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ORIGINAL ARTICLE

Overexpression of the *AtWUSCHEL* gene promotes somatic embryogenesis and lateral branch formation in birch (*Betula platyphylla* Suk.)

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Abstract

Birch (*Betula platyphylla* Suk.) is a deciduous tree with medicinal and ornamental value. During the process of genetic transformation, somatic embryos do not easily develop into transgenic plants, which is a limitation in genetic breeding. The *Arabidopsis thaliana WUSCHEL* (*AtWUS*) gene, which is a transcription factor, plays an important role in maintaining and regulating stem cell characteristics, which determines whether the stem cell population is differentiated. To explore methods for inducing somatic embryogenesis (SE) in birch, we overexpressed the *AtWUS* gene and transferred it to birch. The expression of *AtWUS* increased the SE rate from 101.4 to 717.1%. The expression of the *AtWUS* gene led to the downregulation of *BpWUS* gene expression in both calli and globular embryos as well as bud meristems. The expression of a few genes, i.e., *BpLEC1* (*LEAFY COTYLEDON 1*), *BpLEC2* (*LEAFY COTYLEDON 2*) and *BpFUS3* (*FUSCA 3*), was upregulated during both embryogenesis and bud meristem development. However, *BpABI3* (*ABSCISIC ACID INSENSITIVE 3*) gene expression was upregulated only in calli embryos, while *BpSTM* (*SHOOT MERISTEMLESS*) and *BpCUC2* (*CUP-SHAPED COTYLEDON 2*) gene expression was upregulated only in bud meristems. This result indicated that overexpression of the *AtWUS* gene promoted SE by increasing the expression of SE-related genes. In conclusion, this study focused on the role of the *AtWUS* gene in birch SE and the molecular mechanism by which SE was promoted.

Key message

This work indicates that overexpression of the WUSCHEL gene from Arabidopsis thaliana in birch can promote somatic embryogenesis and increase the development of lateral branches and buds.

Keywords Somatic embryogenesis · Transformation · Transcription factor · Embryogenic potential · Growth regulators

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Abbreviations

SAM	Shoot apical meristem
6-BA	6-Benzylaminopurine;
NAA	Naphthalene acetic acid
IBA	Indole-3-butyric acid
CIM	Callus induction medium
SIM	Shoot induction medium
ABA	Abscisic acid
GA	Gibberellic acid
ABI3	Abscisic acid insensitive 3
AGL15	Agamous-like 15
BBM	Baby boom
FUS3	Fusca 3
LEC1	Leafy cotyledon 1
LEC2	Leafy cotyledon 2
SE	Somatic embryogenesis
WUS	Wuschel
STM	Shoot meristemless
CLV3	Clavata3
CUC1	Cup-shaped cotyledon 1
CUC2	Cup-shaped cotyledon 2
PIN1	Pin-formed 1

Introduction

Birch (*Betula platyphylla* Suk.) is one of the most important hardwood trees in northern China. Its wide distribution, high adaptability, and rapid growth make birch a popular timber species for crafts, boards and furniture manufacture. Somatic embryogenesis (SE) is the key to asexual plant reproduction and regeneration, and SE is controlled by the coregulation of various transcription factors, small RNAs and hormones (Moon and Hake 2011; Jha et al. 2020; Tian et al. 2020; Wojcik et al. 2020; Salaun et al. 2021). The breeding and improvement of birch depend on the development of a large number of plant embryos, and the development of plant embryos depends on the expression of the *WUS* gene. Promoting SE has been reported in many plants, such as *Panax ginseng* (Kim et al. 2019), *Carica papaya* (Solorzano-Cascante et al. 2018), *Gossypium hirsutum* (Zheng et al. 2014), *Holm oak* (Martinez et al. 2019) and *Medicago truncatula Gaertn* (Rose 2019; Kadri et al. 2021).

In many plants, various genes involved in SE have been identified. These include *LEAFY COTYLEDON1* (*LEC1*), *LEAFY COTYLEDON2* (*LEC2*), *FUS3* (AP2/B3-like transcription factor family protein, *FUSCA3*), *ABSCISIC ACID INSENSITIVE3* (*ABI3*), *AGAMOUS-LIKE 15* (*AGL15*) and *BABY BOOM* (*BBM*). *FUS3* and *LEC1* jointly regulate the differentiation of stem cells and play a role in the formation of cotyledons (Gaj et al. 2005). The BBM protein directly acts upstream of the *LAFL* (*LEC1*, *ABI3*, *FUS3* and *LEC2*) gene to activate the *LAFL* network to induce SE (Roscoe et al. 2015; Horstman et al. 2017). The LAFL/AGL15 protein is necessary for the promotion of SE by BBM because the overexpression of BBM in *lec1*, *lec2*, *fus3* and *agl15* mutants reduces or eliminates the ability of seedlings to form somatic embryos. The *abi3* mutant exhibits the same maturation defects as other LAFL mutants (Parcy 1994; Gazzarrini et al. 2004; To 2006; Jia et al. 2014).

WUSCHEL (*WUS*), *SHOOT MERISTEMLESS* (*STM*) and *CLAVATA3* (*CLV3*) are essential for the stem cell division and differentiation of SAM (Reddy 2008). The expression of *WUS* is limited to the region composed of 500 cells below the central region of the apical meristem, *WUS* interacts with *CLV*, and regulation of *WUS* gene expression determines the identity of the meristem (Endrizzi et al. 1996; Mayer et al. 1998; Brand et al. 2000; Schoof et al. 2000). In *wus* mutants, the apical meristem cannot maintain the characteristics of stem cells (Laux et al. 1996). In *Arabidopsis*, ectopic expression of the *WUS* gene promotes the formation of meristems in roots (Gallois et al. 2004; Negin et al. 2017). *GhWUS* promotes the regeneration of embryoids and buds of *Gossypium hirsutum* (Xiao et al. 2018).

Both *WUS* and *STM* encode homeodomain proteins that control the rate of cell division and differentiation in meristematic regions (Long et al. 1996). *STM* is an important component for maintaining the characteristics of stem cells in meristematic tissues, preventing cells from prematurely committing to the differentiation pathway. A study of strong *stm* (*shoot meristemless*) mutants found that meristems disappear in an early stage of embryogenesis and that stem cell characteristics cannot be maintained in meristematic tissues (Barton and Poethig 1993). Ectopic expression of the *WUS* gene was observed in *Arabidopsis thaliana*, indicating the formation of somatic embryos without the induction of exogenous hormone expression (Zuo et al. 2002). In *Coffea canephora*, heterologous expression of the transcription factor *WUS* increases SE, induces callus formation, and increases somatic embryo yields by 400% (Arroyo-Herrera et al. 2008). However, no reports have described the expression of the *Arabidopsis WUSCHEL* gene in birch to increase the SE rate. *CUP-SHAPED COTYLEDON 1* and *2* (*CUC1* and *CUC2*) are genes that are essential for SAM maintenance during embryo development or SAM maintenance after embryo development (Takada et al. 2001). *stm* and *wus* (*wuschel*) double mutants or *cuc1* and *cuc2* double mutants cannot form normal SAMs. *CUC1* and *CUC2* are thought to act upstream of *STM* and are localized to cells at the top of the globular embryo to determine the identity of SAM. Overexpression of *CUC1* itself induces ectopic bud formation in transgenic plants (Aida 1997; Takada et al. 2001). Overexpression of the *WUS* gene in *Arabidopsis* is sufficient to induce SE in shoots and root tips (Chatfield et al. 2013).

Birch is an economically important tree species. The efficient in vitro regeneration of embryogenic calli contributes

to the development of somatic embryos in birch (Yang et al. 2021). There are no reports on lateral branch development in birch. Previous studies have reported that the *AtWUS* gene can promote the SE of other plants. We assume that the overexpression of the *AtWUS* gene can significantly change the SE of birch callus and form a large number of bud tissues. Therefore, the *AtWUS* gene promotes the development of somatic embryos, lateral branches and bud formation.

Materials and methods

Plant materials and growth conditions

Birch seeds were collected from Northeast Forestry University (Harbin, China, 45°43′45.83″ northern latitude, 126°38′11.14″ eastern longitude). Birch seeds were sterilized in 75% ethanol for 2 min, washed with sterile water, and seeded on basal medium consisting of MS (Murashige and Skoog 1962), sucrose (30 g L^{-1}), and agar (Macklin, product code: A800728) (6 g L^{-1}), MS medium adjusted to pH = 5.8. The seedlings were grown in a culture chamber at 22 °C ± 2 °C with six LED lights that generated a light intensity of 30 μmol m^{-2} s^{-1}. When the birch seedlings grew to 6 cm, 4–5 mm stem segments were cut for explants and cultured on woody plant medium (WPM) to yield calli. The medium was supplemented with 6-BA (6-benzylaminopurine, 0.8 mg L^{-1}) and NAA (naphthalene acetic acid, 0.6 mg L^{-1}). The calli were transferred to WPM differentiation medium supplemented with 6-BA (1.0 mg L^{-1}) (Zeng et al. 2010). Finally, the samples were transferred to WPM rooting medium supplemented with IBA (indole-3-butyric acid, 0.5 mg L^{-1}). All the media used in our experiments were adjusted to pH 5.8 and autoclaved at 115 °C for 20 min. Leaves were selected for DNA and RNA isolation for the identification of transgenes. Birch buds were fixed with FAA for 24 h, embedded in paraffin and sectioned for observation.

Gene cloning and vector construction

RNA was extracted from Columbia *Arabidopsis thaliana,* and the full-length CDS of *the AtWUS* gene was cloned. The overexpression vector pH7WG2D-WUS was prepared using a Gateway clone series (Nakagawa et al. 2007) (http://gateway.psb.ugent.be), and the gene was expressed under the control of the CaMV35S promoter. The cloned fragments in all the vectors were confirmed by PCR. The plasmids were transformed into *Agrobacterium* GV3101 cells. Details of the primers used for this purpose are provided in Table S2. The full-length sequence of the *AtWUS* gene was derived from NCBI (Accession No. At2g17950).

RNA extraction and quantitative real-time PCR *(qRT–PCR)*

Plant material was collected and frozen in liquid nitrogen, and total RNA and DNA were extracted according to the CTAB method (Murray and Thompson 1980; Gambino et al. 2008). First strand cDNA was synthesized using the Takara™ First Strand cDNA Synthesis Kit (Takara, Dalian, China, product code: RR047A). The PCR amplification reaction mix included 2 μL of cDNA, which was equivalent to 50 ng total RNA, and 1X PCR buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.05 U Taq DNA polymerase (Invitrogen®) and 0.2 μM of each primer in a final volume of 50 μL (Table S2). qRT–PCR was performed in 96-well plates with each reaction volume (20 μl) including 10 μl 2 × SYBR Premix Ex Taq™, 6.8 μL PCR-grade water, 2 μL cDNA template, 0.4 μL 50 × ROX reference dye I and 0.4 μL each of the forward and reverse primer (10 μM). The thermal cycling conditions were as follows: denaturation at 95 °C for 5 min followed by 40 cycles of amplification at 95 °C for 8 s, 58 °C for 30 s and 72 °C for 20 s. The gene sequences analyzed by qRT–PCR were obtained from the birch transcriptome database (http://birch.genomics.cn/page/species/index.jsp). The specific primers are listed in the attached table S2. Three biological replicates and three technical replicates were performed for each of the analyzed genes. The relative transcript levels of each gene were calculated with the comparative cycle threshold (ddCt) method (Livak and Schmittgen 2001).

Genetic transformation of birch

In the present study, genetic transformation was performed as per the procedure prescribed by Zeng et al. 2010. Birch seeds were inoculated on 1/2 MS medium after disinfection. The stem segments (Fig. 1b) of birch (approximately 2 cm) were used as explants for inoculation on WPM (Woody Plant Medium) supplemented with 6-BA (0.8 mg L^−1) and NAA (0.6 mg L^{-1}) for 30 days to allow callusing. The excess bacterial solution on the surface of the callus was dried with sterile filter paper and inoculated on the coculture medium, which was cocultured in the dark at 28 °C for 2 days. To remove bacterial growth, cocultured calli were incubated in sterile water containing 700 mg L^{-1} cephalosporin. The explants were separated from Agrobacterium by light shaking, and the surface liquid of the explants was removed with sterile filter paper. Furthermore, these explants were inoculated on transgenic plant selection medium comprising WPM supplemented with 6-BA (0.8 mg L^{-1}), NAA (0.6 mg L^{-1}), hygromycin (50 mg L^{-1}) and cephalomycin (500 mg L^{-1}). After 60 days, the explants were transferred to the medium to screen transgenic plants, namely, induction medium comprising WPM supplemented with 6-BA

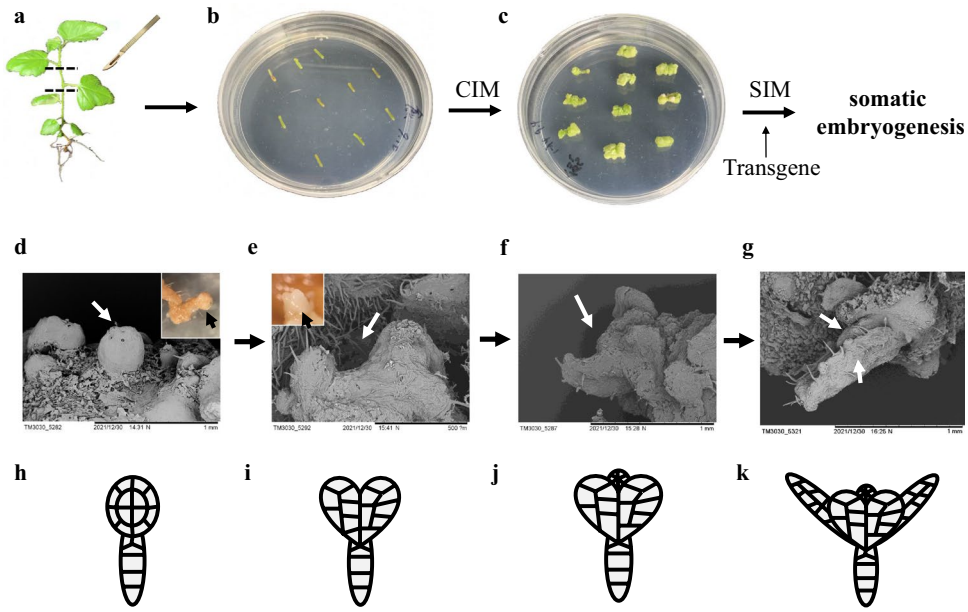

Fig. 1 The process of somatic embryogenesis in birch. **a**-**c** The stem segments of birch seedlings were cut to generate explants to establish a genetic transformation system. **d**, **h** Globular embryo. **e**, **i** Heart-shaped embryo. **f**, **j** Torpedo embryo. **g**, **k** Cotyledon embryo. (Scale bar in d, f, g = 1 mm, e = 500 μm)

(1.0 mg L^{-1}), hygromycin (50 mg L^{-1}) and cephalomycin (500 mg L^{-1}).

DNA extraction and identification of transgenic plants

A plant DNA extraction kit (Takara, Dalian, China, product code: 9765) was used to extract total genomic DNA from leaves according to the manufacturer's instructions to analyze the putative *AtWUS* gene. *AtWUS* gene-specific primers were used to amplify the full-length AtWUS-CDS in the present study to identify transgenic plants, and the sequences are listed in Table S2. We used *AtWUS* gene-specific primers to amplify the 882-bp *WUS* fragment. Primers were also designed for the identification of the 35S promoter fusion with the *AtWUS* gene, which had an expected amplicon length of 1200 bp 35S:WUS fusion region. The PCR products were analyzed by electrophoresis on a 1% agarose gel. We used a fluorescence microscope to detect GFP expression in the roots of transgenic plants under excitation light.

Southern blotting analysis

The genomic DNA samples (10 μg/sample) of wild-type and transgenic plants were digested with *Eco*RI or *Bam*HI, resolved on a 0.7% agarose gel, and imprinted on a charged nylon membrane. Probe labeling of the *AtWUS* gene with digoxigenin and southern hybridization were performed according to the manufacturer's instructions (Roche, http://www.rocheapplied-science.com). Blocking reagent, anti-DIG-AP and NBT/BCIP were purchased from Sigma. Digoxigenin-labeled probes were used for Southern hybridization and detection. *AtWUS* gene probe binding was analyzed by electrophoresis (Figure S5).

Statistical analysis

All the experiments, including germination rate and branching number statistical analyses, were completely randomized and repeated three times. In each treatment, 30 calli explants or transgenic plants were used, and the EC rate in the table indicates the number of somatic embryos formed by each callus/ the number of calli. After 40 days of culture in SIM (shoot inducing medium), we repeatedly measured the weight of the embryos derived from the calli in *35S:WUS* and CK with three techniques. We conducted a t test to determine significant differences ($p < 0.05$ or $p < 0.01$, depending on the experiment). The average number of branches was determined by statistically analyzing the results of 30 transgenic plants (Fig. 4d). To statistically analyze plant height, plant height was measured upward from the upper part of the root (Fig. 4e). The average number of meristems of each branch of 150-day-old plants was calculated based on the number of branch meristems in the stem region 2 cm from the ground (Fig. 4 h). SPSS software v 19.0 was used to analyze the data.

The sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: WUS (At2g17950), ABI3 (At3g24650), LEC1 (At1g21970), FUS3 (At3g26790), STM (At1g62360), PIN1 (At1g73590), LEC2 (At1g28300), and CUC2 (At5g53950). We compared the nucleotide sequence of *Arabidopsis thaliana* with the *Betula platyphylla* database at http://birch.genomics.cn/page/species/index.jsp.

Results

SE process of birch

The SE process of birch was determined. First, sterile birch seedlings were cultured (Fig. 1a), and 1–2 cm stem nodal explants of birch were inoculated into callus formation medium (Fig. 1b). SE was induced in differentiation medium after callus formation (Fig. 1c). Globular embryos (Fig. 1d, h), heart-shaped embryos (Fig. 1e, i), torpedo embryos (Fig. 1f, j) and cotyledon embryos (Fig. 1g, k) were observed by scanning electron microscopy (h–k is the model figure). The structure of birch during embryonic development is clearly visible (arrows).

Overexpression of AtWUS resulted in abnormal SE

Transgenic birch plants overexpressing (OEx) *AtWUS* were established, and the results are shown in Fig. 2. High levels of AtWUS expression were observed in the transgenic lines (Fig. 2b, d–f, h–j). WT calli exhibited difficulty in SE and bud formation (Fig. 2a, c, g). However, the explants overexpressing the *AtWUS* gene showed increased SE (Fig. 2d, e, f), and clustered buds were also formed on the callus (Fig. 2g, h, i). This phenomenon indicated that overexpression of heterologous *AtWUS* promoted the development of somatic embryos and formation of buds. In addition, there were no obvious differences in other parts of the transgenic plants compared with the WT plants, except for a higher SE rate.

The SE rate was investigated in explants. We observed that transgenic line 3 (L3) exhibited the highest SE rate, reaching 717.1%, which was approximately 7 times higher than the 101.4% of wild-type plants (Table 1). The SE rates of L1 and L2 were 615.2% and 371.6%, respectively (Table 1), which were higher than those of the wild type. These results suggested that overexpression of *AtWUS* can increase the SE rate in birch.

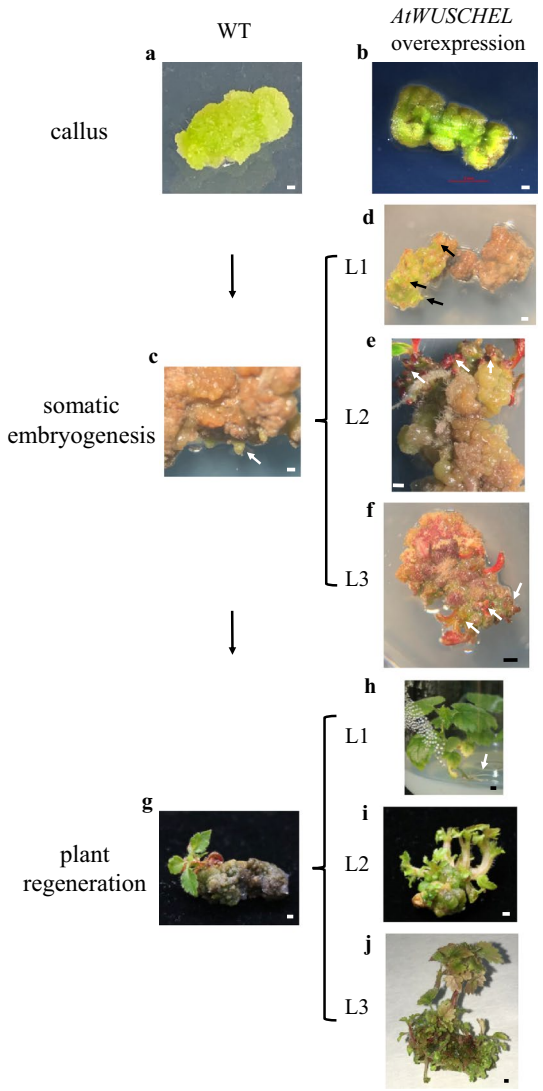

Fig. 2 Somatic embryogenesis in wild-type and *AtWUS*-overexpressing tissues. Wild-type (**a**) and *AtWUS*-overexpressing tissues (**b**) in the callus stage. Wild-type **c** and *AtWUS*-overexpressing tissues in the L1d, L2e, and L3f stages of somatic embryogenesis. Wild-type (**g**) and *AtWUS*-overexpressing tissues in the L1h, L2i, and L3j stages of plant regeneration. (Scale bar = 1 mm)

AtWUS gene overexpression promotes vegetative-to-embryogenic transition

We examined the expression patterns of 8 genes (including *BpLEC1*, *BpLEC2*, *BpFUS3*, *BpABI3*, *BpSTM*, *BpPIN*, *BpWUS* and *BpCUC2*) in calli and globular embryos in WT and transgenic birch. We characterized the effects of *AtWUS* overexpression on SE development in birch somatic embryos. We analyzed the overexpression of *AtWUS* in different transgenic lines, and the expression of L3 was higher than that of L1 and L2 (Fig. S1a). We analyzed the expression of several candidate genes in transgenic and wild-type *AtWUS* L3 plants, including the callus and globular embryo stages of SE (Fig. 3a–d). This observation suggested that the expression of the *BpWUS* gene in calli and globular embryos was lower than that in WT samples, which may be due to the high homology and similar structure of *BpWUS* and *AtWUS*. The expression of the endogenous *BpWUS* gene was inhibited (the amino acid sequence alignment of the gene in this paper is shown in Fig. S3). The *BpSTM* gene was highly expressed during the formation of globular embryos, but there was no significant difference between the wild type and the callus. This indicated that the overexpression of the *AtWUS* gene and *BpSTM* gene interacted to control the

Table 1 Somatic embryo induction rates of wild-type and transgenic birch

Replication			C[a]	SE[b]	SE rate (%)	Average (%)
35S:WUS	L1	1	195	1167	598.5	615.2**
		2	191	1149	601.6	
		3	185	1197	647	
	L2	1	179	692	386.6	371.6**
		2	192	726	378.1	
		3	195	708	363.1	
	L3	1	188	1389	738.8	717.1**
		2	194	1362	702.1	
		3	191	1358	711	
CK		1	192	181	94.3	101.4
		2	193	199	103.1	
		3	181	194	107.2	

*Indicates $P < 0.05$; **Indicates $P < 0.01$; ***Indicates $P < 0.001$, and ns indicates no significant difference)

[a]Number of explants forming a callus

[b]Number of SEs (somatic embryos) forming from a callus. (P values were calculated by Student's *t* test

formation of the meristem and promote the generation of somatic embryos.

LEC1, *LEC2*, *ABI3* and *FUS3* are essential for SE. In calli and globular embryos, the expression of *AtWUS* induced the high expression of *BpLEC1* and *BpLEC2* and further induced the high expression of *BpABI3* and *BpFUS3*. This explains the abnormal increase in SE in transgenic birch. *CUC2* is the gene required for *STM* expression. However, there was no difference in the *BpSTM* expression of transgenic birch in the calli, but *BpCUC2* expression in transgenic birch was higher than that in WT birch, and the gene expression in globular embryos showed the opposite trend. This may be due to the disordered endogenous gene regulation caused by overexpression of the *AtWUS* gene. However, the auxin transport gene *BpPIN1* was highly expressed in globular embryos, but there was no difference in the calli. The reason was that after SE development began, the auxin concentration gradient activated the expression of the *BpPIN1* gene (Fig. S1b).

After 40-day quality statistical analysis of abnormally increased calli in transgenic and WT birch, it was found that SE exhibited significant quality differences in these samples. These poor qualities represent increased somatic embryos (Table S1).

Overexpression of AtWUS led to an abnormal increase in the number of lateral branches and bud meristems of birch

Through the cultivation of transgenic plants, it was found that transgenic plants had developed lateral branches (Fig. 4a). The average lateral branch number was 5.38 ± 0.49. During 150 days of culture, we monitored plant height every 10 days and performed statistical analysis (Fig. 4a–c, e). In the first 50 days, there was little difference in plant height between wild-type plants and transgenic plants. With the growth and development of plants, the plant height of wild-type plants increased faster than that of transgenic plants. This may be caused by nutritional limitations. More lateral branches require more nutrition, which limits the development of transgenic plants.

We counted the number of buds 2 cm above the stem; in the WT plants, the number was 1.29 ± 0.69, and in the transgenic birch plants, the number was 5.28 ± 1.25. This suggests that overexpression of *AtWUS* resulted in increased bud numbers (Fig. 4f–h). Interestingly, the buds change from single to axisymmetric (Fig. 4f, g).

To further investigate the expression of *AtWUS*, we identified transgenic birch. The PCR results of the *AtWUS* transgenic plants showed that they had a 882 bp product (Fig. 5a). We used 35S:WUS gene fusion PCR detection to detect a 1200 bp fusion amplification fragment (Fig. 5b). We used a digoxigenin-labeled *AtWUS* probe for southern blotting (Fig. S5). Southern blotting of *AtWUS* L3 expression showed that this gene was successfully transferred into the plants (Fig. 5c). The identification of GFP (green fluorescent protein) in birch roots showed that *AtWUS* was successfully expressed (Fig. 5d).

Bud structure and expression of SE-related genes in birch

To further explain the abnormal increase in bud numbers on the branches of transgenic birch, the characteristics of the shoot meristem in WT and transgenic plants were observed in frozen section (Fig. 6a–d). No difference was found in buds (Fig. S4). Further analysis of the expression of genes related to the *AtWUS* gene in buds showed that

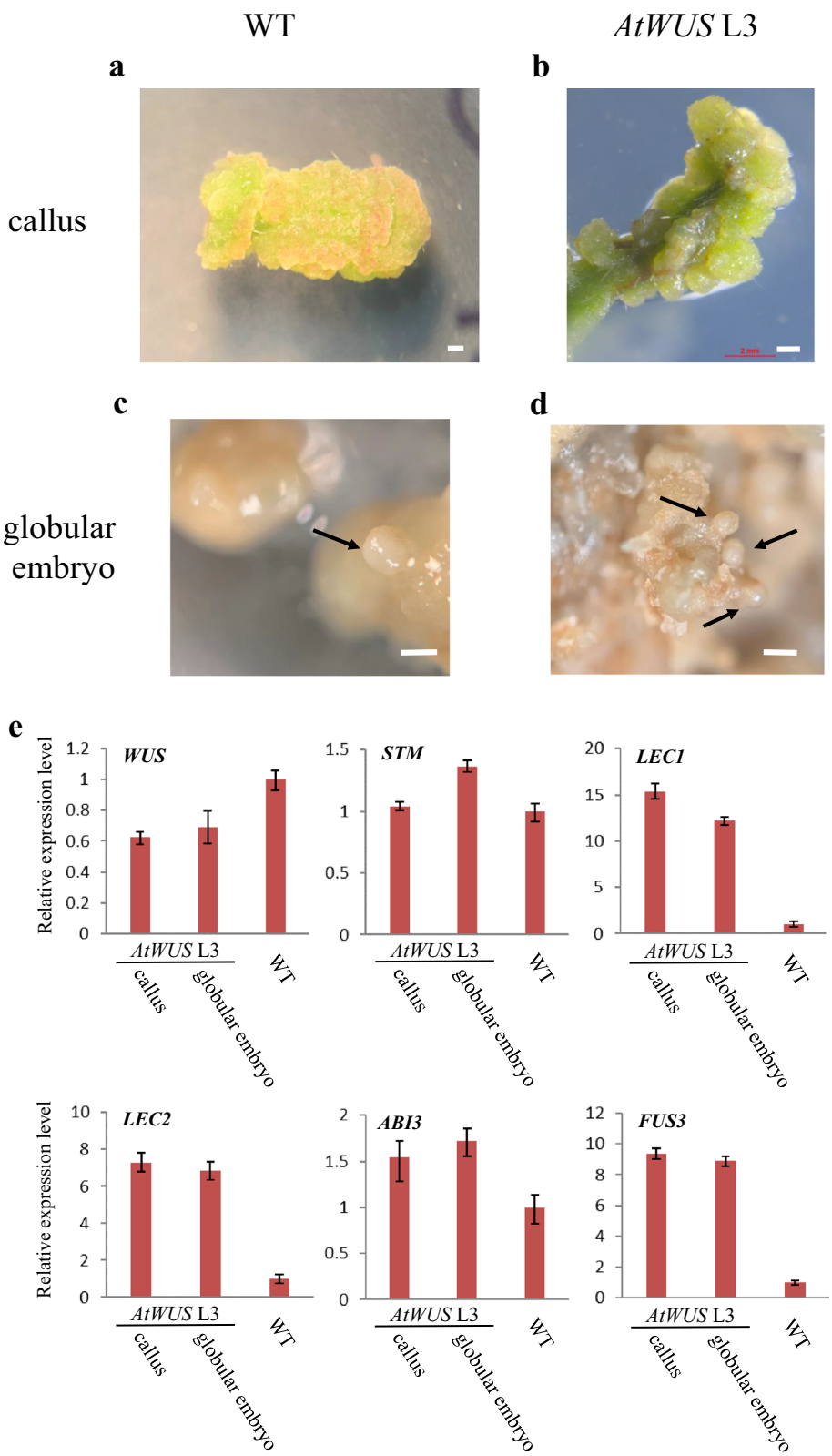

Fig. 3 Overexpression of the *AtWUS* gene promotes embryonic development and increases SE. Morphology of wild-type and *AtWUS* L3-overexpressing plants in the callus (**a**, **b**) and globular embryo (**c**, **d**) stages. **e** Gene expression analysis of transgenic calli and globular embryos expressing *AtWUS*. (Scale bar = 1 mm; P values were calculated by Student's *t* test, * is < 0.05, and ** is P < 0.01)

the expression of the *BpWUS* gene was downregulated, but the expression of *BpSTM* was upregulated. The expression levels of *BpLEC1*, *BpLEC2*, *BpFUS3*, *BpPIN1* and *BpCUC2* were upregulated, and the expression of *BpABI3* was not significantly different (Fig. 6e). The above experimental results showed that the overexpression of *AtWUS* activated

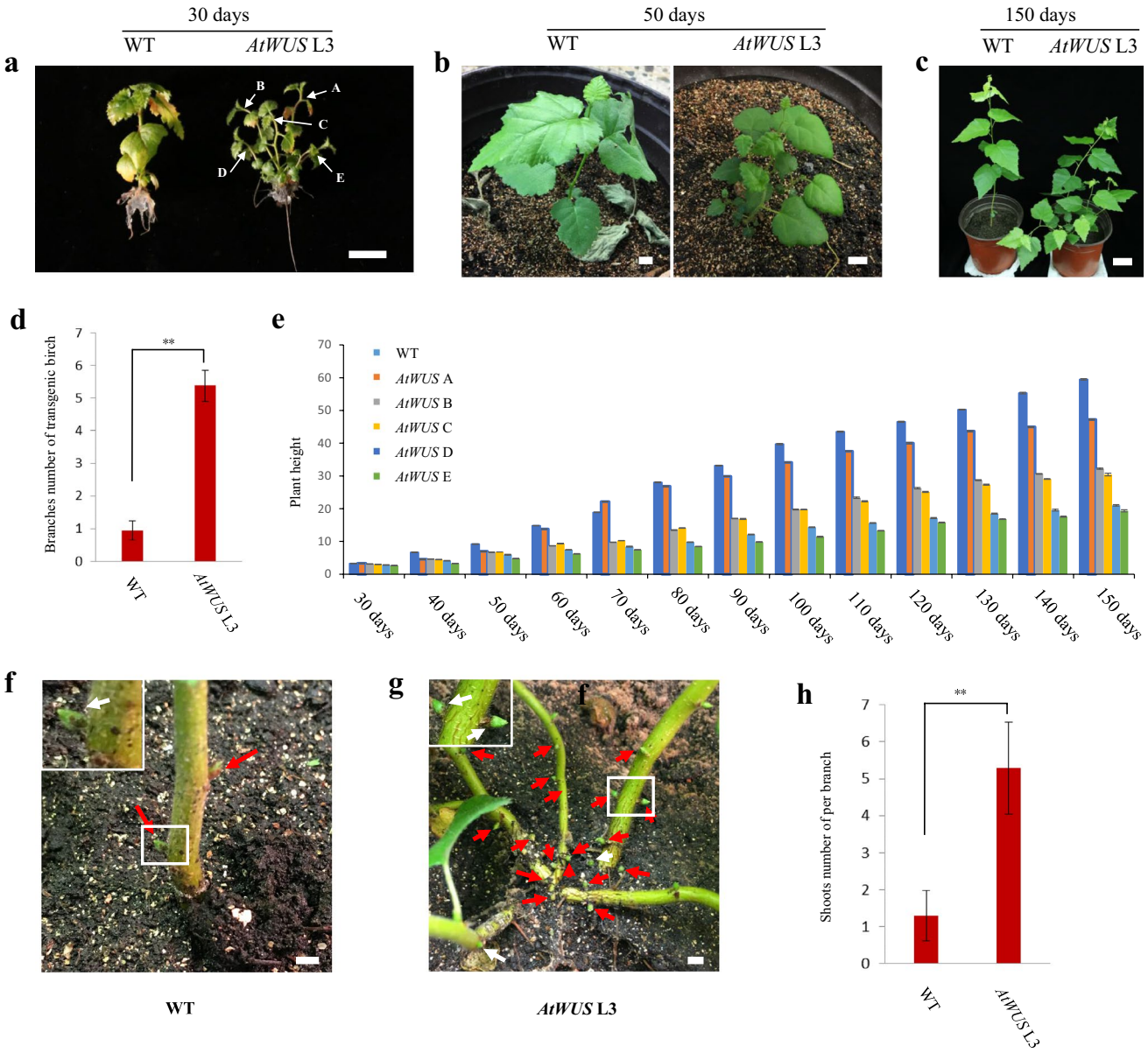

Fig. 4 Overexpression of *AtWUS* increased the number of lateral branches and bud meristems in birch. Morphological characteristics of transgenic birch at 30 days (**a**), 50 days (**b**), 150 days (**c**). **d** Number of lateral branches quantified as the mean ± SE. **e** Plant height of wild-type and transgenic plants within 150 days. **f**, **g** Wild-type and *AtWUS* L3 cells were cultured for 150 days, and the branch bud meristem number of the plant stems 2 cm from the ground. **h** Number of bud meristems are presented as the mean ± SE. (Scale bar in a, b = 1 cm; c = 10 cm; f, g = 5 mm; P values were calculated by Student's *t* test, * is < 0.05, and ** is P < 0.01)

BpWUS-related regulatory genes and promoted the increase in bud numbers by upregulating *BpLEC2* expression (Fig. S2).

Discussion

In this study, *AtWUS* transgenic plants were generated to elucidate the molecular mechanism underlying SE. Regulation of embryonic development and bud meristem formation by plant stem cells is a complex process. Environmental signals, auxin, cytokinin (CKs), ethylene, abscisic acid (ABA) and epigenetic mechanisms that are involved in chromatin remodeling have become the key factors of SE (Mendez-Hernandez et al. 2019; Wojcik et al. 2020; Jha et al. 2020; Salaun et al. 2021). The *WUS* gene exerted an antagonistic effect on STM and was negatively associated with *CLV3* (Schoof et al. 2000; Gallois et al. 2002; Reddy 2005; Yadav et al. 2010). Our results showed that *AtWUS* promoted the expression of *BpSTM* (Fig. 3e and Fig. 6e). Overexpression

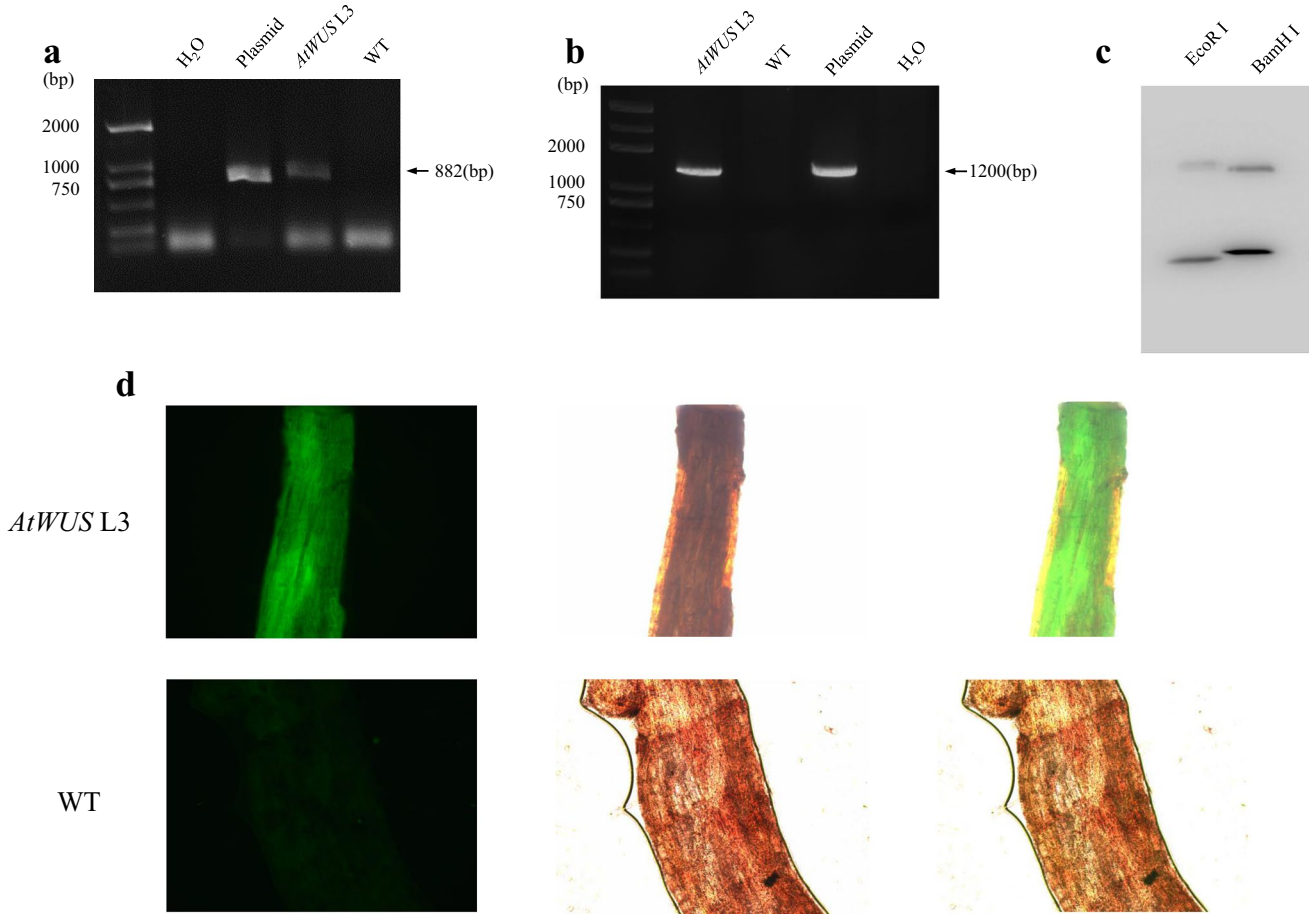

Fig. 5 Identification of transgenic birch plants. **a** Specific amplified 882 bp fragment of the *AtWUS* gene. **b** The 1200 bp amplification fragment was the result of the detection of the 35S:WUS gene fusion gene. **c** Southern blotting results of *AtWUS* L3. **d** Identification of GFP in birch roots showed successful expression of *AtWUS*. (Scale bar in c, d = 10 cm; and b = 1 cm)

of the *WUS* gene has been performed to promote SE in many plants, such as *Gossypium hirsutum* (Zheng et al. 2014), *Coffea* (Arroyo-Herrera et al. 2008), *Panax ginseng* (Kim et al. 2019) and *Medicago truncatula Gaertn* (Kadri et al. 2021). We also observed the same results.

The plant SAM (shoot apical meristem) plays a role in controlling tissue differentiation and corresponding external developmental signals, and the differentiation rate is regulated by multiple genes (Lenhard et al. 2002; Carles and Fletcher 2003; Baurle 2005; Kieffer 2006). The *WUS* gene promotes transformation from the vegetative stage to the embryonic stage and promotes the formation of somatic embryos. Overexpression of the *AtWUS* gene in *Arabidopsis* promotes the development of somatic embryos and increases the size of embryos (Brand et al. 2000; Schoof et al. 2000; Zuo et al. 2002; Arroyo-Herrera et al. 2008). Our results were more exciting (Fig. 2 and Table 1). Expression of the *AtWUS* gene in plants increased the SE rate of birch, resulting in the formation of multiple lateral branches and multiple buds. The great difference in SE development quality between transgenic plants and wild-type plants may be due to the increase in the SE rate of birch induced by *AtWUS* gene transfer, and transgenic plants had more buds than WT plants.

In birch, *PIN* plays critical roles in SE. The globular embryo moves auxin from the tip to the bottom of *PIN1 (PIN-FORMED 1)* to establish an auxin concentration gradient. During somatic embryo development, auxin accumulates in apical cells, is carried to cotyledon primordia, and is finally detected in the radicle (Friml et al. 2003; Wisniewska et al. 2006; Su et al. 2009). Overexpression of *AtWUS* promotes *PIN* gene expression and further promotes SE.

In our study, the *AtWUS* gene induced *BpLEC2* and *BpLEC1* gene expression in the calli and buds of birch and further induced *BpFUS3* gene expression. The high expression of these genes affected the growth and development of plants through AUXIN, GA and ABA signaling (Fig. 7). *LEC* plays an important role in embryonic development (Braybrook and Harada 2008). The BBM protein directly targets the upstream activation of the LAFL network of the

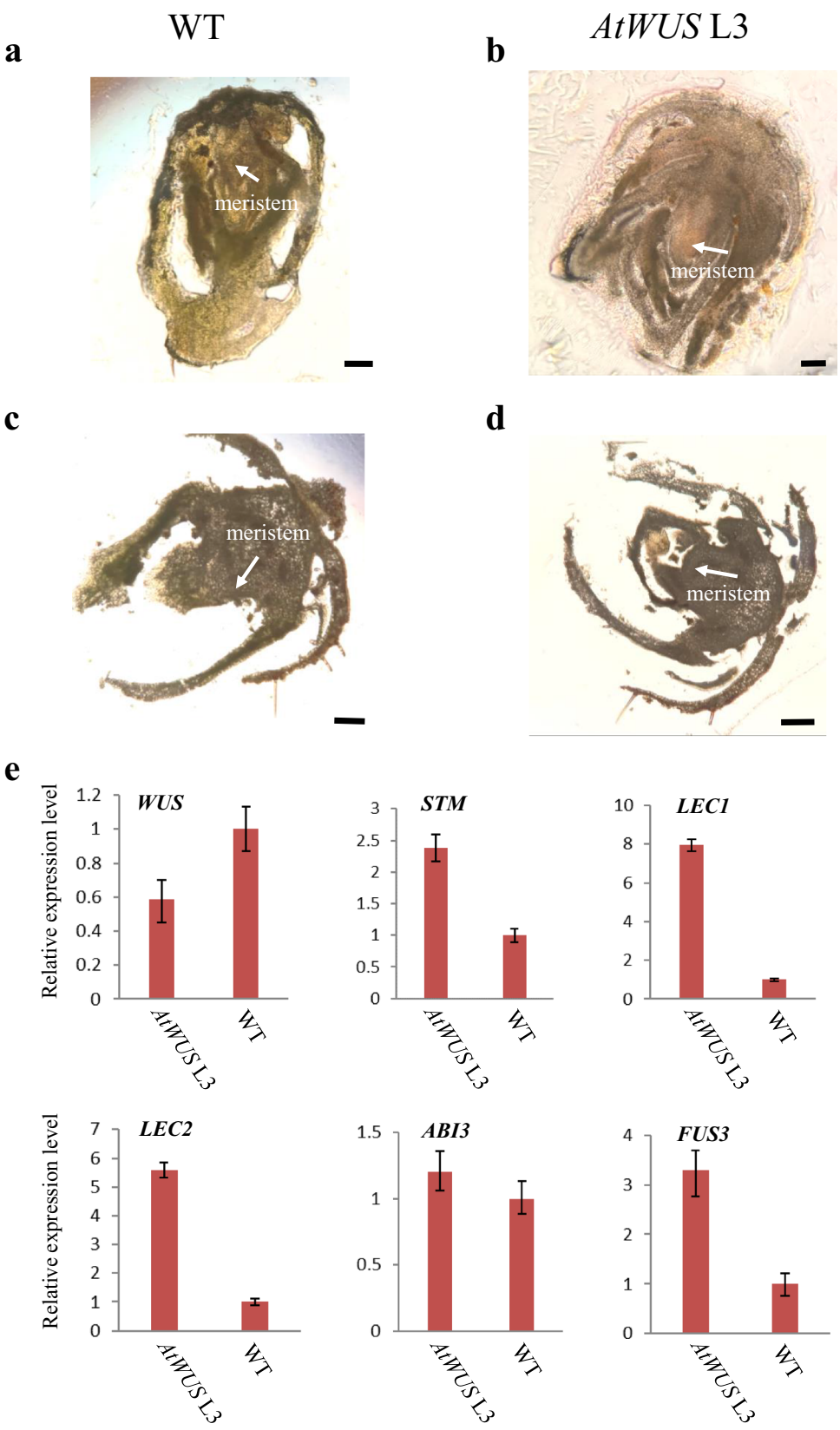

Fig. 6 Overexpression of the *AtWUS* gene promotes embryonic development-related gene expression and promotes increased bud meristem. Morphology of wild-type and *AtWUS* L3-overexpressing plants in resting buds (**a**, **b**) and buds (**c**, **d**) stages. **e** Gene expression analysis of transgenic buds carrying *AtWUS*. (Scale bar = 300 μm)

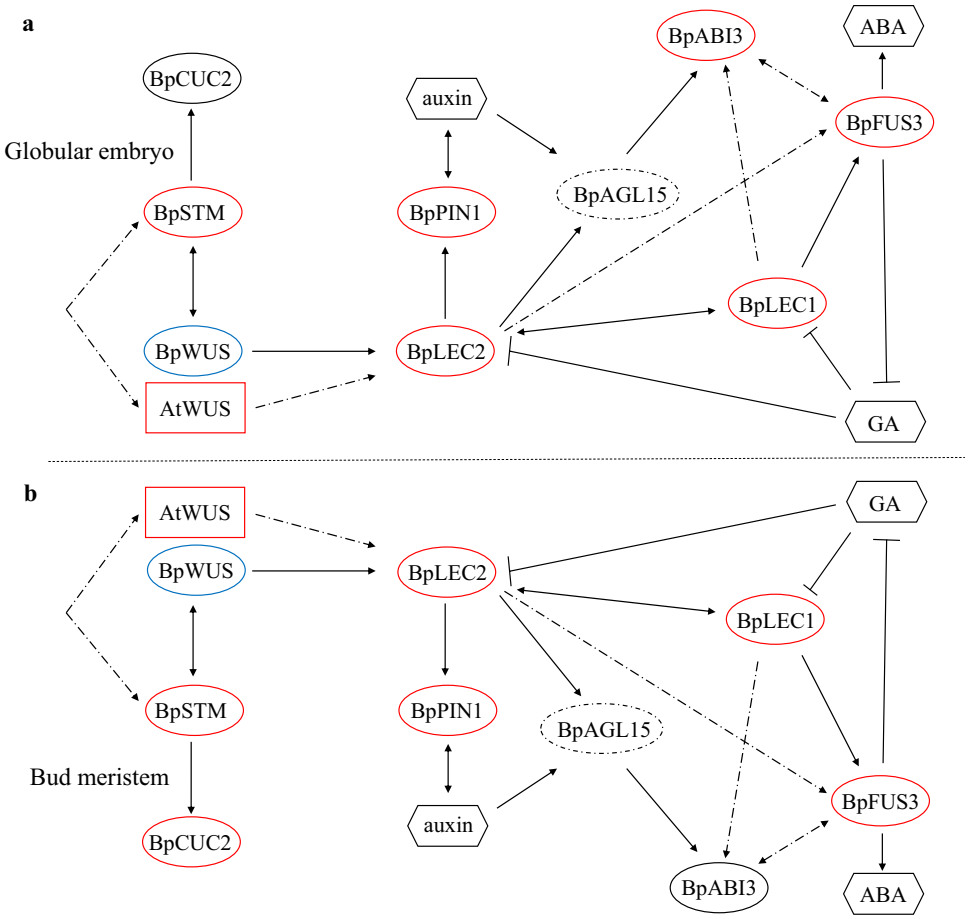

Fig. 7 Interaction between key regulatory factors of SE. Red, upregulated genes. Blue, downregulated genes. Black, genes with unchanged expression. Real lines represent reported effects, and virtual lines are possible interactions

LAFL (*LEC1*, *ABI3*, *FUS3* and *LEC2*) gene to induce SE (Roscoe et al. 2015; Horstman et al. 2017). The expression of *AtLEC2* and *AtIPTs* in *Arabidopsis* promotes tobacco embryogenic callus formation and bud regeneration (Guo et al. 2013; Li, et al. 2019). Changes in the expression of *LEC1* and *FUS3* affect microspore embryogenesis in *Brassica napus* (Elahi et al. 2016). *LEC2* is an important *WUS* response factor that promotes SE in cocoa (Fister, et al. 2018). *FUS3* and *LEC1* play roles in cotyledon formation, and *BpPIN1* expression is related to the formation of cleft leaves (Gaj et al. 2005; Qu et al. 2020). Our results showed that the expression of the *AtWUS* gene increased the expression of the *BpPIN1* gene during SE. *BpCUC1* or *BpCUC2* act upstream of *BpSTM* and are expressed in cells at the top of globular embryos; these genes determine whether the identity of SAM is closely related to plant SE (Aida 1997; Takada et al. 2001; Ikeda-Iwai et al. 2002; Su et al. 2009).

In summary, our study provides a new method for the genetic transformation of birch. increasing SE by *AtWUS* gene transformation. Similarly, the lateral branches of transgenic birch developed abnormally, which changed the normal morphology of birch and transformed it from an arbor morphology to shrub morphology. This method provides new varieties for landscaping in northern China.

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Author contributions All the authors read and approved the final manuscript. HL, LS and QJX designed the experiments and wrote the manuscript. HL, YTH, WZW and ZYC analyzed these data. Others participated in the experiments. All the authors read and approved the final manuscript.

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Data availability The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have not disclosed any competing interests.

Ethical approval Not applicable.

Consent for publication Not applicable.

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