of LD exposure. Values represent the mean of the bud score measure of n = 10–15 plants. Significant differences between overexpressing (OE) and wild type (WT) were analyzed using Tukey test, *p < 0.05. Top panels indicate photoperiodic and temperature conditions used.

DISCUSSION

MADS-box homologs to Arabidopsis SOC1 have been identified in trees; however, their function remains very little understood. Arabidopsis SOC1 is expressed in the shoot apical meristem and plays a key role during floral transition, integrating vernalization, photoperiod, GAs, and the aging pathways (Amasino, 2010; Lee and Lee, 2010). Furthermore, Arabidopsis SOC1-related genes, AGL14, AGL19, AGL42, AGL71, and AGL72, are also implicated in the control of the floral transition (Schonrock, 2006; Dorca-Fornell et al., 2011; Pérez-Ruiz et al., 2015). Our phylogenetic

respect to WT, while only GA2ox4 is found to be significantly repressed (Figure 4A). GA2ox3 shows inconsistent differences in MADS12 OE3 and OE5 with respect to WT (Figure 4A). These results point out that an increase in bioactive GAs could cause the accelerated growth resumption. Accordingly, the mid-winter to mid-spring RNAseq analysis showed an opposite temporal expression pattern, with GA2ox4 and GA2ox5 being greatly downregulated when MADS12 is highly expressed (Figure 4B). This result supports the antagonistic role of MADS12 over GA2ox4 within the temporal events that lead to poplar bud break.
FIGURE 4 | GA2ox4 is downregulated in MADS12 overexpressing lines during bud dormancy release. (A) qRT-PCR analysis of FT1, GA2ox3, GA2ox4, and GA2ox5 genes in ecodormant MADS12 overexpressing OE3 and OE5 and wild-type (WT) apices collected after 5 days in long day (LD) and 22°C treatment. Ubiquitin7 is used as the housekeeping gene. Plotted values and error bars are fold-change means ± s.d. of two biological replicates. Asterisks (*) represent statistical differences assessed by one-way ANOVA followed by Tukey post-hoc test (p < 0.05). (B) Heatmap showing RNAseq expression data of MADS12, FT1, GA2ox3, GA2ox4, and GA2ox5 genes in apical buds from mid-winter f2 to mid-spring f1. (C) Schematic representation of MADS12 and GA2ox4 antagonist expression pattern during bud dormancy release.

tree of poplar and Arabidopsis MADS-box proteins identifies a SOC1 clade including seven poplar SOC1-like family members (Figure 1A). These seven poplar SOC1-like genes show a specific expression pattern during mid-winter to mid-spring, suggesting their role in dormancy release (Figure 2A). Among them, MADS12 and Potri.015G098400 do not conserve a C-terminal SOC1 motif, suggesting that MADS12 C-terminal domain undergoes a divergent evolution (Figure 1B). The functional
importance of C-terminal SOC1 motif is still unclear. Structure-function analysis of Arabidopsis with intragenic mutations in the SOC1 gene shows that deletion of C-terminal and part of the K domain renders weak suppression of early flowering of FRIGIDA mutants (Lee et al., 2000, 2008). This observation and the fact that AGL14, also lacking the C-terminal SOC1 motif, is required for Arabidopsis flowering (Pérez-Ruiz et al., 2015) indicate that the C-terminal SOC1 motif is not essential for SOC1 and its homologs function in flowering.

Functional analyses of three SOC1 homolog genes in the kiwifruit tree, which exhibited a C-terminal SOC1 motif, resulted in only AcSOC1i overexpression that promoted early bud break under ecodormant conditions (Voogd et al., 2015). MADS12 OE lines also display an accelerated bud break of ecodormant poplars. Moreover, the seasonal expression pattern of AcSOC1i resembles one of the MADS12 showing a seasonal induction at the end of the winter (Figures 2A,B; Voogd et al., 2015). These two studies point out that AcSOC1i and MADS12 play identical functions during dormancy phenology, despite C-terminal SOC1 motif differences. Similar to our hybrid poplar MADS12 OE lines, overexpression of Arabidopsis SOC1 in poplar did not show phenotypical differences during vegetative shoot growth, but phenological assays were not performed (Bruegmann and Fladung, 2019). Additional functional studies of poplar SOC1-like genes are needed.

This work shows that chilling requirement fulfillment is necessary for MADS12 activation, as it does for FTI, EBI, and DML10, already described as bud break promoter genes in poplar (Hsu et al., 2011; Yordanov et al., 2014; Conde et al., 2017a). Our results show that MADS12 participates in ecodormancy release, concurring with a single-nucleotide polymorphism’s (SNP’s) presence associated with bud flush (Evans et al., 2014). We find that ecodormant MADS12 OE lines resume growth under LDs and warm temperatures 10 days earlier than WT, consistent with its possible function as a growth inducer during ecodormancy. Our phenological assays indicate that bud break of MADS12 OE poplars has no differences to WT after chilling fulfillment. Possibly, MADS12 OE phenotype would have been masked by the induction of endogenous MADS12 after chilling. To test this hypothesis, bud break analysis of MADS12 knockout lines should be performed. Another possibility is that the levels of a transcriptional coregulator limit the activity of MADS12 OE plants in vivo. MADS-box transcriptional regulation operates forming heterocomplexes (de Felter et al., 2005). We found that AGAMOUS-LIKE MADS-BOX PROTEIN AGL16 (AGL16-like) TF coexpressed with MADS12 (Supplementary Figures 5, 6A, and Supplementary Table 3). Moreover, AGL16-like expression is not induced in MADS12 OE lines (Supplementary Figure 6B). Thus, we proposed that chilling dependent bud break in MADS12 OE lines could be conditioned by a limiting factor such as AGL16-like. Future protein–protein interaction and coexpression assays in transgenic poplar will sort this out.

During the ecodormancy stage before bud break, reactivation of growth correlates with upregulation of GA biosynthetic genes and simultaneous downregulation of GA catabolic genes, particularly GA20ox4 (Karlb erg et al., 2010). Likewise, bud break correlates with downregulation of GA2 oxidases in Vitis vinifera and sweet cherry (Zheng et al., 2018; Vimont et al., 2019). Exogenous addition of GAs to winter buds promoted poplar bud break (Rinne et al., 2011). A functional study demonstrated that the overexpression of a Gibberellin Oxidase gene caused delayed bud break pointing to GA signaling involvement during bud break (Singh et al., 2018). Recent work showed that MADS12 activates PIN5b in Populus deltoides × eurameriana stem tissue during full-growth conditions (Zheng et al., 2020). Unexpectedly, our qRT-PCR analysis showed that PIN5b expression is significantly repressed in both MADS12 OE3 and OE5 than WT, suggesting an impaired polar auxin efflux in MADS12 OE ecodormant buds (Supplementary Figure 7). The differences in seasonal stages or Populus ecotypes might explain this contrasting activity of MADS12. Arabidopsis PIN5 localizes in endoplasmic reticulum (ER) membrane and mediates polar auxin efflux from the cortex to ER (Mrvacev et al., 2009). This intracellular auxin transport plays a role in regulating auxin homeostasis by compartmentalizing cellular auxin pools (Barbez and Kleine-Vehn, 2013). Thus, repression of PIN5b in ecodormant buds could increase cytoplasmic auxin pool. That could contribute to the faster shoot growth resumption capacity observed in MADS12 OE plants, along with GAs. Phenological assays of PIN5b RNAi lines are necessary to investigate its role during winter-to-spring transition.

An increasing amount of evidence points out that dormancy and flowering transition sharing conserved regulatory elements (Maurya and Blharaou, 2017; Triozzi et al., 2018; Falavigna et al., 2019). In Arabidopsis shoot apical meristem, SOC1 is the major hub in regulatory networks underlying flowering (Immink et al., 2012). SOC1 antagonizes SVP repressive action over GA20 oxidases 2 (GA20ox2), promoting GA biosynthesis and flowering induction at the shoot apex (Andrés et al., 2014). It is unknown if this model could be conserved during dormancy to growth transition. An inspection of public mid-winter to mid-spring RNAseq data indicates that SVL shows an opposite expression pattern with SOC1-like genes encoded by Potri.001G112400 and Potri.012G13300, and GA20 oxidases 3, 5, and 6, but not with MADS12 (Supplementary Figure 8). This observation suggests a possible interplay between SOC1-like genes and SVL to modulate GA biosynthesis in poplar during dormancy to growth transition. However, MADS12 might operate in an SVL-independent manner.

Our results revealed that ecodormant MADS12 OE poplars showed accelerated bud break and GA20ox4 repression. Moreover, the RNAseq mid-winter to mid-spring profiling demonstrates that MADS12 and GA2ox4 show opposite expression patterns during dormancy release. In silico analysis of GA2ox4 promoter identified 10 potential MADS-box-binding elements, supporting the idea that GA2ox4 could be transcriptionally repressed by MADS12 (Supplementary Table 2). Future transactivation assays should test this hypothesis. It has been shown that RNAi downregulation of GA2ox4 increased shoot growth in poplar; however, it is unknown whether GA2ox4 RNAi lines could reactivate earlier shoot growth of ecodormant plants (Gou et al., 2011; Singh et al., 2018). Phenological assays of GA2ox4 RNAi lines are critical to sort the importance of downregulation of GA catabolism during winter-to-spring
transition. We propose that the seasonal function of MADS12 is to promote growth reactivation during ecodormancy by downregulating GA2ox4 (Figure 4C). Activation of GA signaling promotes reactive oxygen species (ROS) signaling and bud break in Japanese apricot (Zhuang et al., 2013). A transitory activation of oxidative stress has been proposed to play a pivotal role in dormancy in several plant models (Beavieux et al., 2018). Whether downregulation of GA2ox4 by MADS12 promotes GAs and ROS signaling should be explored in poplar.

DATA AVAILABILITY STATEMENT
The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the Corresponding authors.

AUTHOR CONTRIBUTIONS
DG-S, JR-S, DA, DC, PT, and MP performed the experiments. All authors participated in the design of the experiments and in the discussions described elsewhere. DG-S, MP, and IA wrote the manuscript.

FUNDING
This study was supported by grants AGL2014-53352-R and PGC2018-093922-B-I00, awarded to IA and MP; and SEV-2016-0672 (2017-2021) to the CBGP Severo Ochoa Programme for Centres of Excellence in R&D from the Agencia Estatal de Investigación de Spain. The work of MP was supported by the Ramón y Cajal MINECO program (RYC-2012-10194), of whom DG-S has an FPI fellowship (PRE2019-089312) from Ministerio de Ciencia e Innovacion de España.

SUPPLEMENTARY MATERIAL
The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2021.670497/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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