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 the value of medicinal and ornamental greening. Plant somatic embryogenesis is a limiting step in birch genetic breeding. As a transcription factor, the *Arabidopsis thaliana WUSCHEL* (*AtWUS*) gene plays an important role in maintaining and regulating stem cell characteristics. It determines whether the stem cell population is differentiated. To explore the method of inducing somatic embryogenesis in birch. We overexpressed the *AtWUS* gene and transferred it into birch. The expression of *AtWUS* increased the somatic embryogenesis rate from 101.4% to 717.1%. The expression of the *AtWUS* gene in calli and globular embryos led to the downregulation of the *BpWUS* gene. The *BpLEC1*, *BpLEC2*, *BpFUS3* and *BpABI3* genes were upregulated. In addition, overexpression of *AtWUS* increased the number of lateral branches and bud meristem in birch. Similarly, the *BpWUS* gene was downregulated in the bud meristem. The *BpLEC1*, *BpLEC2*, *BpFUS3*, *BpSTM* and *BpCUC2* genes were upregulated. This result indicated that overexpression of the *AtWUS* gene promoted somatic embryogenesis (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 of promoting SE.

Key Message

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

Introduction

Somatic embryogenesis (SE) is the key to asexual plant reproduction and regeneration and is controlled by the coregulation of various transcription factors, small RNAs and hormones (Jha, et al. 2020; Salaun, et al. 2021; Tian, et al. 2020; Wojcik, et al. 2020; Moon and Hake 2011). Promoting somatic embryogenesis 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* (Kadri, et al. 2021; Rose 2019).

Many plant somatic embryogenesis genes 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 somatic embryogenesis (Roscoe et al. 2015; Horstman et al. 2017). LAFL/AGL15 protein is necessary for BBM to promote somatic embryogenesis 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*), *SHOOTMERISTEMLESS* (*STM*) and *CLAVATA3* (*CLV3*) are essential for the stem cell division and differentiation of SAM (Reddy 2008). The expression of *WUS* was limited to the region composed of 500 cells below the central region of the apical meristem, interacted with *CLV*, and regulated the expression of the *WUS* gene to determine 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 WUS promoted the formation of meristems in roots (Gallois, et al. 2004; Negin, et al. 2017). *GhWUS* promoted 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, regulating the role of cell division in meristematic regions (Long et al. 1996). *STM* is an important component in maintaining the characteristics of stem cells in meristematic tissues, preventing cells from prematurely concentrating into the differentiation pathway. A study of strong *stm* mutants found that meristems disappeared early in the embryogenesis stage and that stem cell characteristics could not 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 hormones (Zuo et al. 2002). In *Coffea canephora*, heterologous expression of the transcription factor *WUS* increases SE, induces callus formation, and increases somatic embryo yield by 400% (Arroyo-Herrera et al. 2008). However, there is no report on expressing the *Arabidopsis WUSCHEL* gene in birch to increase the SE rate. *CUP-SHAPED COTYLEDON 1* and *2* (*CUC1* and *CUC2*) are genes essential for SAM maintenance during embryo development or SAM maintenance after embryo development (Takada et al. 2001). *stm* and *wus* double mutants or *cuc1* and *cuc2* double mutants cannot form normal SAMs. *CUC1* or *CUC2* is thought to act upstream of *STM* and is concentrated in 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 important economic tree species. 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 the lateral branch development in birch. Overexpression of the *WUS* gene increased the SE rate from 101.4% to 717.1%. Similarly, overexpression of the *WUS* gene also increased lateral branch formation and bud meristem development. Somatic embryogenesis and the bud meristem may be due to *WUS* gene-induced regulation of *BpSTM*, *BpPIN1*, *BpLEC1*, *BpLEC2*, *BpABI3*, *BpFUS3*, *BpWUS* and *BpCUC2* gene expression. 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 taken from Northeast Forestry University (Harbin, China, 45°43'45.83" northern latitude, 126°38'11.14" eastern longitude). Birch seeds were sterilized in 75% alcohol for 2 minutes, rinsed with sterile water, and inoculated on a basal medium consisting of MS (Murashige and Skoog 1962), sucrose (30 g L^{-1}), and agar (6 g L^{-1}). The seedlings were grown in a culture chamber at 22°C ± 2°C with a light intensity of 30 μmol m^{-2} s^{-1}. When the birch seedling grew to 6 cm, a 4-5 mm stem segment was cut for explants and cultured on woody plant medium (WPM) to yield calli. The medium was supplemented with 6-BA (0.8 mg L^{-1}) and NAA (0.6 mg L^{-1}). Calli were transferred to WPM differentiation medium supplemented with 6-BA (1.0 mg L^{-1}). Finally, the samples were transferred to WPM rooting medium supplemented with IBA (0.5 mg L^{-1}). All media used in our experiments were pH adjusted to 5.8 and autoclaved at 115°C for 20 minutes. Leaves were selected for DNA and RNA isolation for the identification of transgenes.

Gene cloning and vector construction

The full-length sequence of the *AtWUS* gene is from NCBI (Accession No. At2g17950). 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 expressed by the CaMV35S promoter. The cloned fragments were checked by PCR in all vectors. The plasmid was transformed into *Agrobacterium* GV3101 cells. The primers used for cloning and connection are shown in Table S2.

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). PCR used 2 μL of cDNA, equivalent to 50 ng total RNA, in a final volume of 50 μL, 1X PCR buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, Taq DNA polymerase (Invitrogen®) 0.05 U and 0.2 μM of each primer (Table S2). qRT-PCR was performed on an Applied Biosystems 7500 real-time PCR system. The qPCR experiment was conducted according to the guidelines of the SYBR Premix Ex Taq™ kit (Takara, product code: RR390L). QPCR was performed in 96-well plates. The total volume was 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 forward and reverse primer (10 μM). The thermal cycling conditions were as follows: denaturation at 95°C for 5 minutes followed by 40 cycles of amplification at 95°C for 8 seconds, 58°C for 30 seconds and 72°C for 20 seconds. Gene sequences for qRT-PCR analysis were obtained from the birch transcriptome database (http://birch.genomics.cn/page/species/index.jsp). Specific primers are listed in the attached table S2. Each treatment or control used three biological replicates. Three biological replicates and three technical replicates were performed for each of the analyzed genes. Relative transcript levels of each gene were calculated with the comparative cycle threshold (ddCt) method (Livak and Schmittgen, 2001).

Genetic Transformation of Birch

Reference Zeng et al ' s method (Zeng, et al. 2010). Inoculate birch seeds in 1/2 MS medium after disinfection. Cut the stem fragments of birch into approximately 2 cm after 6-8 cm. Growth on callus induction medium (WPM + 6-BA 0.8 mg L^{-1} + NAA (0.6 mg L^{-1})). After 30 days callus formation. The engineering strain was prepared and infected, and the plant explants were immersed in the engineering bacterial liquid for 5 min. 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. Then, remove bacteria. The explants were incubated in sterile water containing 700 mg L^{-1} cephalosporin. The explants were separated from engineering bacteria by light shaking, and the surface liquid of explants was dried by sterile filter paper. Transgenic plant selection medium (WPM + 6-BA (0.8 mg L^{-1}) + NAA (0.6 mg L^{-1}) + hygromycin (50 mg L^{-1}) + cephalomycin (500 mg L^{-1})) was inoculated. After 60 days of transfer to induction medium (WPM+ 6-BA (1.0 mg L^{-1}) +hygromycin (50 mg L^{-1}) + cephalomycin (500 mg L^{-1})).

DNA extraction and identification of transgenic plants

According to the manufacturer's instructions. A plant DNA extraction kit (Takara, Dalian, China, product code: 9765) was used to isolate total genomic DNA from the leaves of transformed plants and control plants. We used *AtWUS* gene primers to amplify the 882 bp *WUS* fragment. For the identification of transgenic plants, the primers are shown in Table S2. We designed primers for 35S fusion of the *AtWUS* gene to identify the amplified 1200 bp *35S:WUS* fragment. For the identification of transgenic plants, the primers are shown in Table S2. The PCR products were analyzed by electrophoresis on a 1% agarose gel. We selected transgenic birch and wild-type birch roots for GFP detection to identify transgenic plants.

Southern blot analysis

The DNA extracted above was quantified using a NanoDrop2000. The genomic DNA of wild-type and selected transformed plants was digested with EcoRI restriction endonuclease or BamHI for 10 μg, separated on a 0.7% agarose gel and imprinted on a charged nylon membrane. According to the manufacturer's instructions (Roche, http://www.rocheapplied-science.com), blocking reagent (product code: 11096176001), anti-DIG-AP (product code: 11093274910), and NBT/BCIP (product code: 11681451001) were purchased from Sigma. digoxin-labeled probes were used for Southern hybridization and detection. *AtWUS* gene probe electrophoresis Fig. S5

Statistical analysis

All experiments were completely randomized and repeated three times. In each treatment, 30 callus explants or transgenic plants were used. EC rate (number of somatic embryos formed by callus/number of callus). After 40 days of culture in SIM (Shoot Inducing Medium), we repeatedly measured the weight of abnormal embryos of callus in *35S:WUS* and normal cotyledon embryos in CK by three techniques. We conducted a t test to determine the significant difference ($p < 0.05$ or $p < 0.01$, depending on the

experiment). The average number of branches was the statistical result of 30 transgenic plants (Fig. 4d). For plant height statistics, height measurement starts from the upper part of the root, excluding the root (Fig. 4e). The average meristem number of each branch was 150-day-old plants, and the meristem number of branches in the 2 cm part of stems on the ground (Fig. 4 h). SPSS software v 19.0 was used to analyze the data.

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).

Results

Somatic embryogenesis process of birch

To observe the somatic embryogenesis of birch trees, we used microscopic techniques. First, sterile birch tissue culture seedlings were cultured (Fig. 1a). Cut 1-2 cm stem fragments of birch into callus induction medium□CIM□(Fig. 1b). SE was induced in differentiation medium after callus formation (Fig. 1c). Through the induction of somatic embryos in birch, we observed the development of somatic embryos. globular embryo (Fig. 1d, h), heart-shaped embryo (Fig. 1e, i), torpedo embryo (Fig. 1f, j) and cotyledon embryo (Fig. 1g, k). The microstructure showed that the developmental structure of somatic embryos was relatively close, and it was difficult to separate a single embryo.

Overexpression of *AtWUS* resulted in abnormal somatic embryogenesis

To understand the role of the *AtWUS* gene in stem cell regulation and growth control in birch. We constructed *AtWUS* overexpression vector. The expression was under the cauliflower mosaic virus coat protein promoter, resulting in the universal expression of adjacent genes. We observed the difficulty of somatic embryogenesis and bud formation in nontransgenic calli (Fig. 2a, c, g). However, the explants overexpressing the *AtWUS* gene showed more somatic embryogenesis (Fig. 2d, e, f). Clustered buds were also formed on the callus (Fig. 2h, i, g). This phenomenon indicated that overexpression of heterologous *AtWUS* promoted the development of somatic embryos and formed more buds.

It has been reported that the *AtWUS* gene can promote SE, but the efficiency of promoting somatic embryogenesis is different. According to the somatic embryogenesis rate of explants. We observed that the highest transgenic line 3 (L3) reached 717.1%, which was approximately 7 times higher than the 101.4% of wild-type plants (Table 1). The somatic embryogenesis rates of L1 and L2 were 615.2% and 371.6%, respectively (Table 1), which were higher than those of the wild type. This indicates that overexpression of *AtWUS* can increase the incidence of somatic embryos in birch.

Transformation from calli to somatic embryos depends on *AtWUS* overexpression

To characterize the effect of *AtWUS* gene overexpression on somatic embryogenesis in birch. We analyzed the expression of several candidate genes in transgenic and wild-type *AtWUS* L3 plants. We isolated callus and early globular embryo stages of somatic embryogenesis and analyzed gene expression (Fig. 3a-d). qPCR was used to determine the expression level of *AtWUS* in birch. The calli of transgenic plants (L1, L2, L3) cultured in SIM were analyzed, and wild-type (WT) birch calli were used as controls. The data showed that *AtWUS* was overexpressed in different transgenic lines (Fig. S1a).

Expression of the *AtWUS* gene induces the lateral wall of roots to form bud meristems(Gallois, et al. 2004; Negin, et al. 2017). The expression of the *BpWUS* gene in calli and globular embryos was lower than that in control plants, 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). *STM* acts upstream of *WUS*, *WUS* and *STM* to complement each other to activate cell division and control the formation of bud meristem (Endrizzi, et al. 1996; Gallois, et al. 2002; Lenhard, et al. 2002). The *BpSTM* gene was highly expressed in 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 formation of the meristem and promote the generation of somatic embryos.

LEC1, *LEC2*, *ABI3* and *FUS3* are essential for SE (Horstman, et al. 2017). The expression of *LEC1* and *FUS3* can induce microspore embryogenesis (Ikeda-Iwai, et al. 2002). Our results showed that the expression of *AtWUS* activated the high expression of *BpLEC1* and *BpLEC2* in calli and globular embryos (Fig. 3e). The high expression of *BpABI3* and *BpFUS3* was further activated (Fig. 3e). This explains the abnormal increase in somatic embryos. *CUC2* is the gene required for *STM* expression, but there was no significant difference in *BpSTM* expression between calli and controls, and c *BpCUC2* was highly expressed. The opposite is true in globular embryos. This may be due to the disorder of endogenous gene regulation caused by overexpression of the *AtWUS* gene. Auxin plays an important regulatory role in plant embryonic development. We studied auxin transport *BpPIN1*. The *BpPIN1* gene was highly expressed in globular embryos but not in calli. The expression of the PIN1 gene was activated by an auxin concentration gradient after somatic embryogenesis(Su, et al. 2009) (Fig. S1b).

We counted the quality of transgenic birch and wild-type control for 40 days. Abnormal proliferation of somatic embryos was found to have significant quality differences. These quality differences represent the abnormal proliferation of somatic embryos (Table S1).

The overexpression of *AtWUS* led to an abnormal increase in the number of lateral branches and bud meristem of birch

Through the cultivation of transgenic plants, we found that transgenic plants had developed lateral branches (Fig. 4a). The average lateral branch was 5.38 ± 0.49. Through 150 days of culture, we monitored plant height every 10 days for statistical analysis (Fig. 4a-c, e). The plant height difference between the initial wild-type and transgenic plants was small. As plants grew, wild-type plants grew faster than transgenic plants. This may be caused by nutritional limitations. More lateral branches require more

nutrition, which limits the development of transgenic plants. We cultured the plants for 150 days to determine the shoot meristem number of the 2 cm part of the stem. The statistical results showed that the wild type was 1.29 ± 0.69. However, the transgene was 5.28 ± 1.25. This indicated that overexpression of *AtWUS* resulted in an abnormal increase in bud meristem (Fig. 4f-h). Buds change from a single form to an axisymmetric form (Fig. 4f, g).

To further analyze the causes of abnormal increases in lateral branch number and bud meristem of birch. We identified transgenic birch. The PCR results of the *AtWUS* gene in transgenic plants showed that 882 bp had a specific amplification fragment of the *AtWUS* gene (Fig. 5a). We used *35S:WUS* gene fusion PCR detection to show a 1200 bp fusion amplification fragment (Fig. 5b). Fig. 5c shows the southern blot results of *AtWUS* L3. We used digoxin-labeled *AtWUS* gene probes (see Fig. S5 for electrophoresis). The identification of GFP in birch roots showed that *AtWUS* was successfully expressed (Fig. 5d).

AtWUS regulated gene expression resulting in abnormal shoot meristem increase

To further explain the abnormal increase in bud meristem on birch transgenic paper. We observed the shoot meristem characteristics of wild-type and transgenic plants using frozen sections (Fig. 6a-d). The bud meristems were all single, and there were no abnormal differences (Fig. S4). The expression of genes related to the *BpWUS* gene in the bud meristem was analyzed. The results showed that the expression of the *BpWUS* gene was downregulated. The expression levels of *BpLEC1*, *BpLEC2* and *BpFUS3* were upregulated, and the expression of *BpABI3* was not significantly different (Fig. 6e). The expression levels of *BpSTM* and *BpCUC2* were upregulated. The auxin transport-related gene *BpPIN1* was significantly upregulated. The results showed that the overexpression of *AtWUS* activated *BpWUS*-related regulatory genes and induced the formation of meristems on shoots (Fig. S2).

Discussion

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 in chromatin remodeling have become the key factors of SE (Jha, et al. 2020; Mendez-Hernandez, et al. 2019; Salaun, et al. 2021; Wojcik, et al. 2020). The *WUS* gene has been identified as a key transcription factor that maintains the identity and quantity of stem cells, antagonizes STM, and provides negative feedback regulation with *CLV3*, together determining stem cell division and differentiation (Schoof et al. 2000; Gallois et al. 2002). Overexpression of the *WUS* gene has been used in many plants to promote SE, 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). For overexpression of the *CLV3* gene in *Arabidopsis thaliana*, 82% of the shoot meristems stopped organogenesis after the initial leaf grew, indicating that the *CLV3* gene inhibits the expression of the *WUS* gene (Brand et al. 2000). The *wus* mutant showed no protrusions of meristematic tissue, and over time, there were only individual stamens and no pistils. The *clv1* mutant had normal meristematic processes, a larger volume than the wild type, and an additional carpel. The *clv3* phenotype was similar to the *clv1*

phenotype. Neither the *wus* and *clv1* double mutants nor the *wus* and *clv3* double mutants formed normal meristems (Schoof et al. 2000). Short-term downregulation of *CLV3* leads to the lateral growth of stem cells in the central zone (CZ), and peripheral zone (PZ) cells regain stem cell characteristics (Reddy 2005). The transient downregulation of *CLV3* increases the expression of the *WUS* gene, thereby promoting an increase in the number of stem cells (Yadav et al. 2010).

Plant SAM plays a role in controlling tissue differentiation and the corresponding external developmental signals. The number of cells with stem cell characteristics in the SAM region is stable, and the division of these cells with the differentiation of daughter cells requires the coregulation of multiple genes (Carles and Fletcher 2003; Baurle 2005; Kieffer 2006). The *WUS* gene promotes the transformation from plant nutrition to an embryonic stage and promotes the formation of somatic embryos. This regulation is useful in heterologous applications. Overexpression of the *AtWUS* gene in *Coffea canephora* promotes the development of somatic embryos and increases embryo size. Low expression of the *AtWUS* gene allows a small number of cells to undergo SE (Brand et al. 2000; Schoof et al. 2000; Zuo et al. 2002; Arroyo-Herrera et al. 2008). We obtained the same result (Fig. 2 and table 1). The *AtWUS* gene resulted in a large number of somatic embryos in birch, and the formation of multilateral branches and bud stem cells increased. Quality statistics conducted on the embryonic development process of birch indicated that the difference in quality between the transgenic plants and wild-type plants during somatic embryo development was significant. There are two possible reasons for this result. (1) Transformation of the Arabidopsis *AtWUS* gene increases the growth and mass of birch somatic embryos. (2) Transformation of the Arabidopsis *AtWUS* gene resulted in a large number of birch cell embryos, with transgenic birch producing more bud meristems than wild-type birch. For technical reasons, we were not able to isolate the size of somatic birch embryos at various stages of development. However, the number of bud meristems in transgenic birch abnormally increased, and the increased number of bud stem cells makes us convinced that the second hypothesis is correct.

Auxin and cytokinin have significant effects on plant regeneration. For many endangered plants, regenerated plants can be obtained through tissue culturing. Such asexual reproduction methods depend on the action of plant hormones. During early SE, auxin aggregates in apical cells and has an essential influence on the formation of embryonic structures. 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).

For SE-related genes such as *LEC1*, *LEC2*, *FUS3* and *ABI3*, the regulation of the *WUS* gene in birch somatic embryos is not clear. *FUS3* and *LEC1* play roles in cotyledon formation(Gaj, et al. 2005). The BBM protein directly acts on the upstream activation of the LAFL network of the LAFL (*LEC1*, *ABI3*, *FUS3* and *LEC2*) gene to induce somatic embryogenesis (Roscoe et al. 2015; Horstman et al. 2017). The expression of *AtLEC2* and *AtIPTs* in Arabidopsis promoted tobacco embryogenic callus formation and bud regeneration (Li, et al. 2019). The difference in induced expression of *AtLEC1* and *AtLEC2* promoted somatic embryogenesis in transgenic tobacco plants (Guo, et al. 2013). The expression changes of *LEC1* and

FUS3 affected microspore embryogenesis in *Brassica napus* (Elahi, et al. 2016). *LEC2* is an important *WUS* response factor. In cocoa, the expression of *TcLEC2* regulated by glucocorticoid receptors triggers somatic embryogenesis in cocoa leaf tissue (Fister, et al. 2018). Double mutants *stm* and *wus* or double mutants *cuc1* and *cuc2* cannot form normal SAMs. *CUC1* or *CUC2* is thought to act upstream of *STM* and is concentrated in 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).

In our study, the *AtWUS* gene activated the *BpLEC2* and *BpLEC1* genes in the callus and bud meristem of birch and further activated the *BpFUS3* gene. The upregulation of these genes affects plant growth and development through auxin, GA and ABA signals (Fig. 7). The *LEC* gene plays an important role in embryonic development (Braybrook and Harada 2008). It has been reported that the expression of *BpPIN* is related to IAA levels and the formation of lobes (Qu, et al. 2020). Our results showed that the regulation of the *WUS* gene stimulated the high expression of *BpPIN1* during SE development. However, no difference was found in leaves.

Birch is a deciduous tree widely used in eastern Asia. Due to its intense cold tolerance, birch is widely cultivated in northern China. The overexpression of *AtWUS* in birch resulted in abnormal proliferation of somatic embryos, which provided a new method for genetic transformation of birch. Similarly, the lateral branches of transgenic birch developed abnormally, which changed the normal morphology of birch and transformed it from arbor morphology to shrub morphology. This transgenic approach will achieve our goals and provide a new breed for landscaping in the northern region of China.

Abbreviations

SAM, shoot apical meristem; 6-BA, 6-benzylaminopurine; NAA, naphthalene acetic acid; IBA, Indole-3-Butytric 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, SHOOTMERISTEMLESS; CLV3, CLAVATA3; CUC1, CUP-SHAPED COTYLEDON 1; CUC2, CUP-SHAPED COTYLEDON 2; PIN1, PIN-FORMED 1.

Declarations

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

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

Ethics and consent of participate Not applicable

Consent for publication Not applicable

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Tables

Table 1. SE induction rate 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	

[a] Number of explants forming a callus. [b] Number of SE forming from a callus. (P values were calculated by Student's *t* test, * is P < 0.05, ** is P<0.01, *** is P<0.001, and ns indicates no significant difference).

Figures

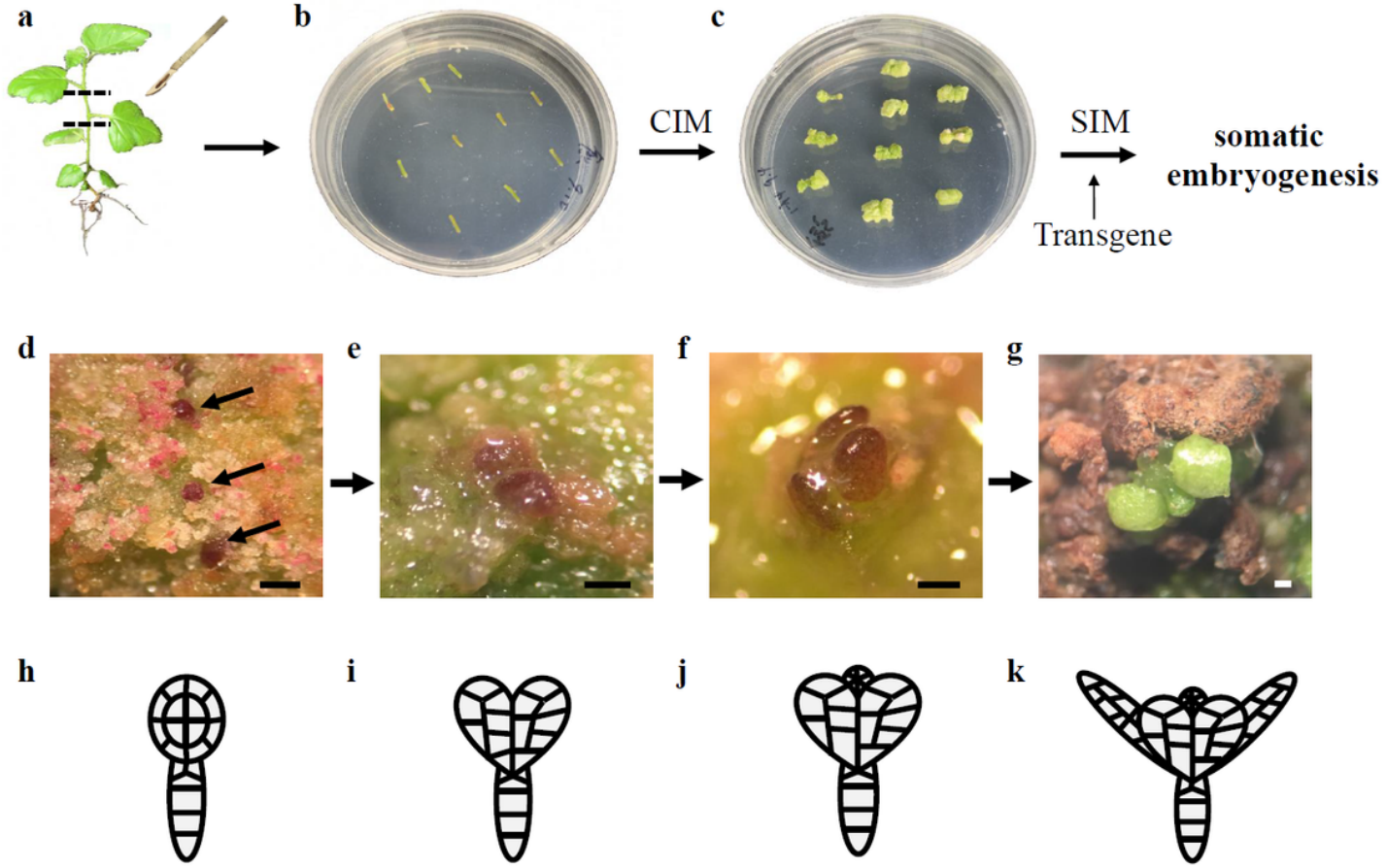

Figure 1

The process of somatic embryogenesis in birch. a-c The stem segments of birch seedlings were cut as 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-g = 1 mm)

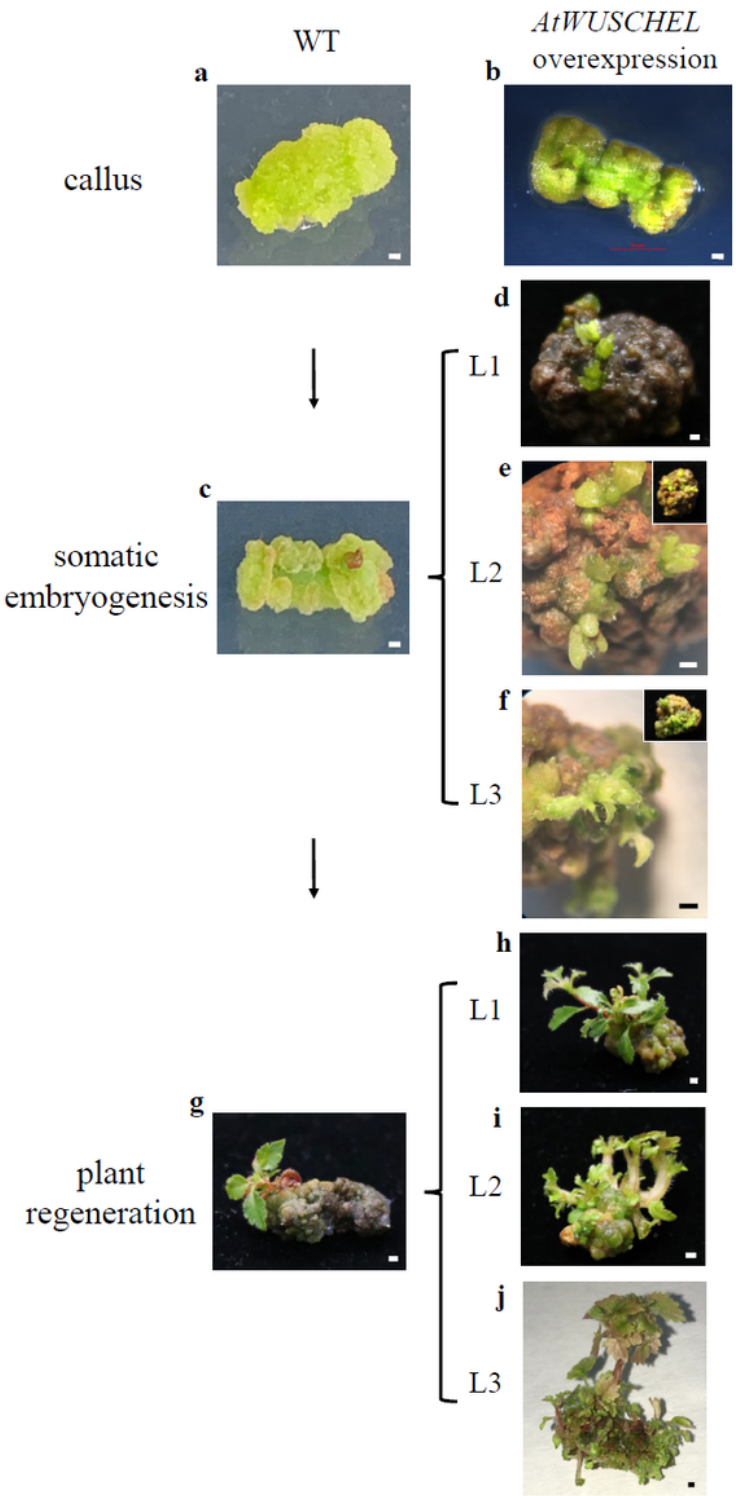

Figure 2

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 L1d, L2e, and L3f in somatic embryogenesis. Wild-type g and AtWUS-overexpressing tissues L1h, L2i, and L3j in plant regeneration. (Scale bar = 1 mm)

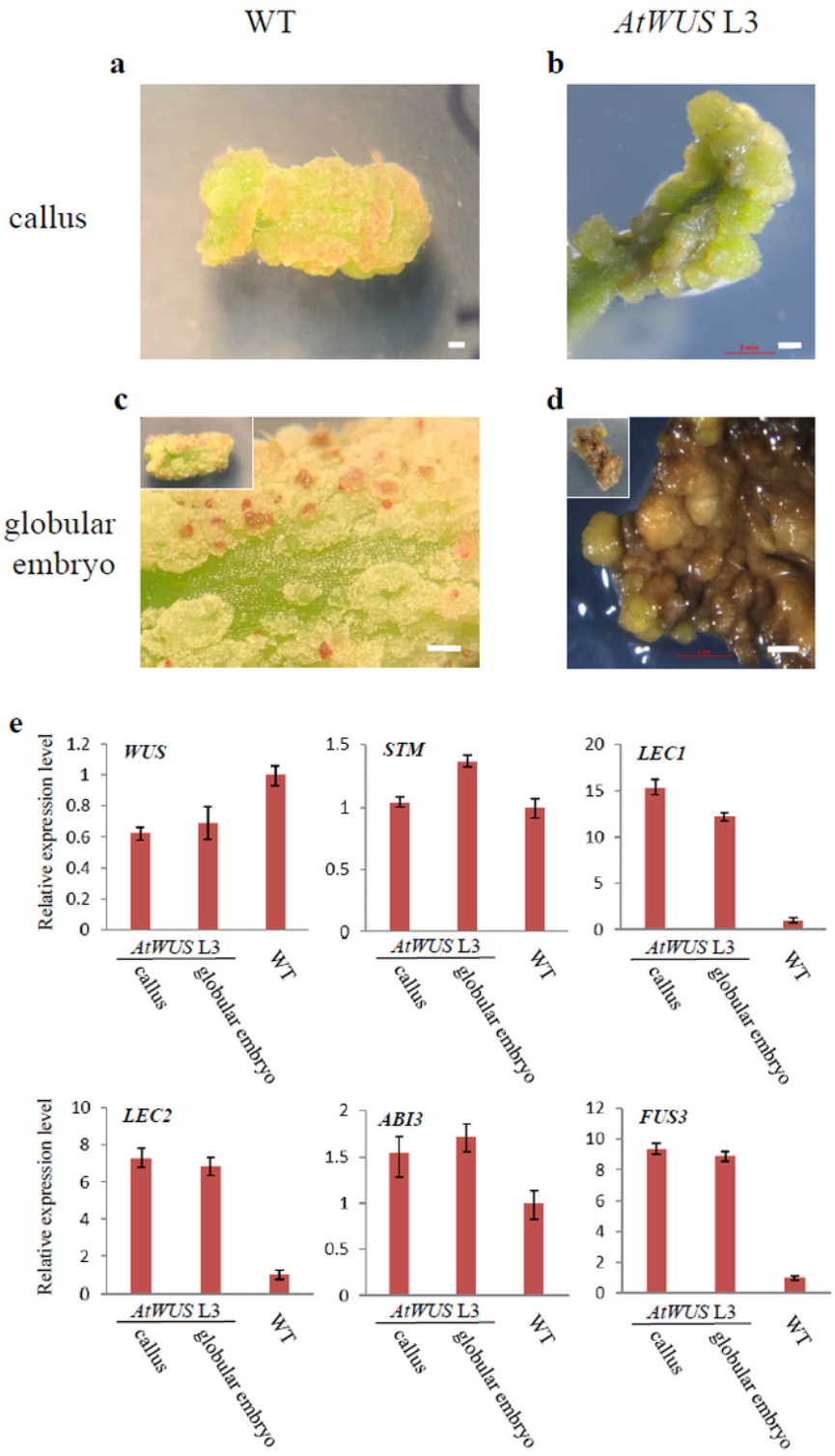

Figure 3

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

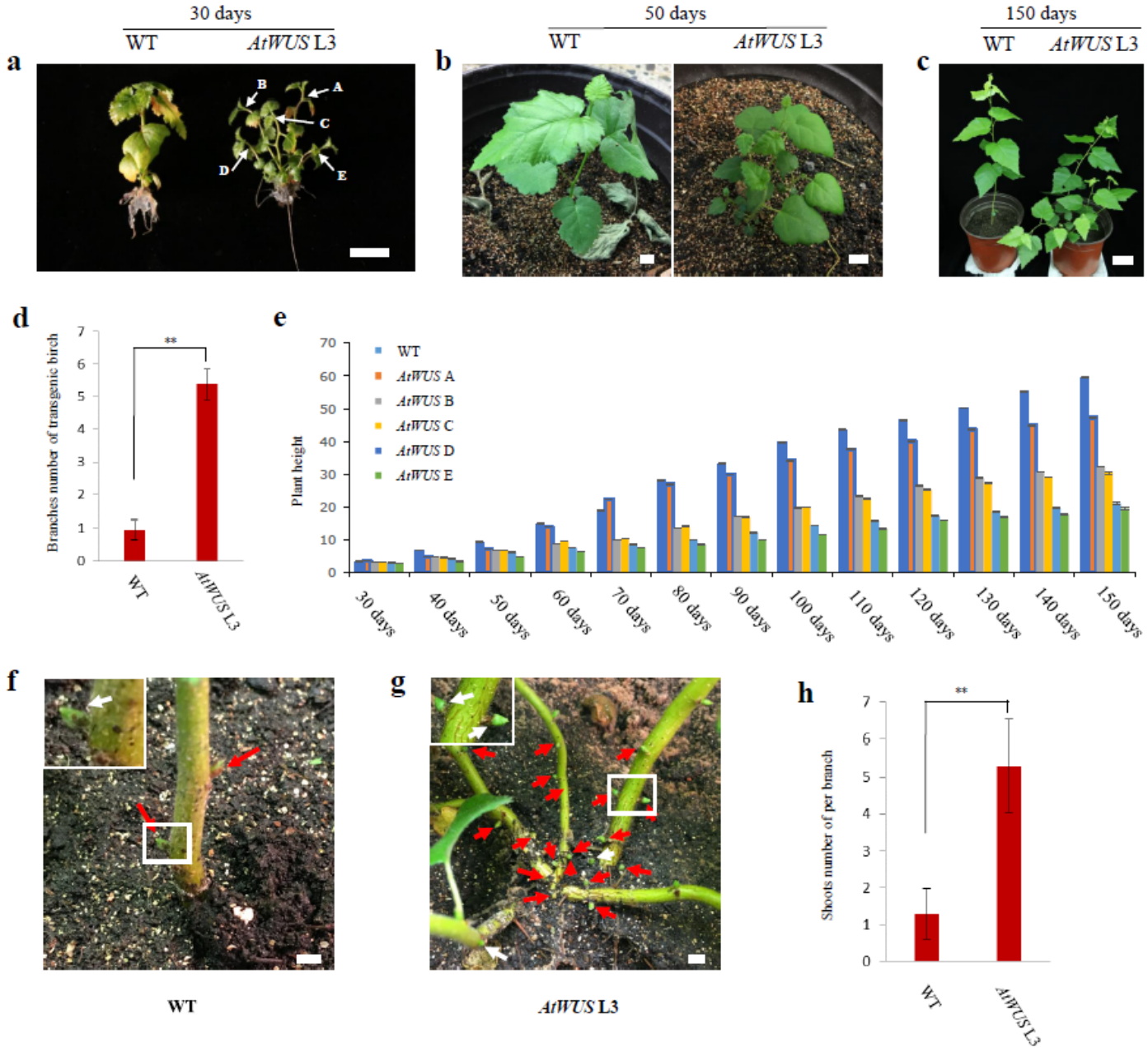

Figure 4

Overexpression of AtWUS increased the number of lateral branches and bud meristem 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 2 cm stems in plant ground. h Number of bud meristem quantified 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)

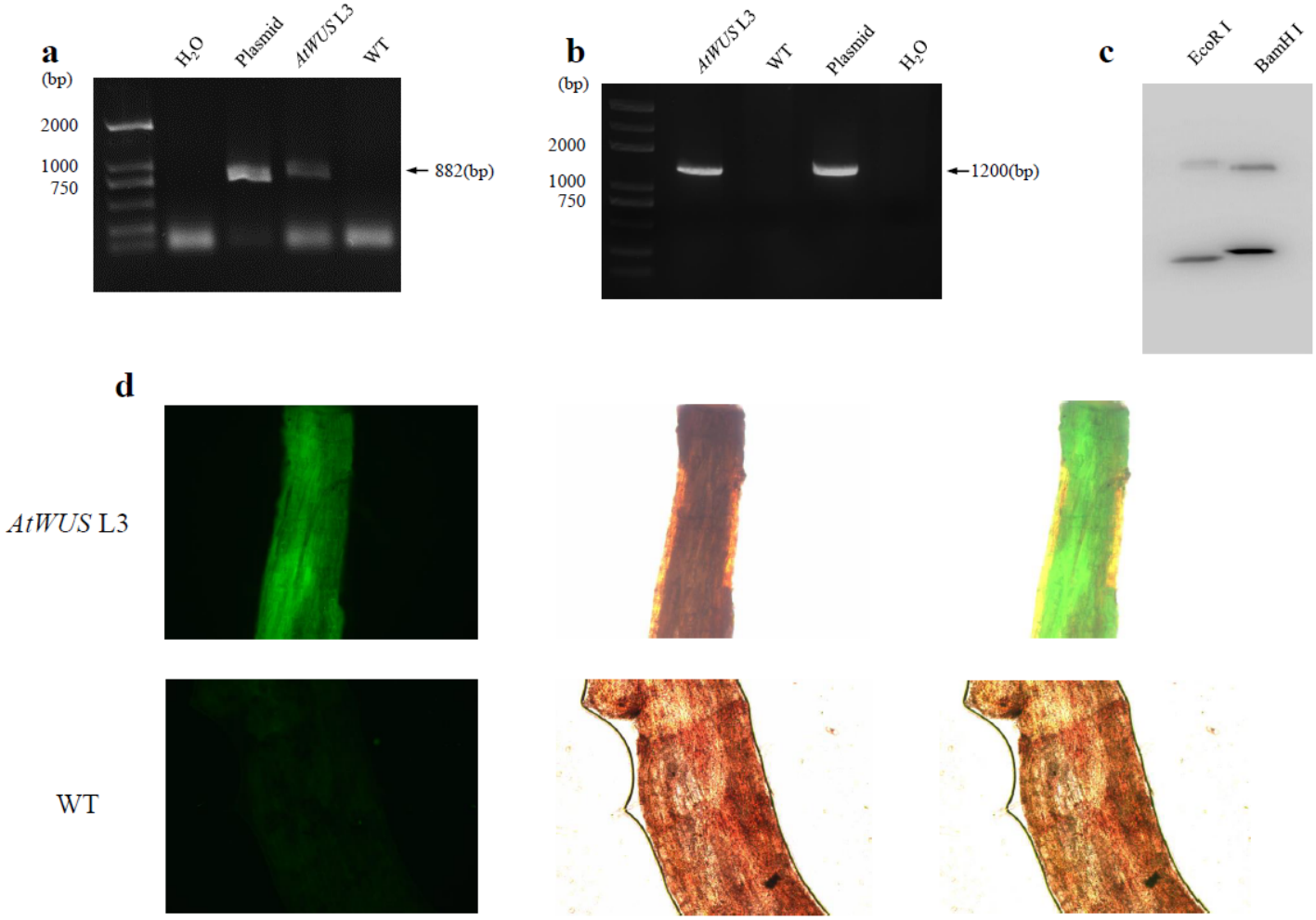

Figure 5

Identification of Transgenic Plants of birch. a Specific amplified fragment of AtWUS gene at 882 bp. b The 1200 bp amplification fragment was the detection result of the 35S:WUS gene fusion gene. c Southern blot 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)

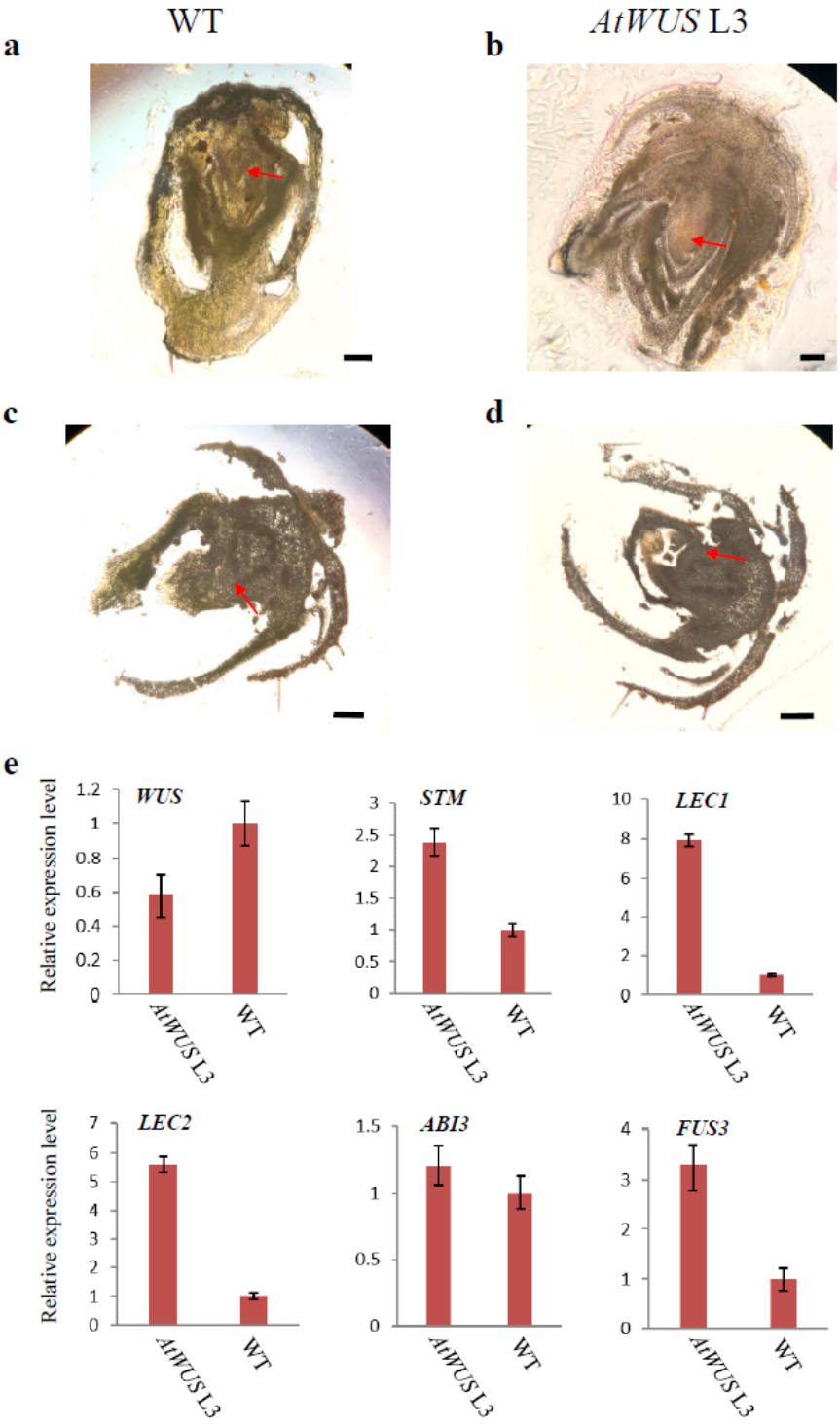

Figure 6

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 stage. e Gene expression analysis of transgenic buds carrying AtWUS. (Scale bar = 300 μm)

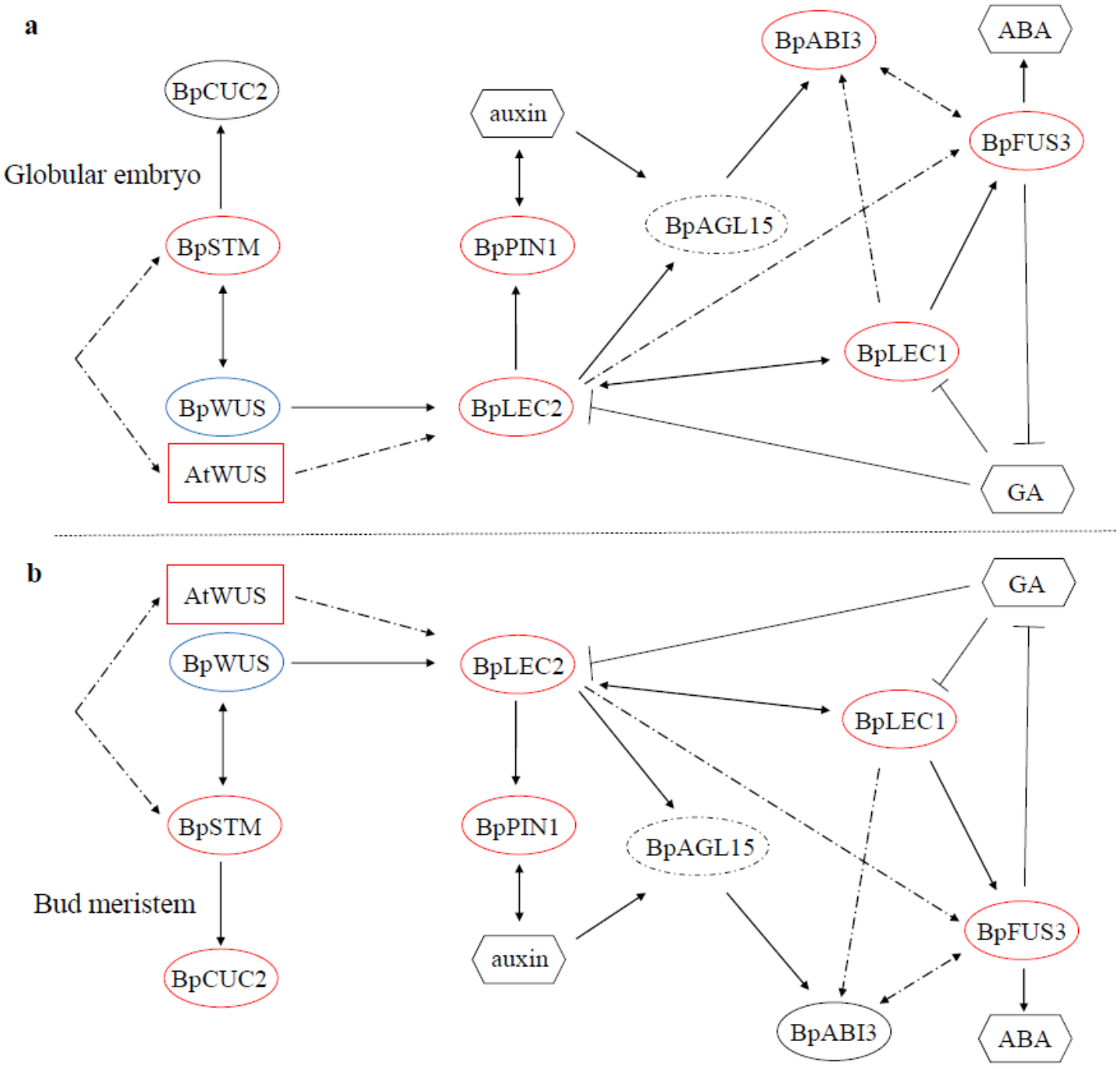

Figure 7

Interaction between key regulatory factors of SE. Red, upregulated genes. Blue, downregulated genes. Black, gene expression unchanged. Real lines represent reported effects, and virtual lines are possible interactions (Braybrook and Harada 2008; Feher 2015; Gordon-Kamm, et al. 2019; Jha, et al. 2020; Mendez-Hernandez, et al. 2019; Rose 2019; Salaun, et al. 2021; Tian, et al. 2020; Wojcik, et al. 2020).

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