

The bp-miR156c-BpSPL2 module positive regulates drought tolerance by mediating lateral root development and reactive oxygen species scavenging in *Betula platyphylla*

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Research Article

Keywords:

Posted Date: March 19th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-9102551/v1>

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Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at Plant Cell Reports on September 8th, 2026. See the published version at <https://doi.org/10.1007/s00299-026-03924-4>.

Abstract

Drought is one of the major abiotic stress factors affecting plant growth and productivity. The miR156/SPL module plays a crucial role in plant growth, development, and responses to abiotic stress; however, its regulatory mechanism in mediating drought adaptation in woody plants such as birch remains incompletely understood. In this study, we used transgenic plants overexpressing *bp-miR156c* and *BpSPL2* as experimental materials and employed GUS staining, RNA-seq, yeast one-hybrid assay, ChIP-PCR and dual-luciferase reporter assays to investigate the mechanism by which the miR156/SPL module regulates drought tolerance in birch. GUS staining results indicated that *BpSPL2* is a target gene of *bp-miR156c* and subject to its cleavage. Compared with wild-type plants, *bp-miR156c*-overexpressing transgenic plants exhibited reduced drought tolerance under drought stress, whereas plants overexpressing its target gene *BpSPL2* showed enhanced drought resistance. Specifically, *BpSPL2*-OE lines under drought stress displayed alleviated photodamage in both PSII and PSI, along with reduced oxidative damage. Moreover, overexpression of *BpSPL2* significantly promoted root system development, particularly lateral root growth, in birch. RNA-seq analysis revealed that, compared with the wild type, differentially expressed genes (DEGs) in *BpSPL2*-OE plants under drought stress were significantly enriched not only in photosynthesis-related pathways but also in tryptophan metabolism, redox processes, and glutathione metabolism. We speculate that the alleviation of photosynthetic inhibition and oxidative damage in birch leaves under drought stress by *BpSPL2* may be related to its regulation of ROS metabolism, while the promotion of lateral root development may be associated with activation of the tryptophan metabolic pathway and subsequent accumulation of IAA. Further studies demonstrated that the *BpSPL2* transcription factor recognizes the GTAC motif and binds to the promoters of glutathione-S-transferase *BpGSTF3* and the key rate-limiting enzyme in tryptophan synthesis gene *BpASA1*, thereby enhancing their transcription. On one hand, this upregulates GST and antioxidant enzyme activities, mitigating drought-induced photodamage and oxidative injury; on the other hand, it promotes IAA accumulation, stimulating lateral root formation and ultimately improving drought tolerance in birch. In summary, our findings demonstrate that the miR156/SPL module enhances drought tolerance in birch by modulating ROS homeostasis and lateral root development, providing novel molecular insights and a theoretical foundation for drought resistance research in birch trees.

Introduction

Drought is one of the major abiotic stress factors that limit plant growth, development, yield and quality(Jaleel et al. 2009; Gupta, Rico-Medina, and Caño-Delgado 2020). Currently, approximately 45% or more of global agricultural land is affected by persistent or frequent drought as a result of climate change(Liang et al. 2024). Drought stress affects all aspects of plant growth, development, and reproduction, encompassing morphological, physiological, and biochemical changes as well as the regulation of specific genes(Hussain et al. 2018; Ji et al. 2025). Plants primarily employ two strategies to cope with drought stress: drought escape and drought tolerance. Drought tolerance, in particular, is enhanced through multiple mechanisms. These include morphological adjustments, especially increased biomass allocation to the shoot; active accumulation of osmolytes to promote water uptake;

reduction of stomatal conductance to improve water use efficiency; enhanced antioxidant enzyme activity to mitigate reactive oxygen species (ROS) damage; activation of hormone signaling pathways; and various transcriptional regulations and post-translational protein modifications(Sun et al. 2025; Chen et al. 2025). In the aforementioned biological processes of drought response, the regulation by specific miRNAs and transcription factors plays a pivotal role. During evolution, plants have developed multiple regulatory networks to cope with adverse environmental conditions, with the miRNA/SPL regulatory network being one of them(Shriram et al. 2016), it can adapt to environmental stresses by regulating SPL transcription factor genes via miR156 under various stress conditions(Wang and Wang 2015). SPLs are key inducers of plant stress resistance, mediating the plant's ability to withstand various stresses, such as drought and low temperature(Ma et al. 2021; Qi et al. 2025). However, the regulatory mechanism of the miR156/SPL module in the drought tolerance process of birch remains poorly understood.

MicroRNAs are a class of small non-coding RNAs, typically 20–24 nucleotides in length, that play important roles in processes such as leaf development, flower development and resistance to both abiotic and biotic stresses(Chen 2004; Mallory et al. 2004; Sunkar and Zhu 2004; Khraiwesh, Zhu, and Zhu 2012; Sunkar, Li, and Jagadeeswaran 2012). Recently, an increasing number of studies have revealed that miRNAs are involved in plant tolerance to a wide range of abiotic and biotic stresses, including pathogen infection(Jones-Rhoades and Bartel 2004), salinity, drought, cold, oxidative stress(Li et al. 2017; Covarrubias and Reyes 2010; Yin et al. 2015; Koroban et al. 2016), UV radiation(Zhou, Wang, and Zhang 2007), nutrient deficiency(Panda and Sunkar 2015), and heavy metals(Huang et al. 2009), among others. In plants, miRNAs play a crucial regulatory role in response to drought stress, particularly the well-conserved miR156/SPL module. This module contributes to drought tolerance by participating in phytohormone signaling pathways, synthesis of osmoprotectant metabolites, enhancement of antioxidant enzyme activities and maintenance of ROS homeostasis. For instance, in Cassava (*Manihot esculenta* Crantz), *MeSPL9* reduces drought resistance by regulating JA signaling, whereas the miR156-targeted resistant line *rMeSPL9-SRDX* confers drought tolerance without compromising overall plant growth(Li et al. 2022). In tomato (*Solanum lycopersicum* L.), under drought conditions, the overexpression of *miR156* and strigolactone treatment lead to enhanced drought tolerance, which is associated with ABA accumulation and reduced stomatal conductance(Visentin et al. 2020). *MicroRNA156ab* regulates plant growth and drought resistance by targeting the transcription factor *MsSPL13*; heterologous expression of *MsSPL13* reduces auxin content and inhibits its growth in *Arabidopsis* under both normal and stress conditions(Feng et al. 2023). Osmoprotectant metabolites serve as key osmoregulators: in cassava, the *rMeSPL9-SRDX* line confers drought tolerance not only through JA signaling but also by promoting the synthesis of osmoprotectant metabolites such as proline and anthocyanins(Li et al. 2022). *Csn-miR156f-2-5p* and *CsSPL14* are involved in the drought response in tea (*Camellia sinensis*) plants. Knockdown of *csn-miR156f-2-5p* significantly reduces both the maximum photochemical efficiency of PSII (*Fv/Fm*) and chlorophyll content in tea leaves, while lead to a substantial accumulation of ROS(Wen et al. 2024).

Photosynthesis is one of the processes most sensitive to drought in plants. Drought stress leads to chlorophyll degradation and reduced photosynthetic capacity(Bhusal, Han, and Yoon 2019; Ji et al.

2025), causing damage to the reaction centers of both Photosystem II (PSII) and Photosystem I (PSI) in most plants and inhibiting electron transfer along the photosynthetic electron transport chain (Yao et al. 2023; Ohashi et al. 2006). When photosynthesis is suppressed under stress, the excess energy and electrons in chloroplasts are a major cause of reactive oxygen species (ROS) burst. ROS are by-products formed during normal oxygen metabolism and play crucial roles in cellular signaling and homeostasis maintenance; however, their excessive accumulation can cause damage to plant cells, with severe cases potentially leading to cell death. The homeostasis of ROS levels in plants is maintained by antioxidant enzymes that scavenge the excess ROS produced under stress conditions. The plant ROS scavenging system primarily consists of an enzymatic system, including superoxide dismutase (SOD), catalase (CAT) and peroxidases (POD), as well as enzymes in the ascorbate-glutathione (AsA-GSH) cycle such as ascorbate peroxidase (APX) and glutathione peroxidases (GPX), and peroxiredoxins (Prxs) in the thioredoxin-thioredoxin peroxidase (Trx-Prx) pathway (Wang et al. 2023; Zhang et al. 2020). In the non-enzymatic antioxidant system, small molecules such as GSH, AsA, phenolic compounds, flavonoids and carotenoids play important roles in protecting plant cells from oxidative damage (Pan et al. 2020). Studies have shown that the miR156/SPL module can participate in plant resistance to abiotic stress by regulating photosynthesis or ROS balance. In alfalfa (*Medicago sativa* L.) *SPL13RNAi* plants, the maintained or slightly increased transcripts related to photorespiration may act as an energy sink to prevent over-reduction of NADPH and photoinhibition (Feyissa et al. 2020). Transgenic *Arabidopsis thaliana* overexpressing apple *MsSPL13* exhibited inhibited antioxidant enzyme activities, resulting in reduced drought tolerance (Feng et al. 2023). Overexpression of *TaSPL6-A* increased sensitivity to drought stress in wheat (*Triticum aestivum* L.); after drought treatment, transgenic plants showed significantly higher leaf water loss, malondialdehyde, and ROS content, along with markedly lower antioxidant enzyme activities compared to wild-type plants. Conversely, silencing of the *TaSPL6* gene enhanced drought tolerance in wheat, reflected in better growth performance (Zhao et al. 2024). Variation in *OsSPL10* confers drought tolerance in rice (*Oryza sativa* L.) by directly regulating the expression of *OsNAC2* and ROS production (Li et al. 2023).

Glutathione-S-transferases (GSTs; EC 2.5.1.18) are a class of important multifunctional enzymes involved in detoxification, defense against biotic and abiotic stresses, and transport of secondary metabolites (Hu et al. 2024). They detoxify both endogenous and exogenous compounds by conjugating glutathione (GSH) to hydrophobic substrates (Öztetik 2008), a process that involves three phases: transformation, conjugation, and compartmentalization (Light et al. 2005). GSTs influence plant growth and development by participating in physiological metabolism, stress resistance, and cellular signaling (Song et al. 2021). Functionally similar to glutathione peroxidases, GSTs can reduce peroxide production during oxidative processes, thereby enhancing plant adaptability to environmental stresses. A series of studies have shown that the expression of GST genes is induced by drought stress in various plant species, including *Hordeum vulgare* L. (Rezaei et al. 2013), *Solanum lycopersicum* L. (Xu et al. 2015), *Triticum aestivum* L. (Wang et al. 2019), *Solanum tuberosum* L. (Islam et al. 2018), *Capsicum annuum* L. (Islam et al. 2019) and *Oryza sativa* L. (Jain, Ghanashyam, and Bhattacharjee 2010). Overexpression of GST in tobacco (*Nicotiana tabacum* L.) increased GST enzyme activity and GSH content under low

temperature, high temperature, and salt stress, alleviating oxidative damage caused by abiotic stresses (P et al. 2000). In wheat, *TaERF3* activated *TaGST6* expression is enhanced by ABA in response to salt and drought stress (Rong et al. 2014). Heterologous overexpression of a pear *GST* gene in tobacco improved the scavenging capacity for O_2^- under drought conditions, thereby enhancing drought tolerance (Liu et al. 2013). Overexpression of the *Tamarix chinensis* L. *GST* gene (*ThGSTZ1*) improved drought resistance by enhancing ROS scavenging ability (Yang et al. 2014). Transgenic Arabidopsis plants overexpressing tomato *LeGSTU2* exhibited increased resistance to drought stress through elevated antioxidant enzyme activities that scavenge excess ROS (Xu et al. 2015). In tea plants, *CsWRKY48* was found to activate the expression of *CsGSTU8* by directly binding to the W-box region in its promoter; overexpression of *CsGSTU8* in Arabidopsis improved ROS scavenging capacity and enhanced drought tolerance (Zhang et al. 2021). *OsGSTU4* is induced by ABA and participates in ABA-dependent processes that confer stress resistance in transgenic plants (Sharma et al. 2014).

To cope with water deficit induced by drought, plants have evolved a series of adaptive strategies. Among these, the plasticity of root system architecture (RSA), particularly the proliferation and development of lateral roots (LRs), is crucial for enhancing water and nutrient uptake (Ranjan et al. 2022; Comas et al. 2013; Koevoets et al. 2016; Karlova et al. 2021). Lateral root formation not only expands the root surface area (Du, Spalding, and Gray 2020), but is also precisely regulated by endogenous hormone networks, especially the synthesis, transport, and distribution of auxin (Teale, Paponov, and Palme 2006; Lavenus et al. 2013). Notably, auxin in roots is not only derived from polar transport from the shoots (Grieneisen et al. 2007; Zhao 2018), but is also significantly produced through local biosynthesis in root tissues (Ljung et al. 2005; Chen et al. 2014; Zhao 2018). Anthranilate synthase (AS) is a key rate-limiting enzyme in the tryptophan (Trp) biosynthesis pathway, catalyzing the conversion of chorismate to anthranilate (ANT) (Zhao and Last 1996; Niyogi and Fink 1992), where Trp serves as the major precursor for auxin biosynthesis in plants (Cohen, Slovin, and Hendrickson 2003; Zhao 2010; Tu et al. 2021). Research indicates that local auxin synthesis mediated by the AS α -subunit 1 (*ASA1*) plays a central role in root stress responses. Under arsenate stress, the expression of *ASA1* in root tips is activated, promoting auxin synthesis and cooperating with transporters such as *AUX1/PIN2* to regulate auxin transport from the root tip to the elongation zone, thereby influencing root growth and development (Tu et al. 2021). In Arabidopsis leaf explants, jasmonic acid (JA) biosynthesis occurs within two hours after wounding, and the transcriptionally activated ERF109 protein upregulates *ASA1* expression, contributing to auxin biosynthesis and promoting de novo root organogenesis (Zhang et al. 2019). Under drought stress, the *Populus euphratica* transcription factor *PeFUS3* positively regulates lateral root growth and enhances drought resistance by activating the expression of auxin transport genes *PIN2*, *PIN6a*, and *AUX1* (Liu et al. 2025). Additionally, drought signals regulate lateral root formation through crosstalk between ABA and auxin pathways; for instance, ABA receptors *PYL8* and *PYL9* (Zhao et al. 2014; Xing et al. 2016), as well as transcription factors such as *MYB96* and *WRKY46* (Seo et al. 2009; Ding et al. 2015), have been shown to integrate ABA and auxin signaling to control LR development. These findings suggest that *ASA1* may act as a molecular hub linking drought stress signaling to root morphological remodeling, mediating lateral root initiation by modulating local auxin synthesis and thereby improving

plant drought tolerance. Previous studies have reported that the miR156/SPL module participates in lateral root formation. Overexpression of apple *MdmiR156n* significantly increased primary root length and lateral root number in transgenic *Arabidopsis* plants under drought stress (Chen et al. 2023), although its molecular mechanism remained unclear. Here, our findings demonstrate that in birch, the miR156/SPL module promotes lateral root development by activating the expression of the *ASA1* gene, thereby influencing drought tolerance.

While the mechanisms by which the miR156/SPL module influences abiotic stress tolerance have been reported in species such as alfalfa (Arshad, Feyissa, et al. 2017; Arshad, Gruber, et al. 2017), rice (Cui et al. 2014), and wheat (Zhao et al. 2024), its regulatory role in the molecular basis of drought tolerance in woody plants, particularly birch, remains unclear. In this study, we employed genetic engineering techniques to obtain transgenic birch plants overexpressing *bp-miR156c* and *BpSPL2*. By analyzing the physiological and biochemical changes in these transgenic plants under drought stress and conducting RNA-seq to investigate the molecular regulatory mechanisms mediated by *BpSPL2*, we found that the miR156/SPL module enhances drought tolerance in birch by activating the expression of the *BpGSTF3* and *BpASA1* genes. This activation increases antioxidant enzyme activity, alleviates photoinhibition under drought stress, and promotes lateral root formation. Our study reveals the potential value of the *bp-miR156c/BpSPL2* module for the genetic improvement of drought resistance in woody plants and provides a theoretical foundation for further refining the drought stress response network.

Materials and methods

Plant materials and treatment

Transgenic birch plants overexpressing *bp-miR156c* and *BpSPL2*, preserved from previous laboratory work, were subjected to subculture. The growth conditions were as follows: temperature at $24 \pm 2^{\circ}\text{C}$, relative humidity at 65–75%, with light intensity and photoperiod set at $46 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 16 h light/8 h dark, respectively. The culture medium consisted of: 2.14 g/L WPM powder, 20 g/L sucrose, 0.56 g/L calcium salt, 0.4 mg/L IBA, 8 g/L plant agar, adjusted to 1 L with distilled water, and pH adjusted to 5.8 using 5 M NaOH. After 40 days of growth, tissue-cultured seedlings of each transgenic line were transplanted into a soil matrix with a vermiculite:flower soil:peat soil ratio of 1:1:1 (Note: the culture medium on the roots must be thoroughly washed off during transplantation). The plants were then acclimatized and grown indoors until stable growth was achieved (i.e., surviving and growing normally after transfer from the medium to the soil matrix). After three months, both transgenic and wild-type (WT) plants were subjected to natural drought treatment. Prior to treatment, all plant materials were thoroughly watered to achieve saturated soil moisture content. The drought treatment group underwent natural drought stress for 7 days, while the control group was watered every 3 days to maintain normal soil moisture levels. After 7 days of drought treatment, growth phenotypes were observed and photographed, followed by physiological index measurements. Each treatment was performed with 15 biological replicates.

Measurement of chlorophyll fluorescence parameters in BpSPL2 transgenic plants.

The fully expanded third leaf from the apex of both control and treated plants from the wild-type (WT) and *BpSPL2* transgenic lines was selected and dark-adapted for 30 minutes. Subsequently, following the method described by Wang et al. (Wang et al. 2025), the OJIP transients and 820 nm reflection (*MR820*) kinetics of birch leaves under different treatments were measured using a Multifunctional Plant Efficiency Analyzer (M-PEA, Hansatech, UK). The curves were induced by a saturating red light pulse ($1000 \mu\text{mol m}^{-2} \text{s}^{-1}$). Fluorescence signals were recorded starting at 0.01 ms and ending at 2 s. On the OJIP curve, the O, J, I and P steps correspond to time points of 0.01, 2, 30, and 1000 ms, respectively, with fluorescence intensities denoted as F_0 , F_J , F_I and F_m . The following parameters were derived: the maximum quantum yield of PSII photochemistry, $F_v/F_m = 1 - (F_0/F_m)$, and $\Delta I/I_0$, reflecting PSI activity. Here, I_0 represents the maximum value of the *MR820* curve, and ΔI is the difference between the maximum and minimum values of the *MR820* curve. To analyze the changes in relative variable fluorescence at specific characteristic points, the segments of the OJIP transients between O-P, O-J, and O-K were normalized as follows. The normalized curves from the drought-treated plants of the three genotypes were then subtracted from those of their respective controls. The calculation methods are detailed below:

O-P segment normalization: $V_{O-P} = (F_t - F_0) / (F_m - F_0)$, $\Delta V_{O-J} = \Delta[(F_t - F_0) / (F_J - F_0)]$, The relative variable fluorescence at the J step (2 ms) is obtained as: $V_J = (F_J - F_0) / (F_m - F_0)$.

O-J segment normalization: $V_{O-J} = (F_t - F_0) / (F_J - F_0)$, $\Delta V_{O-J} = \Delta[(F_t - F_0) / (F_J - F_0)]$, The relative variable fluorescence at the K step (0.3 ms) is obtained as: $V_K = (F_K - F_0) / (F_J - F_0)$.

O-K segment normalization: $V_{O-K} = (F_t - F_0) / (F_K - F_0)$, $\Delta V_{O-K} = \Delta[(F_t - F_0) / (F_K - F_0)]$, The relative variable fluorescence at the L step (0.15 ms) is obtained as: $V_L = (F_L - F_0) / (F_K - F_0)$.

Determination of ROS content and physiological parameter quantification

Leaves from the same position of each plant were selected for physiological measurements. First, the fresh weight (M1) of the leaves was recorded. The leaves were then immersed in distilled water for 4–6 hours until fully saturated, blotted dry on the surface, and weighed to obtain the turgid weight (M2). Subsequently, the leaves were placed in an oven and dried at 65°C to a constant weight to obtain the dry weight (M3). The leaf relative water content (RWC) was calculated using the formula: $\text{RWC (\%)} = [(M1 - M3) / (M2 - M3)] \times 100\%$.

Other physiological and biochemical indices were measured using commercial assay kits (Griess Biotechnology Co, Ltd, Nanjing, China) according to the manufacturer's instructions. Specifically, the activities of peroxidase (POD), superoxide dismutase (SOD), and glutathione S-transferase (GST), as well as the contents of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and malondialdehyde (MDA), were

determined using the corresponding kits. All measurements were performed with three independent biological replicates.

bp-miR156c targets and cleaves BpSPL2

To validate the targeting and cleavage of *BpSPL2* by *bp-miR156c* in birch, we utilized the online prediction tool psRNATarget (<http://plantgrn.noble.org/psRNATarget/?function=3>) to predict *BpSPL* genes targeted by miR156 (see Supplementary Material). Constructs 35S::BpSPL2-GUS, 35S::bp-miR156c-GUS and 35S::bp-miR156c were generated (primer sequences and schematic diagrams of the constructs are provided in the Supplementary Material). Transient transformation in 4–6 weeks old tobacco leaves was performed following the protocol described by Lu et al. (2017)(Lu et al. 2017). *Agrobacterium tumefaciens* strain GV3101 harboring the respective constructs was cultured in 20 ml of fresh LB liquid medium supplemented with 100 µg/mL kanamycin and 50 µg/mL rifampicin at 28°C with shaking at 200 rpm until the OD600 reached 0.6–0.8. For co-infiltration, *Agrobacterium* cultures containing GV3101-pBI121-bp-miR156c and GV3101-pBI121-BpSPL2-GUS were mixed in equal volumes and injected into tobacco leaves. The infiltrated plants were kept in the dark at room temperature for 48 hours, followed by GUS staining.

Transcriptome analysis of BpSPL2

For each treatment, leaves from the same position of three biological replicate birch seedlings were collected, flash-frozen in liquid nitrogen, and stored at -80°C. RNA sequencing was performed by Shanghai Majorbio Bio-pharm Technology Co., Ltd. on the Illumina NovaSeq 6000 platform. After quality control of the raw data, clean data were obtained for subsequent analysis. Based on the alignment results from HISAT2, transcripts were reconstructed using StringTie, and gene expression levels for each sample were calculated using RSEM. Genes satisfying the criteria of $|\log_2FC| \geq 1$ and adjusted *P*-value < 0.05 were identified as significantly differentially expressed genes. Functional annotation and enrichment analysis of the differentially expressed genes were then conducted using GO and KEGG databases.

Yeast one-hybrid assay

Promoter sequences containing the predicted GTAC-box motif (approximately 200 bp) from the target genes were cloned into the pAbAi vector to generate bait reporter constructs (pAbAi-proBpGSTF3, pAbAi-proBpASA1). The coding sequence of *BpSPL2* (without the stop codon) was cloned into pGADT7-Rec to produce the effector plasmid pGADT7-*BpSPL2*. Subsequently, the minimum inhibitory concentration of Aureobasidin A (AbA) for each bait vector was determined. Yeast one-hybrid assays were performed following the manufacturer's instructions (BD Matchmaker™ Library Construction and Screening Kit User Manual). Yeast cells were plated onto selective dropout medium (SD/-Ura/-Leu) containing the corresponding concentration of AbA to screen for positive clones. Primers used are listed in the Supplementary Materials.

Dual-LUC transient expression assays

To analyze the regulatory role of *BpSPL2* on its target genes, we constructed reporters by cloning the promoters of the target genes to drive the Luc gene in the pGreenII-0800-LUC vector (pGreenII-proBpGSTF3-LUC and pGreenII-proBpASA1-LUC), and used pGreen-62-SK to drive *BpSPL2* as the effector (pGreen-BpSPL2-SK). The effector and reporter plasmids were individually transformed into *Agrobacterium tumefaciens* strain GV3101 harboring the pSoup helper plasmid. Following the method described by Zang et al. (Zang et al. 2017), bacterial cultures carrying the effector and reporter vectors were mixed in the required ratio to prepare the infiltration solution. Healthy, pre-flowering soil-grown tobacco plants at a vigorous growth stage were selected. The prepared bacterial suspension was infiltrated into tobacco leaves using a 1 mL needleless syringe. Each experimental group included at least three biological replicates. After infiltration, plants were kept in the dark for 12 hours and then transferred to normal growth conditions for 48 hours prior to fluorescence observation. Subsequently, the activities of REN and LUC were measured using a Dual-Luciferase Reporter Assay Kit (Beyotime, Shanghai, China).

ChIP-PCR assay

Chromatin immunoprecipitation (ChIP) was performed following the method described by Zhao et al. (Zhao et al. 2020). Proteins and DNA were cross-linked using 3% (w/v) formaldehyde. After nuclei isolation and purification, chromatin was sheared by sonication into fragments of 200–500 bp. Following sonication, chromatin was immunoprecipitated using an anti-GFP antibody (ChIP+). Sonicated chromatin immunoprecipitated with a nonspecific antibody (IgG) served as the negative control (ChIP–). Cross-linking was reversed by digestion with proteinase K, and the ChIP products were purified using the TIANGel Maxi Purification Kit (Qiagen, Hilden, Germany). The ChIP-PCR program was as follows: 94°C for 3 min; 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 20 s; followed by a final extension at 72°C for 5 min and holding at 4°C. All primers used for ChIP-PCR are listed in the Supplementary Materials.

Statistical analysis

Significant differences were analyzed using Student's t-test, one-way ANOVA combined with Tukey's multiple comparison test in GraphPad Prism 9.0. An asterisk “*” indicates a significant difference at $p < 0.05$, and identical letters denote no significant difference. Data are presented as mean $\pm$ standard deviation.

Results

bp-miR156c negatively regulates plant drought tolerance

Under normal conditions, the *bp-miR156c*-OE lines exhibited superior growth compared to the WT, the plant height significantly greater than that of the wild-type (by approximately 20%, Fig. S1A). The leaf size

(measured as the area of the third mature leaf) was also significantly larger than in wild-type plants (by about 52.3%, Fig. S1B). However, there were no significant differences in physiological indicators such as leaf water content, MDA, H₂O₂ and O₂⁻ levels, or in the activities of SOD and POD. After 7 days of drought stress, the leaves of the *bp-miR156c*-OE lines showed obvious wilting and water loss (Fig. 1A). The leaf water content of WT plants was 35.1% higher than that of the *bp-miR156c*-OE lines ($P < 0.001$), and the plants remained upright (Fig. 1A, B). Measurement of oxidative stress-related parameters under drought conditions revealed that the *bp-miR156c*-OE lines had significantly higher levels of MDA, H₂O₂ and O₂⁻ (increased by approximately 29.4%, 70.1% and 39.3%, respectively) compared to the WT lines (Fig. 1C, D, E). Furthermore, the activities of the antioxidant enzymes POD and SOD were significantly lower in the transgenic birch plants after drought stress compared to the WT plants (Fig. 1F, G). These results indicate that overexpression of *bp-miR156c* in birch increases sensitivity to drought and reduces drought tolerance.

BpSPL2 is a direct target of bp-miR156c

While the canonical mechanism of miR156-targeted cleavage of SPL transcripts has been widely validated, the efficiency of cleavage by different miR156 family members on specific *SPL* genes varies considerably across species. In this study, wild-type (WT) tissue-cultured plantlets were subjected to drought stress simulated by 20% PEG treatment. The results showed that with prolonged stress duration, the expression of *bp-miR156c* was significantly down-regulated, whereas the expression pattern of *BpSPL2* exhibited an opposite trend (Fig. 2A). Furthermore, we predicted the target cleavage site (Fig. 2B) and experimentally validated the *bp-miR156c* mediated cleavage of *BpSPL2* using GUS staining assays. The constructs 35S::*bp-miR156c*-GUS, 35S::*BpSPL2*-GUS and 35S::*bp-miR156c* (without GUS tag) were generated. Following agrobacterium mediated transient expression in tobacco leaves and GUS staining, positive controls (35S::*bp-miR156c*-GUS and 35S::*BpSPL2*-GUS fused with the GUS tag) showed blue coloration, while the negative control (35S::*bp-miR156c* without GUS tag) remained colorless. In the experimental group, due to the cleavage of *BpSPL2* by *bp-miR156c*, GUS activity was suppressed, resulting in significantly lighter or nearly undetectable staining compared to the positive controls (Fig. 2C). These results demonstrate that *bp-miR156c* can specifically target and cleave *BpSPL2* transcripts.

BpSPL2 positively regulates drought tolerance in birch

To investigate the drought tolerance phenotype conferred by *BpSPL2*, we compared the physiological performance of two independent 35S::*BpSPL2*-OE lines (OE3 and OE5) with that of WT plants under both normal and drought conditions. Under normal growth conditions, all lines exhibited similar growth phenotypes with no significant difference. However, under drought stress, leaves of WT plants lost water more severely and showed wilting earlier than those of the overexpressing lines (Fig. 3A). Measurement of leaf relative water content revealed that, after drought stress, both OE lines maintained significantly higher water content (approximately 2-fold) compared to the WT plants (Fig. 3B).

Subsequently, we observed and statistically analyzed the belowground root phenotypes of these three lines before and after drought treatment. The results showed that under both normal and drought conditions, the root length and fresh weight of the two OE lines were significantly greater than WT line. Specifically under drought stress, the root lengths of *BpSPL2-OE3* and *OE5* lines were 45.5% and 49.5% longer than that of the WT, respectively, while their root fresh weights were 30.5% and 54.6% higher than the WT, respectively (Fig. 3C, D).

As lateral roots are a major component of the root system, we examined the root architecture of tissue-cultured seedlings (where the root structure is simpler and easier to observe) of *BpSPL2-OE3*, *OE5* and WT lines. The length and density of lateral roots were significantly higher in both OE lines compared to the WT (Fig. 3E, F, G). This explains why the root systems of the two *BpSPL2-OE* lines were more developed than that of the WT during soil-based drought treatment. Collectively, these results indicate that *BpSPL2* positively regulates drought tolerance in birch by promoting lateral root growth.

***BpSPL2* regulates glutathione metabolism in response to drought stress**

RNA-seq analysis of leaves from *BpSPL2* transgenic birch plants before and after drought treatment yielded approximately 70 Gb of clean data. Each sample produced over 5.81 Gb clean data, with Q30 base percentages exceeding 95.86%. Comparative analysis between the drought stressed *BpSPL2-OE* and WT lines identified 5597 differentially expressed genes (DEGs), comprising 2286 upregulated and 2771 downregulated genes (Fig. S2). When comparing the same genotype before and after stress, the WT line exhibited 2946 upregulated and 3324 downregulated DEGs, whereas the *BpSPL2-OE* line showed only 1086 upregulated and 1148 downregulated DEGs (Fig. 4A, B). The substantially less number of DEGs in the OE line indicates it was less perturbed by drought, consistent with its enhanced tolerance.

GO and KEGG enrichment analyses were performed on the DEGs identified between the OE and WT lines under drought conditions. GO enrichment revealed that these DEGs were primarily associated with processes such as photosynthesis (GO:0009765), light harvesting (GO:0009765), and oxidoreductase activity (GO:0016491) (Fig. 4C). KEGG pathway analysis showed significant enrichment for pathways including MAPK signaling (map04016), photosynthesis - antenna proteins (map00196), photosynthesis (map00195), tryptophan metabolism (map00380), glutathione metabolism (map00480), and biosynthesis of secondary metabolites (map00999) (Fig. 4D). These results suggest that *BpSPL2* modulates drought resistance in birch mainly by influencing photosynthesis, hormone signal transduction, and cellular redox regulation. Therefore, we subsequently focused our analysis on key processes: photosynthesis (including light harvesting and electron transport), ROS scavenging related to glutathione metabolism, and the tryptophan metabolism pathway along with its downstream IAA signaling.

Overexpression of *BpSPL2* stabilizes photosynthetic electron transport and the expression of related genes under drought stress

Drought stress leads to water loss and wilting in birch leaves, which significantly impacts the plant's photosystems. We investigated the effects of drought stress on PSII and PSI activity in birch leaves using parameters derived from prompt chlorophyll fluorescence transients (OJIP) and 820-nm reflection (*MR820*). Under normal conditions, the overall shapes of the OJIP fluorescence induction curves and *MR820* kinetics curves showed no significant differences among the WT, *BpSPL2-OE3* and *BpSPL2-OE5* lines. However, drought stress altered the curve morphology, primarily manifested as an increase in the relative fluorescence intensity at the O step, a decrease at the P step of the OJIP curve, and a reduction in the amplitude of the *MR820* curve (Fig. 5A, C). Analysis of the chlorophyll fluorescence kinetics (OJIP) curve via the JIP-test revealed that the maximum quantum yield of PSII photochemistry (F_v/F_m) under drought stress was significantly higher in *BpSPL2* transgenic plants compared to WT plants (Fig. 5B). The *MR820* curve reflects PSI activity. Under drought stress, the curve for WT plants was overall significantly lower than that for *BpSPL2-OE* plants. Quantitative data for $\Delta I/I_0$ showed a significant decrease under drought, but overexpression of *BpSPL2* partially alleviated this suppression (Fig. 5C, D). Under drought conditions, the OJIP curves of birch leaves exhibited significant distortion, mainly characterized by a marked decrease in fluorescence intensity at the P phase. Compared to WT plants, *BpSPL2-OE* transgenic plants partially restored the OJIP curve morphology under drought stress, particularly improving performance at the P phase. Based on the normalized curves (V_{O-P} , V_{O-J} , V_{O-K}) and double-normalized plots derived from the OJIP transients, *BpSPL2* transgenic plants displayed distinct negative peaks, especially at the J (2 ms), K (0.3 ms) and L (0.15 ms) steps. The relative variable fluorescence values V_J , V_K , V_L were also significantly lower in *BpSPL2* transgenic plants than in WT plants (Fig. 5E-J). These results indicate that overexpression of the *BpSPL2* gene can mitigate the drought-induced inhibition of both PSII and PSI, particularly by facilitating photosynthetic electron transport in PSII.

Based on the analysis of the drought transcriptome, we identified significant changes in a total of 18 genes encoding photosynthesis-antenna proteins, including 6 Lhca and 12 Lhcb genes. Under drought stress, all these genes were down-regulated; however, in the *BpSPL2-OE5* line, the extent of down-regulation for most antenna-protein genes was less pronounced compared to the WT plants (Fig. 5K). Moreover, three Lhca-encoding genes (BpChr06G30386, BpChr06G30442, BpChr08G02997) showed significantly lower expression in WT than in OE-5 under drought conditions, while Lhcb-encoding genes overall exhibited the highest expression in OE-5. Additionally, 25 differentially expressed genes related to the photosynthetic photosystem pathway (map00195) were identified in both OE-5 and WT birch after drought treatment. Under non-stress conditions, the expression levels of all PSII and PSI reaction-center genes showed no significant differences. After stress, these genes were expressed significantly higher in OE-5 plants than in WT, although compared to the pre-drought levels, their expression was down-regulated in both lines—yet the down-regulation was less severe in OE-5 plants (Fig. 5K). These results indicate that drought stress suppresses the expression of genes associated with PSI and PSII proteins. The transcriptome data are consistent with the corresponding phenotypic observations, demonstrating that overexpression of *BpSPL2* reduces drought-induced damage to photosynthetic antenna proteins and thereby alleviates photosynthetic inhibition.

The BpSPL2 gene enhances ROS scavenging under drought stress and up-regulates the expression of genes involved in the glutathione metabolism pathway.

Drought stress severely impacts the plant's photosynthetic system, disrupting electron transport balance and leading to excessive accumulation of ROS. In situ staining of O_2^- and H_2O_2 with NBT and DAB, respectively, in birch leaves before and after drought stress showed no difference in staining intensity between *BpSPL2-OE* and WT lines under normal conditions. After stress, however, the blue and brown staining was significantly deeper in WT leaves compared to *BpSPL2-OE* leaves, indicating that overexpression of *BpSPL2* reduced the accumulation of O_2^- and H_2O_2 under drought stress (Fig. 6A). Physiological measurements aligned with the staining results: under drought stress, the contents of O_2^- and H_2O_2 were significantly lower in both *BpSPL2-OE* lines than in the WT (Fig. 6B, C), resulting in MDA content being 45.07% and 45.58% lower in the *BpSPL2-OE3* and *BpSPL2-OE5* lines, respectively, compared to the WT (Fig. 6D).

As indicated by the earlier RNA-seq results, *BpSPL2* likely regulates ROS metabolism in birch leaves under drought stress mainly by affecting cellular redox processes, with the glutathione metabolism pathway being significantly enriched in the KEGG analysis. We performed a heatmap analysis of the transcriptome expression for genes significantly enriched in the glutathione metabolism process. The results revealed that among the 28 differentially expressed genes (DEGs), 24 were *GST* genes, while the remaining included 1 *GPX*, 2 *DHARs*, and 1 *GR*. In the *BpSPL2-OE5* transgenic plants, 10 *GST* members were significantly up-regulated compared to the WT before and after drought stress, with three genes—*BpChr14G12602*, *BpChr05G19178*, and *BpChr05G19184*—showing particularly prominent induction (Fig. 6E). After screening, we selected *BpChr05G19178* (*BpGSTF3*), whose expression was markedly induced by *BpSPL2* and whose promoter region contains multiple GTAC binding motifs for *SPL* transcription factors, for further investigation.

BpSPL2 positively activates BpGSTF3 and BpASA1, enhancing GST enzyme activity and promoting IAA accumulation.

Earlier, transcriptome data analysis predicted potential downstream regulatory pathways of the *BpSPL2* gene under drought stress. We performed expression pattern analysis on key genes within these pathways, including *BpGSTF3*, *BpChr14G12602* and *BpChr05G19184* from the *GST* family, as well as *BpASA1*, a key rate-limiting enzyme gene in the tryptophan synthesis pathway. RT-qPCR results showed tissue-specific expression: *BpGSTF3* and *BpChr05G19184* were significantly induced by *BpSPL2* mainly in leaves, while *BpChr14G12602* expression was significantly higher in roots than in leaves (Fig. 7A, Fig S3. A, B). In contrast, *BpASA1* was primarily and significantly induced in roots (Fig. 7B). Promoter element analysis revealed that their promoters all contain the GTAC-box, a binding motif for *SPL* transcription factors. To determine whether *BpSPL2* can bind these DNA motifs to regulate gene expression, we performed ChIP-PCR assays on fragments of pro*BpGSTF3* and pro*BpASA1* containing the GTAC-box. The results showed that *BpSPL2* binds to the P4 site of pro*BpASA1* and the P3 site of pro*BpGSTF3*, confirming its ability to regulate gene expression by binding these DNA motifs (Fig. 7C, D).

Subsequently, the promoter binding site fragments (200–300 bp) identified by ChIP-PCR were cloned into the pAbAi vector for yeast one-hybrid (Y1H) validation. The results indicated that sequences containing the GTAC-box can be bound by BpSPL2, enabling growth on selective minimal medium containing Aureobasidin A (AbA) (Fig. 7E). Furthermore, dual-luciferase (LUC) reporter assays confirmed that BpSPL2 binds to the proBpGSTF3 and proBpASA1 promoters and activates their expression (Fig. 7F, G, H).

Drought stress leads to excessive ROS production in plants, triggering severe oxidative stress. Previous experiments verified that birch *BpSPL2* can target and regulate *BpGSTF3*, activating its expression and increasing the activity of the antioxidant enzyme GST under drought stress (by approximately 36.7% and 29.6%, Fig. 7J). This enhances ROS scavenging capacity, reduces oxidative damage, and thereby improves drought tolerance in birch. We also measured the activities of other antioxidant enzymes involved in ROS scavenging (POD and SOD). The results showed no significant differences and generally low activity among the lines under normal conditions. However, after drought stress treatment, SOD (by 55%) and POD (by approximately 80%) activities were significantly higher in *BpSPL2*-OE plants compared to WT plants (Fig. 7K, L). These results indicate that *BpSPL2* can increase the antioxidant capacity in birch, reducing the degree of oxidative damage under drought stress, thereby enhancing drought tolerance. On the other hand, we also confirmed that *BpSPL2* regulates *BpASA1*, activating its expression (Fig. 7G, H) and inducing IAA accumulation (Fig. 7I), which in turn promotes lateral root development in birch and enhances its drought tolerance.

Discussion

Drought stress severely restricts plant growth and development, leading to premature leaf drop, delayed growth, and impaired nutrient accumulation, thereby causing significant economic losses (Luo 2010). Plants have evolved complex transcriptional regulatory networks to respond to drought stress, such as the miR156/SPL transcriptional regulatory module, which can be significantly induced under stress conditions to regulate a series of downstream stress responses. This study is the first to demonstrate that in birch, the miR156c/SPL2 module enhances drought tolerance by activating the key rate-limiting enzyme for auxin biosynthesis, *BpASA1* and glutathione-S-transferase, *BpGSTF3*, thereby promoting lateral root development and mediating the scavenging of ROS. While studies in herbaceous plants have largely shown the miR156/SPL module to act as a negative regulator of stress resistance, our findings reveal that *BpSPL2*, targeted by *bp-miR156c*, functions as a transcriptional activator playing a crucial role in the drought stress response of birch. This novel discovery provides a fresh perspective on the drought resistance mechanisms in birch and potentially other perennial woody plants.

The miR156/SPL module is a key regulator that imparts plasticity to plant growth in response to light and other environmental factors (Sang et al. 2023). For instance, in tomato and Arabidopsis seedlings, overexpression of miR156 and suppression of SPL lead to elongated hypocotyls seeking light and activate light-responsive mechanisms (Sang et al. 2023). In response to drought and other environmental stresses, the miR156/SPL module can regulate plant stress tolerance by modulating anthocyanin

production(Cui et al. 2014), auxin content(Feng et al. 2023), antioxidant enzyme activity(Zhao et al. 2024), *Fv/Fm* values, chlorophyll content(Wen et al. 2024), ABA accumulation, and stomatal conductance(Visentin et al. 2020). Drought stress affects both the morphology and physiology of birch. In this study, under PEG-simulated drought stress, the *BpSPL2* gene was significantly induced, while *bp-miR156c* was suppressed (Fig. 2A). This aligns with findings on the miR156/SPL module in apple under stress, where adversity leads to downregulation of miR156, releasing its inhibition of the downstream gene *MdSPL13*, ultimately enhancing the expression of the stress-responsive gene *MdWRKY100* and improving stress resistance in apple(Ma et al. 2021). Interestingly, however, *bp-miR156c-OE* plants exhibited reduced drought tolerance (Fig. 1), whereas *BpSPL2-OE* plants showed enhanced drought tolerance (Fig. 3). This phenotypic observation contrasts with conclusions in herbaceous plants (such as alfalfa and rice), where miR156 often enhances stress resistance by silencing *SPLs*. For example, heterologous expression of *OsamiR156b/c* simultaneously increases both biomass and stress tolerance in alfalfa(Arshad, Feyissa, et al. 2017; Wang et al. 2021). In cassava, a perennial woody starch crop often grown as an annual, *MeSPL9* also negatively regulates drought tolerance(Li et al. 2022). However, in the study by Ning (Ning et al. 2017), birch *BpSPL9* was described to improve ROS scavenging ability under salt and drought stress, although the molecular mechanisms were not investigated in depth. We speculate that this may reflect functional diversity of *SPL* family transcription factors across different species.

To further explore the molecular regulatory mechanisms underlying these morphological and physiological changes, we analyzed the transcriptomes before and after drought stress. A total of 5597 differentially expressed genes (DEGs) were identified between the *BpSPL2-OE* and WT lines after stress. GO and KEGG enrichment analyses of these DEGs revealed that they are primarily involved in pathways such as photosynthesis, oxidoreductase activity, MAPK signaling, tryptophan metabolism, glutathione metabolism, and biosynthesis of secondary metabolites (Fig. 4). Drought stress led to dry and wilted birch leaves, significantly reduced water content (Fig. 3A, B), and inhibited photosynthesis (Fig. 5). When photosynthesis is suppressed, excess electrons in the photosynthetic electron transport chain result in overproduction of ROS in chloroplasts, which in turn exacerbates photosynthetic inhibition, forming a vicious cycle. Moreover, excessive ROS induces lipid peroxidation and damages membranes, proteins, chlorophyll, nucleic acids, ultimately leading to cell death(Scandalios 2005; Smirnoff 1993). By examining the effects of drought on the photosystems and several ROS-related indicators in *BpSPL2-OE* and WT lines, we found that the *BpSPL2-OE* lines better maintained ROS homeostasis (Fig. 6), thereby protecting birch plants and reducing oxidative damage caused by drought stress.

Combining phenotypic and transcriptomic analyses, we identified glutathione metabolism as a key pathway through which *BpSPL2* regulates ROS balance in birch. Expression analysis of related genes in this pathway showed that most DEGs belonged to the *BpGSTs* family, with *BpGSTF3* being particularly prominent. *GSTs* detoxify ROS via glutathione conjugation, and their induced expression under drought stress can reduce peroxide production during oxidation(Hu et al. 2024; Edwards and Dixon 2005), thereby alleviating photoinhibition. Previous studies have reported that *SPL* genes regulate downstream gene expression by recognizing and binding to GTAC motifs(Ma et al. 2021). In this study, Y1H, ChIP and

LUC assays confirmed that *BpSPL2* directly binds to the GTAC motif in the *BpGSTF3* promoter, activating its expression and enhancing GST enzyme activity (Fig. 7). In *BpSPL2-OE* plants, the activities of GST, SOD, and POD were significantly increased (Fig. 7), effectively reducing H₂O₂ and O₂⁻ levels (Fig. 5B, C) and thereby mitigating membrane lipid peroxidation (reduced MDA content, Fig. 5D). This aligns with reports that overexpression of pear *GST* or tamarisk *ThGSTZ1* enhances ROS scavenging capacity (Liu et al. 2013; Yang et al. 2014). *BpSPL2-OE* plants maintained higher PSII efficiency (*Fv/Fm*) and expression of light-harvesting protein genes under drought (Fig. 5), indicating that GST-mediated ROS scavenging alleviated drought-induced photoinhibition, consistent with the protective role of the chloroplastic ROS scavenging system on photosynthetic machinery (Sun et al. 2013). Additionally, studies have shown that *SPL* genes can regulate the accumulation of osmoregulatory substances in plants, suggesting that *BpSPL2* may also enhance drought tolerance in birch through osmotic adjustment. miR156-targeted *SPLs* regulate anthocyanin synthesis (Gou et al. 2011), which acts as both an osmoregulant and a ROS scavenger (Landi, Tattini, and Gould 2015). In cassava, *SPL9* regulates proline and anthocyanin synthesis via JA signaling (Li et al. 2022), implying that *BpSPL2* might participate in osmoprotection through similar pathways, although this requires further experimental validation.

Furthermore, under drought stress, plants finely modulate their root system architecture to enhance water uptake capacity, among which the formation and development of lateral roots (LRs) is a key adaptive response. LR formation is a major determinant of root system architecture, significantly influencing water absorption efficiency, nutrient acquisition, and plant anchorage (Péret et al. 2009). The initiation and development of LRs are inextricably linked to hormonal regulation, with auxin (IAA) being one of the primary regulatory hormones. Anthranilate synthase α -subunit 1 (*ASA1*), a key rate-limiting enzyme in the tryptophan-dependent IAA biosynthetic pathway, plays a central role in IAA biosynthesis (Niyogi and Fink 1992; Zhao and Last 1996). *ASA1* catalyzes the conversion of chorismate to anthranilate, a precursor for tryptophan synthesis, which in turn leads to auxin production via the tryptophan-dependent IPA pathway. This pathway is widespread in plants and plays a crucial role in local auxin accumulation in root tips (Miles 2001; Zhao 2010; Teale, Paponov, and Palme 2006). Under drought stress, auxin synthesis and distribution in roots undergo significant alterations. The upregulation of *ASA1* expression may promote auxin synthesis, thereby stimulating the initiation and development of lateral root primordia, increasing lateral root density and root surface area, and consequently enhancing water absorption capacity. Additionally, auxin synthesized locally in roots also plays a vital role in responding to environmental stress, coordinating plastic root development in concert with shoot-derived auxin (Chen 2004; Zhao 2018). The expression of *ASA1* is regulated by upstream transcription factors. For instance, in leaf explants, jasmonic acid (JA) biosynthesis occurs within two hours after wounding and transcriptionally activates *ERF109*, which encodes a protein that upregulates *ASA1* expression to promote auxin biosynthesis and facilitate de novo root organogenesis (Lee et al. 2024).

The *SPL* transcription factor family, as target genes of miR156, is widely involved in regulating lateral root development. Niu et al (Yu et al. 2015), found that miR156 is differentially expressed in specific cells and tissues of lateral roots in *Arabidopsis* and regulates *SPL3*, *SPL9*, and *SPL10* genes in response to hormone signaling pathways to suppress lateral root growth. In transgenic potatoes overexpressing *Stu-*

miR156, the expression levels of the target gene *StSPL9* were downregulated. Compared to controls, transgenic plants showed significantly reduced plant height and root length but a significant increase in lateral root number (Luo et al. 2024). Apple *MdSPL23* binds to the promoter of *MdNLP7* to inhibit its expression, thereby negatively regulating nitrogen uptake and suppressing lateral root development in apple (Xu et al. 2025). Rice *OsSPL14* activates the expression of *OsSHR1*; the expressed *OsSHR1* further promotes the transcription of *OsSWEET16*, reducing the accumulation of glucose and fructose in tiller buds and decreasing fructose and sucrose in roots, ultimately leading to fewer tillers and crown roots but increased root length (Feng et al. 2025). In reported results, *SPLs* often function as negative regulators of lateral root development. However, we observed that birch *BpSPL2* positively regulates lateral root development (Fig. 3), acting as a positive regulator to activate auxin synthesis and promote lateral root formation. Subsequent results also confirmed that in birch, *BpSPL2* can positively activate the expression of the *BpASA1* gene. The direct activation of *BpASA1* by *BpSPL2* significantly promoted auxin biosynthesis (Fig. 7G), thereby stimulating lateral root formation and enhancing birch's adaptive capacity under drought conditions. This finding not only provides new evidence for the functional diversity of the *SPL* protein family but also reveals the critical role of the *SPL2*-*ASA1*-auxin signaling pathway in birch lateral root development under drought stress. Furthermore, extensive crosstalk exists among multiple hormone signaling pathways in drought stress responses. ABA, a key hormone in drought response, interacts with the auxin signaling pathway through its receptors *PYL8* and *PYL9* to co-regulate lateral root growth and recovery (Zhao et al. 2014; Xing et al. 2016). Transcription factors such as *MYB96* and *WRKY46* also integrate ABA and auxin signals to regulate lateral root initiation under drought conditions (Seo et al. 2009; Ding et al. 2015). The *miR156*-*SPL2*-*ASA1* regulatory module identified in this study may also interact with ABA signaling to coordinate root system plasticity in response to drought, but this requires further investigation. In summary, our study further reveals that in birch, *SPL2* can directly activate *ASA1* expression to promote auxin synthesis and lateral root development, thereby enhancing drought tolerance. This finding expands the understanding of *SPL* family functions and provides a novel perspective on their role in drought resistance mechanisms in tree species.

Conclusion

This study systematically elucidates the molecular mechanism by which the *bp-miR156c/BpSPL2* module enhances drought tolerance in birch by promoting lateral root development and activating the antioxidant pathway. Drought stress significantly suppresses the expression of *bp-miR156c*, thereby releasing its inhibition on the target gene *BpSPL2*. Overexpression of *bp-miR156c* reduces drought tolerance in birch, while its target gene *BpSPL2* directly binds to the GTAC motifs in the promoters of the target genes *BpASA1* and *BpGSTF3*, activating their transcription. On the one hand, it induces IAA accumulation to promote lateral root development, thereby enhancing drought resistance. On the other hand, it increases GST enzyme activity to reduce ROS-induced oxidative damage and alleviate photosynthetic inhibition.

Declarations

Acknowledgements This work was supported by the National Key Research and Development Program of China (No2021YFD2200304); Doctoral Student Independent Innovation Fund (2572025AW49); the Fundamental Research Funds for the Central Universities (No2572022DQ08); the National Natural Science Foundation of China (No32171738).

Author Contributions: H.P. performed most of the experiments, analysed the data and drafted the manuscript. Z.H. carried out the measurement of photosynthetic electron transport related indicators and data analysis. A.J. conducted phenotypic measurements of the growth of birch seedlings, including plant height and leaf area. Y.Z and D.H measured the relevant data of the lateral roots of the birch. Z.H. advised on all the experiments and thoroughly revised the manuscript. L.X. conceived, designed and directed the project, as well as revised and finalised the manuscript. All authors read and approved the final manuscript.

Data availability Data will be made available on request.

Supplementary Information The online version contains supplementary material available

Conflict of interest All authors have no conflict of interest for the publication of the manuscript.

References

1. Arshad, M., Feyissa, B. A., Amyot, L., Aung, B., & Hannoufa, A. (2017). MicroRNA156 improves drought stress tolerance in alfalfa (*Medicago sativa* L.) by silencing SPL13. *Plant Science*, 258, 122–136.
2. Arshad, M., Gruber, M. Y., Wall, K., & Hannoufa, A. (2017). An Insight into microRNA156 Role in Salinity Stress Responses of Alfalfa. *Frontiers in Plant Science*, 8.
3. Bhusal, N., Han, S. G., & Yoon, T. M. (2019). Impact of drought stress on photosynthetic response, leaf water potential, and stem sap flow in two cultivars of bi-leader apple trees(*Malus x domestica* Borkh). *Scientia Horticulturae*, 246, 535–543.
4. Chen, G., Wang, Y. P., Liu, X. L., Duan, S. Y., Jiang, S. H., Zhu, J., Zhang, Y. G., & Hou, H. M. (2023). The MdmiR156n Regulates Drought Tolerance and Flavonoid Synthesis in Apple Calli and Arabidopsis. *International Journal of Molecular Sciences*, 24(7).
5. Chen, H. Z., Song, L. L., Zhou, H. J., Yao, T. T., Zhang, Z., Zhang, H. J., Meng, L., & Zhang, H. H. (2025). ABA signal transduction and ROS metabolic balance play a key role in the drought resistance of safflower. *Plant Growth Regulation*, 105(1), 273–294.
6. Chen, Q. G., Dai, X. H., De-Paoli, H., Cheng, Y. F., Takebayashi, Y., Kasahara, H., Kamiya, Y., & Zhao, Y. D. (2014). Auxin Overproduction in Shoots Cannot Rescue Auxin Deficiencies in Arabidopsis Roots. *Plant and Cell Physiology*, 55(6), 1072–1079.

7. Chen, X. M. (2004). A microRNA as a translational repressor of APETALA2 in Arabidopsis flower. *Science*, 303(5666), 2022–2025.
8. Cohen, J. D., Slovin, J. P., & Hendrickson, A. M. (2003). Two genetically discrete pathways convert tryptophan to auxin: more redundancy in auxin biosynthesis. *Trends in Plant Science*, 8(5), 197–199.
9. Comas, L. H., Becker, S. R., Cruz, V. V., Byrne, P. F., & Dierig, D. A. (2013). Root traits contributing to plant productivity under drought. *Frontiers in Plant Science*, 4.
10. Covarrubias, A. A., & Reyes, J. L. (2010). Post-transcriptional gene regulation of salinity and drought responses by plant microRNAs. *Plant Cell and Environment*, 33(4), 481–489.
11. Cui, L. G., Shan, J. X., Shi, M., Gao, J. P., & Lin, H. X. (2014). The miR156-SPL9-DFR pathway coordinates the relationship between development and abiotic stress tolerance in plants. *Plant Journal*, 80(6), 1108–1117.
12. Ding, Z. J., Yan, J. Y., Li, C. X., Li, G. X., Wu, Y. R., & Zheng, S. J. (2015). Transcription factor WRKY46 modulates the development of Arabidopsis lateral roots in osmotic/salt stress conditions via regulation of ABA signaling and auxin homeostasis. *Plant Journal*, 84(1), 56–69.
13. Du, M. M., Spalding, E. P., & Gray, W. M. (2020). Rapid Auxin-Mediated Cell Expansion. *Annual Review of Plant Biology*, Vol 71, 2020, 71, 379–402.
14. Edwards, R., & Dixon, D. (2005). Plant glutathione transferases. In H. Sies & L. Packer (Eds.), *Gluthione Transferases and Gamma-Glutamyl Transpeptidases* (Vol. 401, pp. 169–186).
15. Feng, C., Zhang, X., Du, B. Y., Xiao, Y. Q., Wang, Y. Y., Sun, Y. T., Zhou, X., Wang, C., Liu, Y., & Li, T. H. (2023). MicroRNA156ab regulates apple plant growth and drought tolerance by targeting transcription factor MsSPL13. *Plant Physiology*, 192(3), 1836–1857.
16. Feng, M., Luo, W. F., Luo, S., Miao, R., Gu, M. P., Li, S., Xing, X. X., Zhang, J. H., Qian, J. S., Liu, X., Zhou, C. L., Sun, Q., Luo, T., Chen, N., Ren, Y. L., Cheng, Z. J., Lei, C. L., Zhao, Z. C., Zhu, S. S.,... Wan, J. M. (2025). Strigolactones Regulate Sugar Allocation to Control Rice Tillering and Root Development via the OsSPL14-OsSHR1-OsSWEET16 Pathway. *Plant Biotechnology Journal*.
17. Feyissa, B. A., Renaud, J., Nasrollahi, V., Kohalmi, S. E., & Hannoufa, A. (2020). Transcriptome-IPMS analysis reveals a tissue-dependent regulatory mechanism in alfalfa drought tolerance. *BMC Genomics*, 21(1).
18. Gou, J. Y., Felippes, F. F., Liu, C. J., Weigel, D., & Wang, J. W. (2011). Negative Regulation of Anthocyanin Biosynthesis in Arabidopsis by a miR156-Targeted SPL Transcription Factor. *Plant Cell*, 23(4), 1512–1522.
19. Grieneisen, V. A., Xu, J., Marée, A. F. M., Hogeweg, P., & Scheres, B. (2007). Auxin transport is sufficient to generate a maximum and gradient guiding root growth. *Nature*, 449(7165), 1008–1013.
20. Gupta, A., Rico-Medina, A., & Caño-Delgado, A. I. (2020). The physiology of plant responses to drought. *Science*, 368(6488), 266–269.
21. Hu, X. Q., Zheng, T., Chen, W. J., Duan, H. L., Yuan, Z. J., An, J. Q., Zhang, H. H., & Liu, X. M. (2024). Genome-wide identification and expression analysis of the GST gene family of *Betula platyphylla*. *Journal of Forestry Research*, 35(1).

22. Huang, S. Q., Peng, J., Qiu, C. X., & Yang, Z. M. (2009). Heavy metal-regulated new microRNAs from rice. *Journal of Inorganic Biochemistry*, 103(2), 282–287.
23. Hussain, H. A., Hussain, S., Khaliq, A., Ashraf, U., Anjum, S. A., Men, S. N., & Wang, L. C. (2018). Chilling and Drought Stresses in Crop Plants: Implications, Cross Talk, and Potential Management Opportunities. *Frontiers in Plant Science*, 9.
24. Islam, M. S., Choudhury, M., Majlish, A. N. K., Islam, T., & Ghosh, A. (2018). Comprehensive genome-wide analysis of glutathione S-transferase gene family in potato (*Solanum tuberosum* L.) and their expression profiling in various anatomical tissues and perturbation conditions. *Gene*, 639, 149–162.
25. Islam, S., Das Sajib, S., Jui, Z. S., Arabia, S., Islam, T., & Ghosh, A. (2019). Genome-wide identification of glutathione S-transferase gene family in pepper, its classification, and expression profiling under different anatomical and environmental conditions. *Scientific Reports*, 9.
26. Jain, M., Ghanashyam, C., & Bhattacharjee, A. (2010). Comprehensive expression analysis suggests overlapping and specific roles of rice glutathione S-transferase genes during development and stress responses. *BMC Genomics*, 11.
27. Jaleel, C. A., Manivannan, P., Wahid, A., Farooq, M., Al-Juburi, H. J., Somasundaram, R., & Panneerselvam, R. (2009). Drought Stress in Plants: A Review on Morphological Characteristics and Pigments Composition. *International Journal of Agriculture and Biology*, 11(1), 100–105.
28. Ji, G. X., Wang, Z. Y., Song, J. Q., Zhang, H. R., Wang, K. X., Xu, J. J., Nan, S., Zhang, T. H., Qi, S. Y., Ding, C. J., & Zhang, H. H. (2025). The Trx-Prx redox pathway and PGR5/PGRL1-dependent cyclic electron transfer play key regulatory roles in poplar drought stress. *Tree Physiology*, 45(2).
29. Jones-Rhoades, M. W., & Bartel, D. P. (2004). Computational identification of plant MicroRNAs and their targets, including a stress-induced miRNA. *Molecular Cell*, 14(6), 787–799.
30. Karlova, R., Boer, D., Hayes, S., & Testerink, C. (2021). Root plasticity under abiotic stress. *Plant Physiology*, 187(3), 1057–1070.
31. Khraiwesh, B., Zhu, J. K., & Zhu, J. H. (2012). Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants. *Biochimica Et Biophysica Acta-Gene Regulatory Mechanisms*, 1819(2), 137–148.
32. Koevoets, I. T., Venema, J. H., Elzenga, J. T. M., & Testerink, C. (2016). Roots Withstanding their Environment: Exploiting Root System Architecture Responses to Abiotic Stress to Improve Crop Tolerance. *Frontiers in Plant Science*, 7.
33. Koroban, N. V., Kudryavtseva, A. V., Krasnov, G. S., Sadritdinova, A. F., Fedorova, M. S., Snezhkina, A. V., Bolsheva, N. L., Muravenko, O. V., Dmitriev, A. A., & Melnikova, N. V. (2016). The Role of MicroRNA in Abiotic Stress Response in Plants. *Molecular Biology*, 50(3), 337–343.
34. Landi, M., Tattini, M., & Gould, K. S. (2015). Multiple functional roles of anthocyanins in plant-environment interactions. *Environmental and Experimental Botany*, 119, 4–17.
35. Lavenus, J., Goh, T., Roberts, I., Guyomarc'h, S., Lucas, M., De Smet, I., Fukaki, H., Beeckman, T., Bennett, M., & Laplaze, L. (2013). Lateral root development in *Arabidopsis*: fifty shades of auxin. *Trends in Plant Science*, 18(8), 455–463.

36. Lee, K., Yoon, H., Park, O. S., & Seo, P. J. (2024). ENHANCER OF SHOOT REGENERATION1 promotes de novo root organogenesis after wounding in Arabidopsis leaf explants. *Plant Cell*, 36(6), 2359–2374.
37. Li, L. H., Yi, H. L., Xue, M. Z., & Yi, M. (2017). miR398 and miR395 are involved in response to SO₂ stress in Arabidopsis. *Ecotoxicology*, 26(9), 1181–1187.
38. Li, S. X., Cheng, Z. H., Li, Z. B., Dong, S. M., Yu, X. L., Zhao, P. J., Liao, W. B., Yu, X., & Peng, M. (2022). attenuates drought resistance by regulating JA signaling and protectant metabolite contents in cassava. *Theoretical and Applied Genetics*, 135(3), 817–832.
39. Li, Y. X., Han, S. C., Sun, X. M., Khan, N. U., Zhong, Q., Zhang, Z. Y., Zhang, H. L., Ming, F., Li, Z. C., & Li, J. J. (2023). Variations in OsSPL10 confer drought tolerance by directly regulating expression and ROS production in rice. *Journal of Integrative Plant Biology*, 65(4), 918–933.
40. Liang, Y. T., Yang, X. Q., Wang, C., & Wang, Y. W. (2024). miRNAs: Primary modulators of plant drought tolerance. *Journal of Plant Physiology*, 301.
41. Light, G. G., Mahan, J. R., Roxas, V. P., & Allen, R. D. (2005). Transgenic cotton (*Gossypium hirsutum* L.) seedlings expressing a tobacco glutathione-transferase fail to provide improved stress tolerance. *Planta*, 222(2), 346–354.
42. Liu, D., Liu, Y., Rao, J., Wang, G., Li, H., Ge, F., & Chen, C. (2013). Overexpression of the glutathione S-transferase gene from *Pyrus pyrifolia* fruit improves tolerance to abiotic stress in transgenic tobacco plants. *Molecular Biology*, 47(4), 515–523.
43. Liu, S. J., Zhang, H., Jin, X. T., Niu, M. X., Feng, C. H., Liu, X., Liu, C., Wang, H. L., Yin, W. L., & Xia, X. L. (2025). Drives Lateral Root Growth Via Auxin and ABA Signalling Under Drought Stress in. *Plant Cell and Environment*, 48(1), 664–681.
44. Ljung, K., Hull, A. K., Celenza, J., Yamada, M., Estelle, M., Nonmanly, J., & Sandberg, G. (2005). Sites and regulation of auxin biosynthesis in Arabidopsis roots. *Plant Cell*, 17(4), 1090–1104.
45. Lu, X., Dun, H., Lian, C. L., Zhang, X. F., Yin, W. L., & Xia, X. L. (2017). The role of peu-miR164 and its target PeNAC genes in response to abiotic stress in *Populus euphratica*. *Plant Physiology and Biochemistry*, 115, 418–438.
46. Luo, H. Y., Yang, J. W., Liu, S. Y., Li, S. G., Si, H. J., & Zhang, N. (2024). Control of Plant Height and Lateral Root Development via Stu-miR156 Regulation of SPL9 Transcription Factor in Potato. *Plants-Basel*, 13(5).
47. Luo, L. J. (2010). Breeding for water-saving and drought-resistance rice (WDR) in China. *Journal of Experimental Botany*, 61(13), 3509–3517.
48. Ma, Y., Xue, H., Zhang, F., Jiang, Q., Yang, S., Yue, P. T., Wang, F., Zhang, Y. Y., Li, L. G., He, P., & Zhang, Z. H. (2021). The miR156/SPL module regulates apple salt stress tolerance by activating MdWRKY100 expression. *Plant Biotechnology Journal*, 19(2), 311–323.
49. Mallory, A. C., Reinhart, B. J., Jones-Rhoades, M. W., Tang, G. L., Zamore, P. D., Barton, M. K., & Bartel, D. P. (2004). MicroRNA control of PHABULOSA in leaf development: importance of pairing to the microRNA 5' region. *Embo Journal*, 23(16), 3356–3364.

50. Miles, E. W. (2001). Tryptophan synthase: a multienzyme complex with an intramolecular tunnel [; Review]. *Chemical record* (New York, N.Y.), 1(2), 140–151.
51. Ning, K., Chen, S., Huang, H. J., Jiang, J., Yuan, H. M., & Li, H. Y. (2017). Molecular characterization and expression analysis of the SPL gene family with transgenic lines found to confer tolerance to abiotic stress in *Betula platyphylla* Suk. *Plant Cell Tissue and Organ Culture*, 130(3), 469–481.
52. Niyogi, K. K., & Fink, G. R. (1992). Two anthranilate synthase genes in *Arabidopsis*: defense-related regulation of the tryptophan pathway [; Research Support, U.S. Gov't, Non-P.H.S.]. *The Plant cell*, 4(6), 721–733.
53. Ohashi, Y., Nakayama, N., Saneoka, H., & Fujita, K. (2006). Effects of drought stress on photosynthetic gas exchange, chlorophyll fluorescence and stem diameter of soybean plants. *Biologia Plantarum*, 50(1), 138–141.
54. Öztetik, E. (2008). A tale of plant Glutathione-transferases:: Since 1970. *Botanical Review*, 74(3), 419–437.
55. P, R. V., A, L. S., K, G. D., R, M. J., & D, A. R. (2000). Stress tolerance in transgenic tobacco seedlings that overexpress glutathione S-transferase/glutathione peroxidase. *Plant & cell physiology*, 41(11), 1229–1234.
56. Pan, J. W., Li, Z., Dai, S. J., Ding, H. F., Wang, Q. G., Li, X. B., Ding, G. H., Wang, P. F., Guan, Y. N., & Liu, W. (2020). Integrative analyses of transcriptomics and metabolomics upon seed germination of foxtail millet in response to salinity. *Scientific Reports*, 10(1).
57. Panda, S. K., & Sunkar, R. (2015). Nutrient- and other stress-responsive microRNAs in plants: Role for thiol-based redox signaling. *Plant Signaling & Behavior*, 10(4).
58. Péret, B., De Rybel, B., Casimiro, I., Benková, E., Swarup, R., Laplaze, L., Beeckman, T., & Bennett, M. J. (2009). lateral root development: an emerging story. *Trends in Plant Science*, 14(7), 399–408.
59. Qi, S. Y., Yao, T. T., Ren, Z. Q., Wang, J. C., Wang, Z. Y., Liu, H. Z., Song, J. Q., Li, H. J., Liu, T. T., Ding, C. J., Hu, Y. B., & Zhang, H. H. (2025). Integrative miRNA-mRNA analysis reveals key regulatory mechanisms of the miR156g-PagSPL1B module in poplar drought tolerance. *Industrial Crops and Products*, 237.
60. Ranjan, A., Sinha, R., Singla-Pareek, S. L., Pareek, A., & Singh, A. K. (2022). Shaping the root system architecture in plants for adaptation to drought stress. *Physiologia Plantarum*, 174(2).
61. Rezaei, M. K., Shobbar, Z. S., Shahbazi, M., Abedini, R., & Zare, S. (2013). Glutathione S-transferase (GST) family in barley: Identification of members, enzyme activity, and gene expression pattern. *Journal of Plant Physiology*, 170(14), 1277–1284.
62. Rong, W., Qi, L., Wang, A. Y., Ye, X. G., Du, L. P., Liang, H. X., Xin, Z. Y., & Zhang, Z. Y. (2014). The ERF transcription factor TaERF3 promotes tolerance to salt and drought stresses in wheat. *Plant Biotechnology Journal*, 12(4), 468–479.
63. Sang, Q., Fan, L. S., Liu, T. X., Qiu, Y. J., Du, J., Mo, B. X., Chen, M., & Chen, X. M. (2023). MicroRNA156 conditions auxin sensitivity to enable growth plasticity in response to environmental changes in. *Nature Communications*, 14(1).

64. Scandalios, J. G. (2005). Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses. *Brazilian Journal of Medical and Biological Research*, 38(7), 995–1014.
65. Seo, P. J., Xiang, F. N., Qiao, M., Park, J. Y., Lee, Y. N., Kim, S. G., Lee, Y. H., Park, W. J., & Park, C. M. (2009). The MYB96 Transcription Factor Mediates Absciscic Acid Signaling during Drought Stress Response in Arabidopsis. *Plant Physiology*, 151(1), 275–289.
66. Sharma, R., Sahoo, A., Devendran, R., & Jain, M. (2014). Over-Expression of a Rice Tau Class Glutathione S-Transferase Gene Improves Tolerance to Salinity and Oxidative Stresses in Arabidopsis. *Plos One*, 9(3).
67. Shriram, V., Kumar, V., Devarumath, R. M., Khare, T. S., & Wani, S. H. (2016). MicroRNAs As Potential Targets for Abiotic Stress Tolerance in Plants. *Frontiers in Plant Science*, 7.
68. Smirnoff, N. (1993). The role of active oxygen in the response of plants to water deficit and desiccation. *The New phytologist*, 125(1), 27–58.
69. Song, W., Zhou, F. K., Shan, C. H., Zhang, Q., Ning, M., Liu, X. M., Zhao, X. X., Cai, W. C., Yang, X. Q., Hao, G. F., & Tang, F. X. (2021). Identification of Glutathione S-Transferase Genes in Hami Melon (*Cucumis melo* var. *saccharinus*) and Their Expression Analysis Under Cold Stress. *Frontiers in Plant Science*, 12.
70. Sun, N., Yang, S. W., Zhang, T. H., Xu, J. J., Wang, K. X., Liang, Y. L., Sun, C. Q., Zhang, X. L., & Zhang, H. H. (2025). MYB37 enhances drought tolerance by maintaining ROS homeostasis and alleviating photosynthetic inhibition in. *Plant Physiology and Biochemistry*, 228.
71. Sun, W. H., Wang, Y., He, H. G., Li, X., Song, W., Du, B., & Meng, Q. W. (2013). Reduction of methylviologen-mediated oxidative stress tolerance in antisense transgenic tobacco seedlings through restricted expression of. *Journal of Zhejiang University-Science B*, 14(7), 578–585.
72. Sunkar, R., Li, Y. F., & Jagadeeswaran, G. (2012). Functions of microRNAs in plant stress responses. *Trends in Plant Science*, 17(4), 196–203.
73. Sunkar, R., & Zhu, J. K. (2004). Novel and stress-regulated microRNAs and other small RNAs from Arabidopsis. *Plant Cell*, 16(8), 2001–2019.
74. Teale, W. D., Paponov, I. A., & Palme, K. (2006). Auxin in action: signalling, transport and the control of plant growth and development. *Nature Reviews Molecular Cell Biology*, 7(11), 847–859.
75. Tu, T. L., Zheng, S. S., Ren, P. R., Meng, X. W., Zhao, J. H., Chen, Q., & Li, C. Y. (2021). Coordinated cytokinin signaling and auxin biosynthesis mediates arsenate-induced root growth inhibition. *Plant Physiology*, 185(3), 1166–1181.
76. Visentin, I., Pagliarani, C., Deva, E., Caracci, A., Turecková, V., Novák, O., Lovisolo, C., Schubert, A., & Cardinale, F. (2020). A novel strigolactone-miR156 module controls stomatal behaviour during drought recovery. *Plant Cell and Environment*, 43(7), 1613–1624.
77. Wang, H., & Wang, H. Y. (2015). The miR156/SPL Module, a Regulatory Hub and Versatile Toolbox, Gears up Crops for Enhanced Agronomic Traits. *Molecular Plant*, 8(5), 677–688.

78. Wang, J. C., Cui, C. C., Qi, S. Y., Wang, Z. Y., Song, J. Q., Ji, G. X., Sun, N., Liu, X. M., & Zhang, H. H. (2025). The NAC transcription factor enhances salt tolerance in poplar by alleviating photosynthetic inhibition. *Plant Physiology and Biochemistry*, 221.
79. Wang, J. C., Song, J. Q., Qi, H. L., Zhang, H. J., Wang, L., Zhang, H. B., Cui, C. C., Ji, G. X., Muhammad, S., Sun, G. Y., Xu, Z. R., & Zhang, H. H. (2023). Overexpression 2-Cys Peroxiredoxin of alleviates the NaHCO₃ stress-induced photoinhibition and reactive oxygen species damage of tobacco. *Plant Physiology and Biochemistry*, 201.
80. Wang, K. X., Liu, Y. R., Teng, F. K., Cen, H. F., Yan, J. P., Lin, S. W., Li, D. Y., & Zhang, W. J. (2021). Heterogeneous expression of Osa-MIR156bc increases abiotic stress resistance and forage quality of alfalfa. *Crop Journal*, 9(5), 1135–1144.
81. Wang, R. B., Ma, J. F., Zhang, Q., Wu, C. L., Zhao, H. Y., Wu, Y. A., Yang, G. X., & He, G. Y. (2019). Genome-wide identification and expression profiling of glutathione transferase gene family under multiple stresses and hormone treatments in wheat (*Triticum aestivum* L.). *BMC Genomics*, 20(1).
82. Wen, S. J., Zhou, C. Z., Tian, C. Y., Yang, N. N., Zhang, C., Zheng, A. R., Chen, Y. X., Lai, Z. X., & Guo, Y. Q. (2024). Identification and Validation of the miR156 Family Involved in Drought Responses and Tolerance in Tea Plants (*Camellia sinensis* L. O. Kuntze). *Plants-Basel*, 13(2).
83. Xing, L., Zhao, Y., Gao, J. H., Xiang, C. B., & Zhu, J. K. (2016). The ABA receptor PYL9 together with PYL8 plays an important role in regulating lateral root growth. *Scientific Reports*, 6.
84. Xu, J., Xing, X. J., Tian, Y. S., Peng, R. H., Xue, Y., Zhao, W., & Yao, Q. H. (2015). Transgenic *Arabidopsis* plants expressing tomato glutathione S-Transferase showed enhanced resistance to salt and drought stress. *Plos One*, 10(9).
85. Xu, R. R., Wang, P., Pang, Y. N., Liu, H. L., Zhang, T. P., Li, Y., & Zhang, S. Z. (2025). Involvement of the miR156/SPLs/NLP7 modules in plant lateral root development and nitrogen uptake. *Planta*, 261(6).
86. Yang, G. Y., Wang, Y. C., Xia, D. A., Gao, C. Q., Wang, C., & Yang, C. P. (2014). Overexpression of a GST gene (ThGSTZ1) from *Tamarix* improves drought and salinity tolerance by enhancing the ability to scavenge reactive oxygen species. *Plant Cell Tissue and Organ Culture*, 117(1), 99–112.
87. Yao, T. T., Ding, C. J., Che, Y. H., Zhang, Z., Cui, C. C., Ji, G. X., Song, J. Q., Zhang, H. B., Ao, H., & Zhang, H. H. (2023). Heterologous expression of *Zygophyllum xanthoxylon* zinc finger protein gene (ZxZF) enhances the tolerance of poplar photosynthetic function to drought stress. *Plant Physiology and Biochemistry*, 199.
88. Yin, F. Q., Qin, C., Gao, J., Liu, M., Luo, X. R., Zhang, W. Y., Liu, H. J., Liao, X. H., Shen, Y. U., Mao, L. K., Zhang, Z. M., Lin, H. J., Lübberstedt, T., & Pan, G. T. (2015). Genome-Wide Identification and Analysis of Drought-Responsive Genes and MicroRNAs in Tobacco. *International Journal of Molecular Sciences*, 16(3), 5714–5740.
89. Yu, N., Niu, Q. W., Ng, K. H., & Chua, N. H. (2015). The role of miR156/SPLs modules in *Arabidopsis* lateral root development. *Plant Journal*, 83(4), 673–685.
90. Zang, D. D., Wang, L. N., Zhang, Y. M., Zhao, H. M., & Wang, Y. C. (2017). ThDof1.4 and ThZFP1 constitute a transcriptional regulatory cascade involved in salt or osmotic stress in *Tamarix hispida*.

Plant Molecular Biology, 94(4–5), 495–507.

91. Zhang, G. F., Zhao, F., Chen, Y. Q., Pan, Y., Sun, L. J., Bao, N., Zhang, T., Cui, C. X., Qiu, Z. Z., Zhang, Y. J., Yang, L., & Xu, L. (2019). Jasmonate-mediated wound signalling promotes plant regeneration. *Nature Plants*, 5(5), 491–497.
92. Zhang, H. H., Xu, Z. S., Huo, Y. Z., Guo, K. W., Wang, Y., He, G. Q., Sun, H. W., Li, M. B., Li, X., Xu, N., & Sun, G. Y. (2020). Overexpression of Trx CDSP32 gene promotes chlorophyll synthesis and photosynthetic electron transfer and alleviates cadmium-induced photoinhibition of PSII and PSI in tobacco leaves. *Journal of Hazardous Materials*, 398.
93. Zhang, Y. H., He, J. Y., Xiao, Y. Z., Zhang, Y. A., Liu, Y. Q., Wan, S. Q., Liu, L., Dong, Y., Liu, H., & Yu, Y. B. (2021). CsGSTU8, a Glutathione S-Transferase from *Camellia sinensis*, Is regulated by CsWRKY48 and plays a positive role in drought tolerance. *Frontiers in Plant Science*, 12.
94. Zhao, H. M., Li, H. Y., Jia, Y. Q., Wen, X. J., Guo, H. Y., Xu, H. Y., & Wang, Y. C. (2020). Building a Robust Chromatin Immunoprecipitation Method with Substantially Improved Efficiency. *Plant Physiology*, 183(3), 1026–1034.
95. Zhao, J., & Last, R. L. (1996). Coordinate regulation of the tryptophan biosynthetic pathway and indolic phytoalexin accumulation in *Arabidopsis* [; Research Support, U.S. Gov't, Non-P.H.S.; Research Support, U.S. Gov't, P.H.S.]. *The Plant cell*, 8(12), 2235–2244.
96. Zhao, Y., He, J. Q., Liu, M. M., Miao, J. N., Ma, C., Feng, Y. J., Qian, J. J., Li, H. H., Bi, H. H., & Liu, W. X. (2024). The SPL transcription factor TaSPL6 negatively regulates drought stress response in wheat. *Plant Physiology and Biochemistry*, 206.
97. Zhao, Y., Xing, L., Wang, X. G., Hou, Y. J., Gao, J. H., Wang, P. C., Duan, C. G., Zhu, X. H., & Zhu, J. K. (2014). The ABA Receptor PYL8 Promotes Lateral Root Growth by Enhancing MYB77-Dependent Transcription of Auxin-Responsive Genes. *Science Signaling*, 7(328).
98. Zhao, Y. D. (2010). Auxin Biosynthesis and Its Role in Plant Development. *Annual Review of Plant Biology*, Vol 61, 61, 49–64.
99. Zhao, Y. D. (2018). Essential Roles of Local Auxin Biosynthesis in Plant Development and in Adaptation to Environmental Changes. *Annual Review of Plant Biology*, Vol 69, 69, 417–435.
100. Zhou, X. F., Wang, G. D., & Zhang, W. X. (2007). UV-B responsive microRNA genes in *Arabidopsis thaliana*. *Molecular Systems Biology*, 3.
101. Zhang, Y. H., J. Y. He, Y. Z. Xiao, Y. A. Zhang, Y. Q. Liu, S. Q. Wan, L. Liu, Y. Dong, H. Liu, and Y. B. Yu. 2021. 'CsGSTU8, a Glutathione S-Transferase From, Is Regulated by CsWRKY48 and Plays a Positive Role in Drought Tolerance', *Frontiers in Plant Science*, 12.
102. Zhao, H. M., H. Y. Li, Y. Q. Jia, X. J. Wen, H. Y. Guo, H. Y. Xu, and Y. C. Wang. 2020. 'Building a Robust Chromatin Immunoprecipitation Method with Substantially Improved Efficiency', *Plant Physiology*, 183: 1026–34.
103. Zhao, J, and R L Last. 1996. 'Coordinate regulation of the tryptophan biosynthetic pathway and indolic phytoalexin accumulation in *Arabidopsis*', *The Plant cell*, 8: 2235–44.

104. Zhao, Y. D. 2010. 'Auxin Biosynthesis and Its Role in Plant Development', Annual Review of Plant Biology, Vol 61, 61: 49–64.

105. Zhao, Y. D. (2018). 2018. 'Essential Roles of Local Auxin Biosynthesis in Plant Development and in Adaptation to Environmental Changes', Annual Review of Plant Biology, Vol 69, 69: 417–35.

106. Zhao, Y., J. Q. He, M. M. Liu, J. N. Miao, C. Ma, Y. J. Feng, J. J. Qian, H. H. Li, H. H. Bi, and W. X. Liu. 2024. 'The SPL transcription factor TaSPL6 negatively regulates drought stress response in wheat', Plant Physiology and Biochemistry, 206.

107. Zhao, Y., L. Xing, X. G. Wang, Y. J. Hou, J. H. Gao, P. C. Wang, C. G. Duan, X. H. Zhu, and J. K. Zhu. 2014. 'The ABA Receptor PYL8 Promotes Lateral Root Growth by Enhancing MYB77-Dependent Transcription of Auxin-Responsive Genes', Science Signaling, 7.

108. Zhou, X. F., G. D. Wang, and W. X. Zhang. 2007. 'UV-B responsive microRNA genes in', Molecular Systems Biology, 3.

Figures

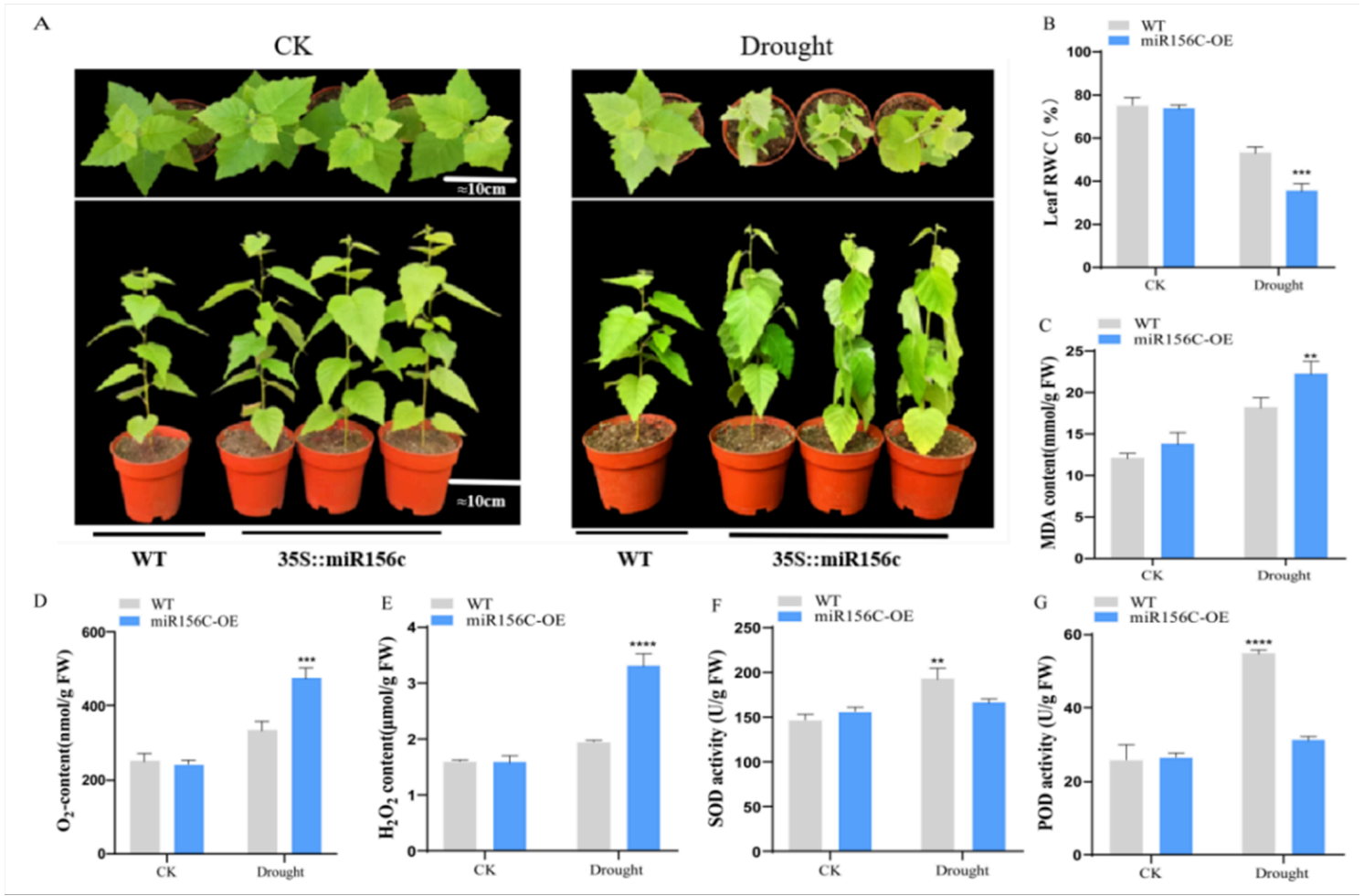


Figure 1

Overexpression of *bp-miR156c* impairs drought tolerance in birch. (A) Phenotypes of wild-type (WT) and *bp-miR156c*-OE birch plants under normal growth conditions and after 7 days of drought stress. (B) Leaf relative water content (RWC). (C) MDA content. (D) O₂⁻ content. (E) H₂O₂ content. (F) SOD activity. (G) POD activity. Note: In panel A, the *bp-miR156c* overexpressing plants shown belong to the same transgenic line. Scale bar ≈ 10 cm. Error bars represent the mean ± SD from three independent biological replicates (each replicate consisted of 15 plants). Asterisks indicate significant differences between the WT and transgenic plants as determined by one-way ANOVA with Tukey's multiple comparisons test. (**P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001).

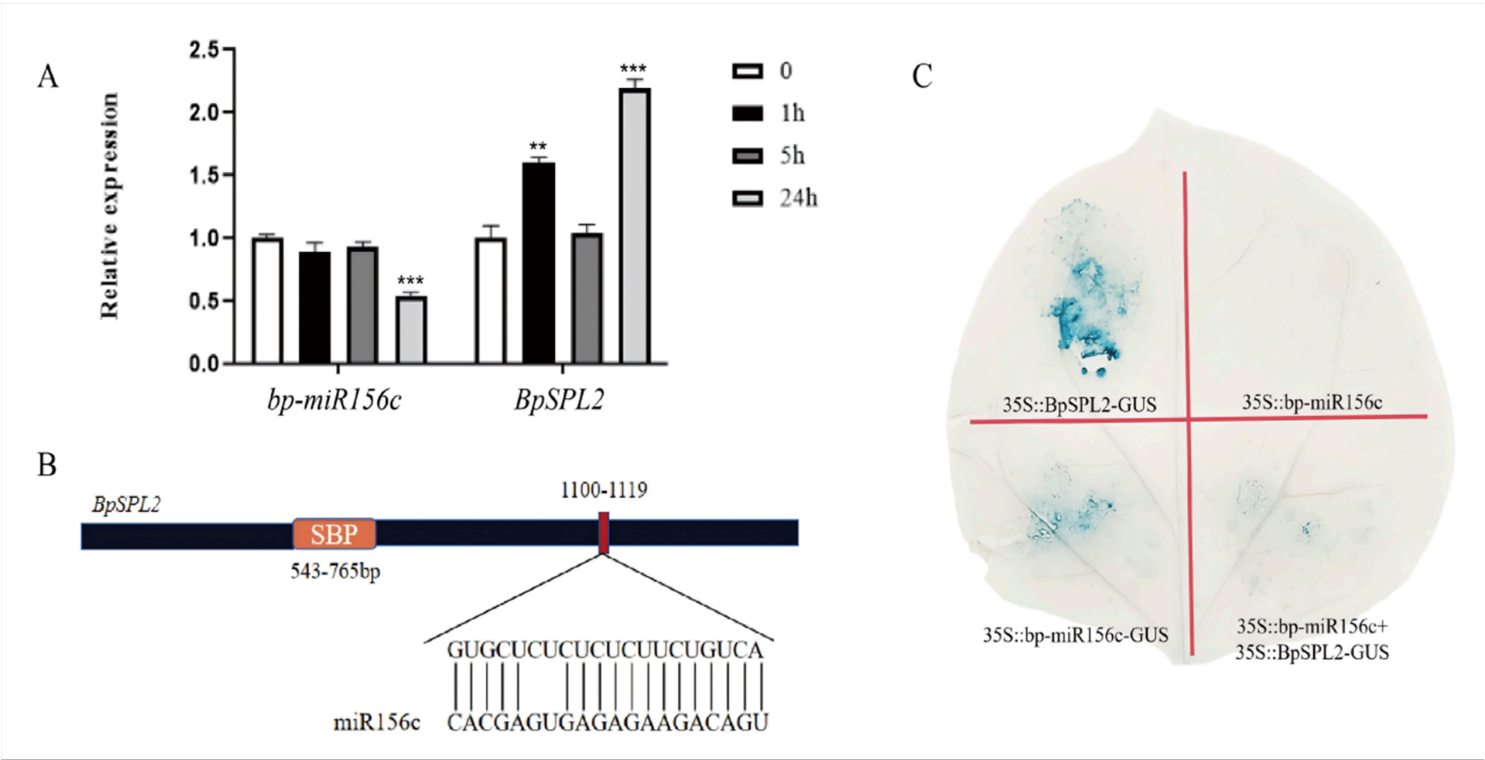


Figure 2

bp-miR156c targets and cleaves *BpSPL2* transcripts. (A) Expression patterns of *bp-miR156c* and *BpSPL2* following PEG-simulated drought treatment. (B) Prediction of the miR156/SPL2 cleavage site. (C) GUS staining assay validating the *bp-miR156c*-mediated cleavage of *BpSPL2*. Error bars represent mean ± SD from three independent biological replicates. Asterisks indicate significant differences as determined by one-way ANOVA with Tukey's multiple comparisons test. (**P* < 0.05).

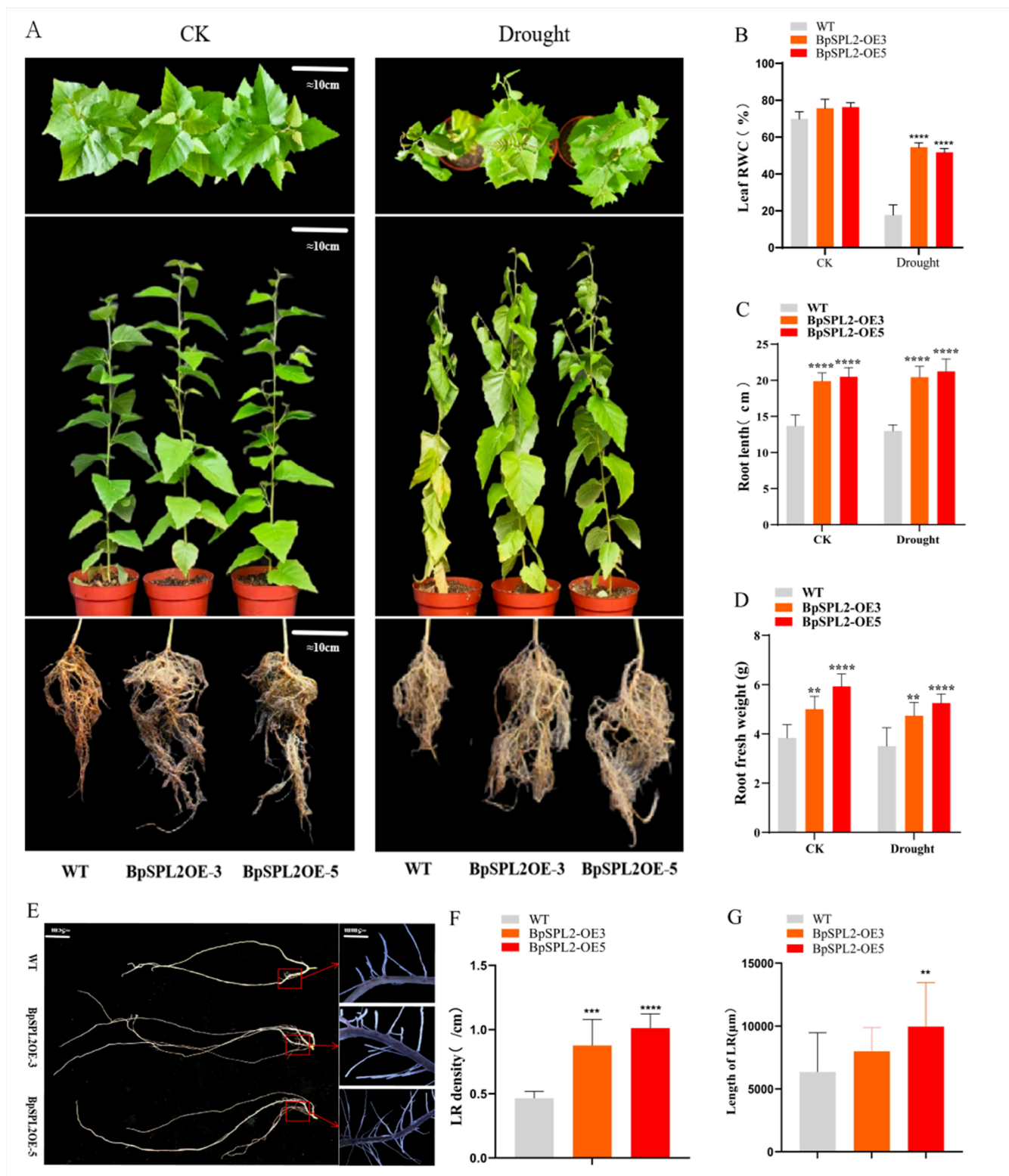


Figure 3

BpSPL2 enhances drought tolerance in birch. (A) Phenotypes of WT and *BpSPL2-OE* transgenic birch plants after 7 days of drought stress. (B) Leaf relative water content. (C) Root length. (D) Root fresh weight. (E) Lateral root phenotypes of tissue-cultured seedlings of WT and *BpSPL2-OE* lines. (F) Lateral root density. (G) Lateral root length. Error bars represent the mean $\pm$ SD from three independent biological replicates. Asterisks indicate significant differences between the WT and transgenic plants as

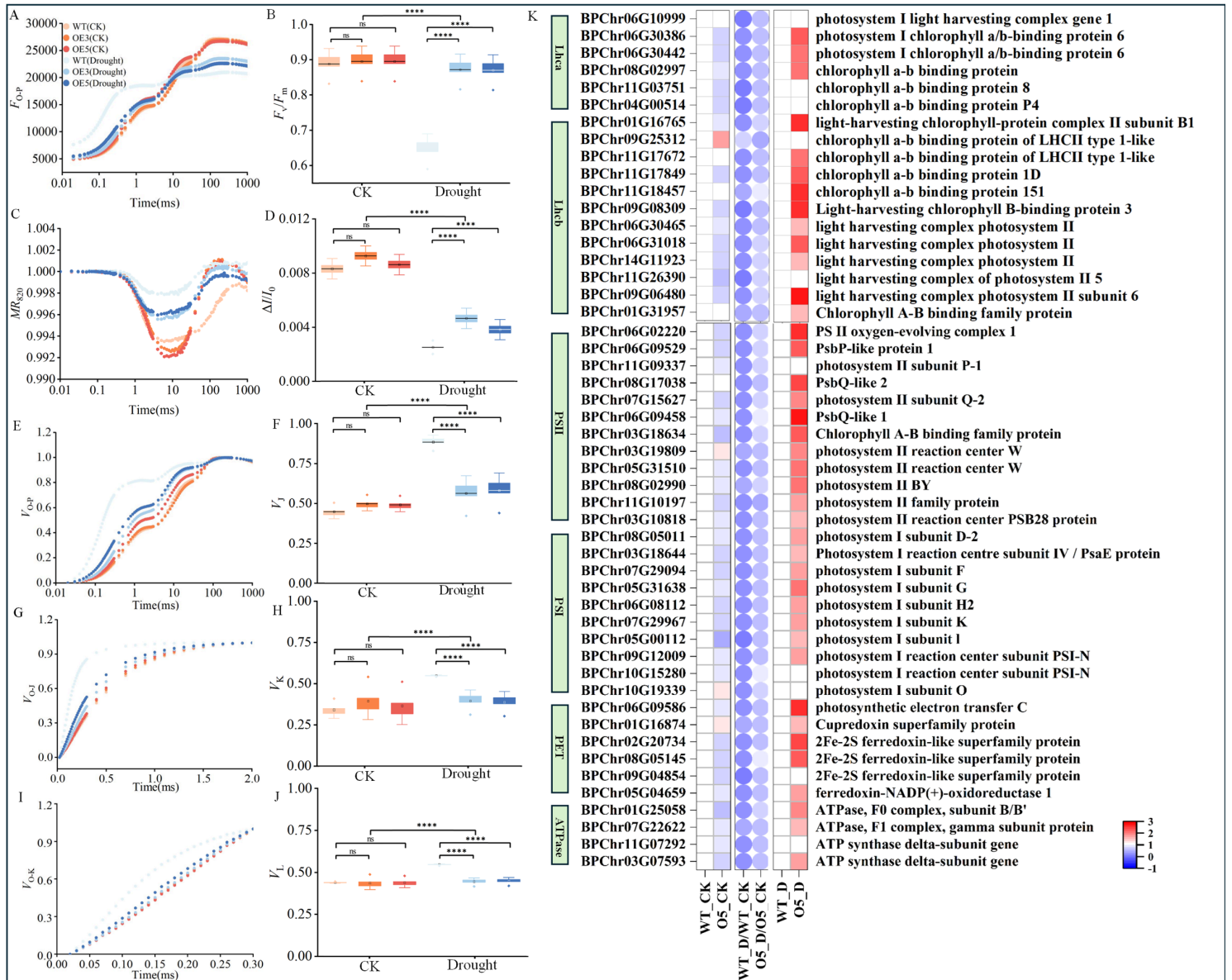


Figure 5 Chlorophyll fluorescence parameters and expression of photosynthetic electron transport-related genes in *BpSPL2* transgenic and WT birch under drought stress. (A) OJIP transients of *BpSPL2*-OE and WT lines before and after drought stress. (B) Maximum photochemical efficiency of PSII (F_v/F_m). (C) MR_{820} kinetics of *BpSPL2*-OE and WT lines before and after drought stress. (D) $\Delta I/I_0$ values. (E) Normalized O-P curves (V_{O-P}). (F) V_J values before and after drought stress. (G) Normalized O-J curves (V_{O-J}). (H) V_K values before and after drought stress. (I) Normalized O-K curves (V_{O-K}). (J) V_L values before and after drought stress. (K) Effects of drought stress on the expression levels of genes encoding light-harvesting antenna proteins and proteins involved in photosynthetic electron transport in leaves of different birch lines. Error bars represent the mean $\pm$ SD from three independent biological replicates. Asterisks indicate significant differences between the WT and transgenic plants as determined by one-way ANOVA with Tukey's multiple comparisons test. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

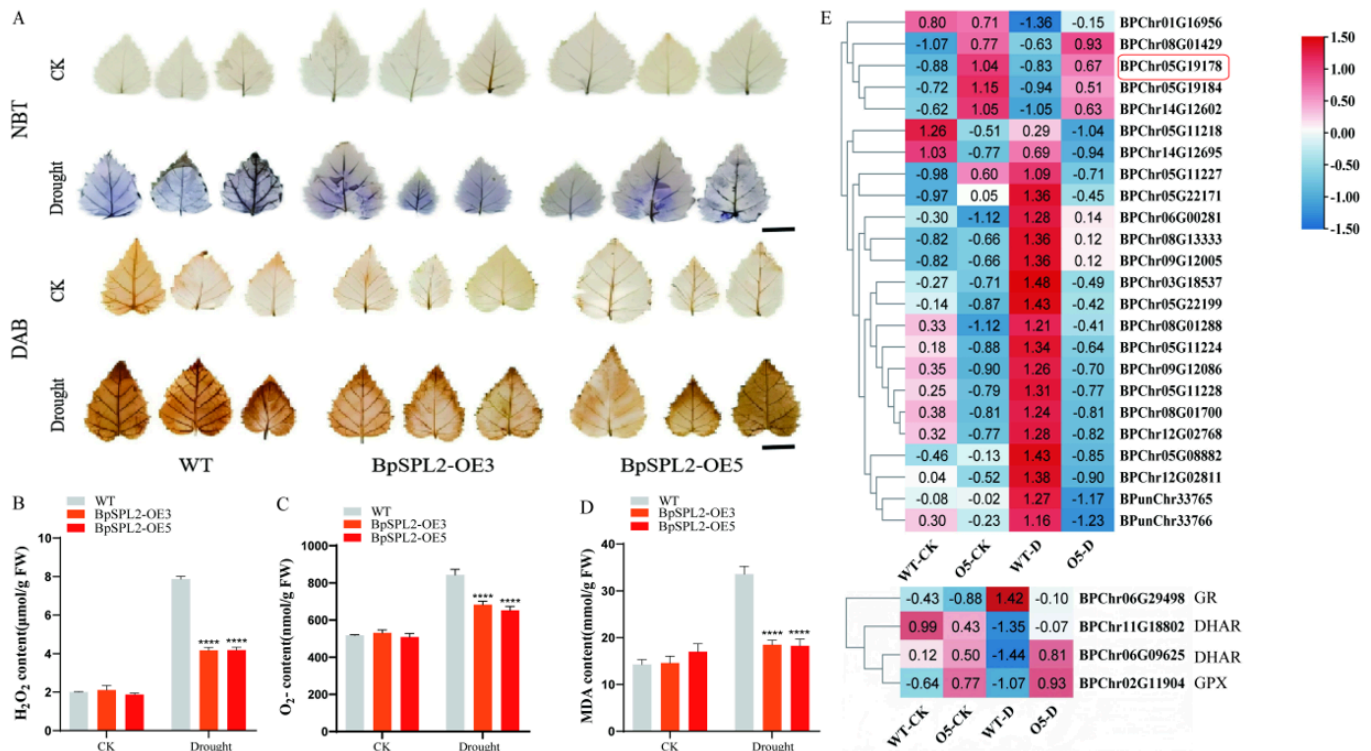


Figure 6

Effect of drought stress on ROS levels in WT and *BpSPL2-OE* birch. (A) NBT and DAB staining for visualizing O₂⁻ and H₂O₂ accumulation, respectively. (B) H₂O₂ content. (C) O₂⁻ content. (D) Malondialdehyde (MDA) content. (E) Heatmap of differentially expressed genes in the glutathione metabolism-related gene families. Scale bar ≈5 cm. Error bars represent mean ± SD from three independent biological replicates. Asterisks indicate significant differences between the WT and transgenic plants as determined by one-way ANOVA with Tukey's multiple comparisons test. (**P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001).

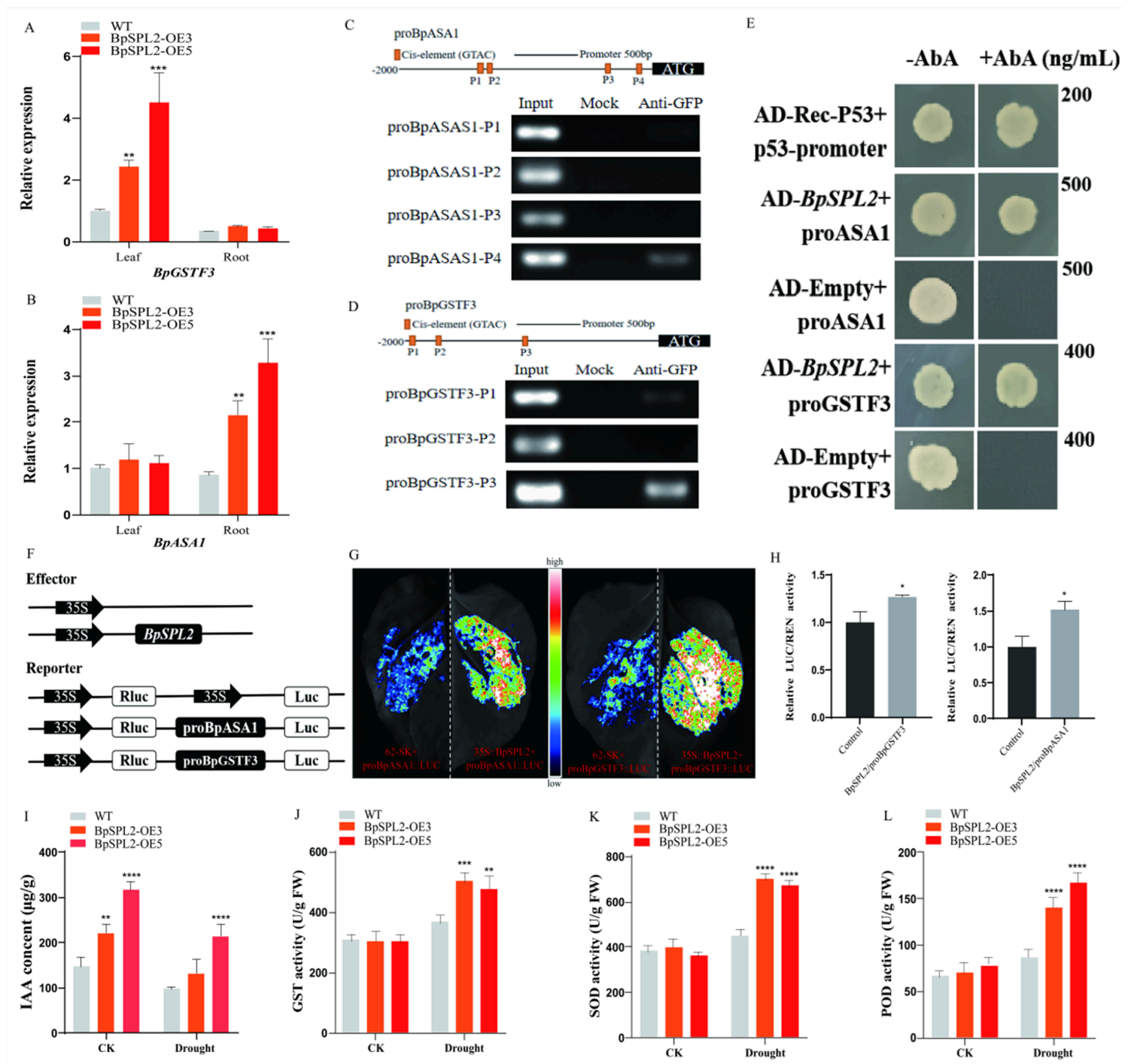


Figure 7

BpSPL2 targets and transcriptionally activates *BpGSTF3* and *BpASA1*. (A, B) *BpGSTF3* and *BpASA1* are significantly induced in *BpSPL2*-OE plants. (C, D) ChIP-PCR assays. (E) Yeast one-hybrid (Y1H) assay. (F), (G), (H) Dual-luciferase reporter assay. (I) IAA content. (J) GST enzyme activity. (K) SOD enzyme activity. (L) POD enzyme activity. Error bars represent mean $\pm$ SD from three independent biological replicates. Asterisks indicate significant differences between the WT and transgenic plants. Data in panel (H) were analyzed using Student's *t*-test (* $P < 0.05$). All other data were analyzed by one-way ANOVA with Tukey's multiple comparisons test. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$).

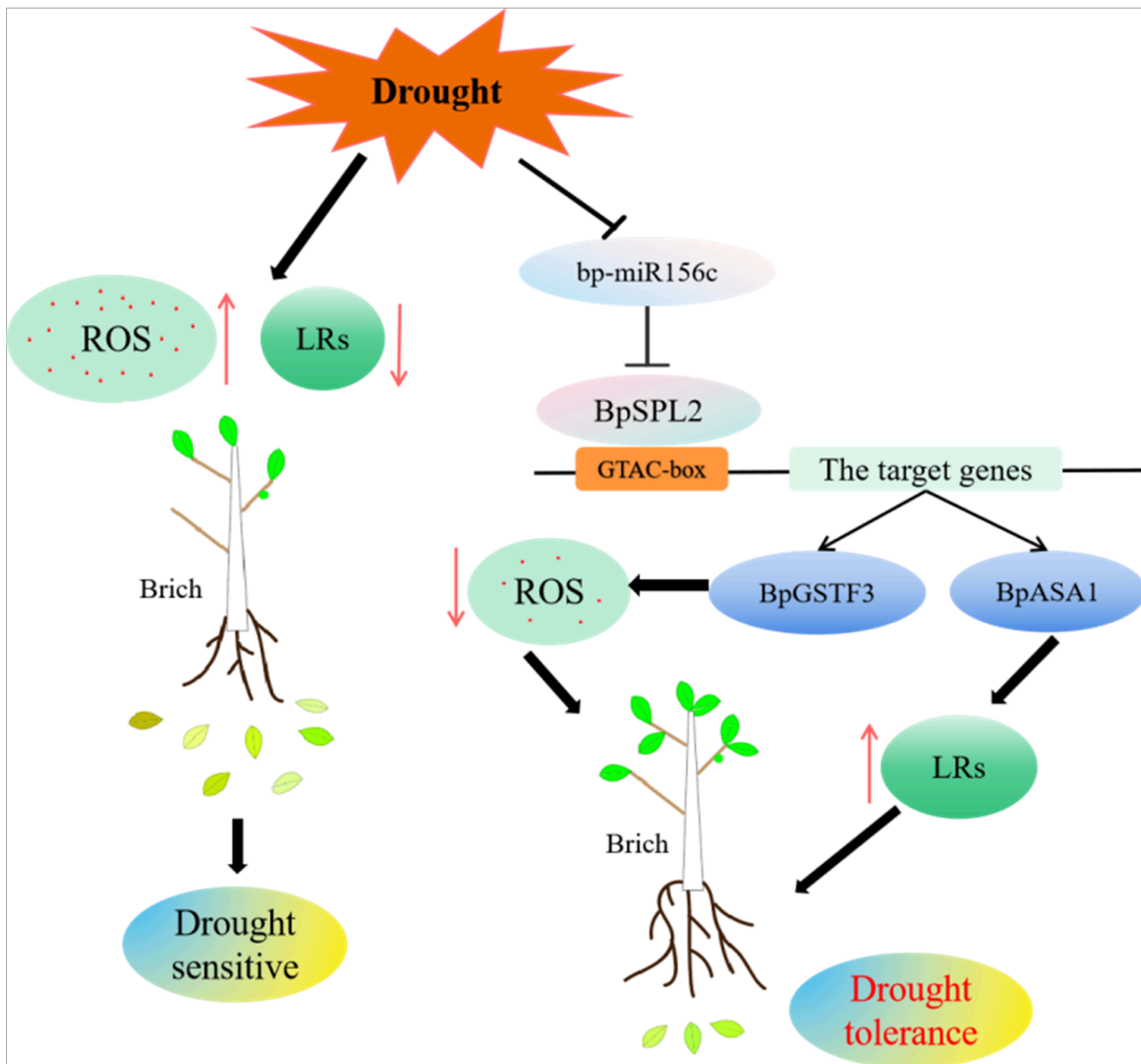


Figure 8

Working model of the *bp-miR156c/BpSPL2* module under drought conditions.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryMaterials.docx](#)