

Increased Absciscic Acid Sensitivity And Drought Stress By Overexpression of Absciscic Acid Receptors In *Arabidopsis Thaliana*

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Research Article

Keywords: ABA, PePYLs, ABA sensitivity, drought stress, transgenic Arabidopsis

Posted Date: July 15th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-703845/v1>

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Abstract

Absciscic acid (ABA) is a key plant hormone that regulates plant growth development and stress response. ABA is recognized and bound by ABA Receptor PYR/PYL/RCAR (referred to as PYLs). However, little is known about the PYLs gene family in *Populus euphratica*. Here, we identified 12 PYLs in *P. euphratica* and named PePYL1-12. Phylogenetic analysis divided the 12 PePYLs into three subfamilies. Subcellular localization showed that PePYL2, PePYL4, PePYL5, PePYL6, and PePYL9 were located in the cytoplasm and nucleus, PePYL10 localized in the nucleus. The promoter of 12 PePYLs contains hormones- and abiotic stress-related cis-acting elements. Moreover, ABA and drought significantly up-regulation the expression of *PePYL6* and *PePYL9*. To study the performance of PePYLs under ABA and drought stress, we generated transgenic *Arabidopsis* plants overexpressing *PePYL6* and *PePYL9*. Compared with wild type, transgenic *Arabidopsis* enhanced ABA sensitivity during seed germination and root growth, improved water use efficiency and drought resistance. Taken together, our results confirmed that PePYL6 and PePYL9 play a positive role in ABA-mediated stress responses in *P. euphratica*.

Introduction

The phytohormone absciscic acid (ABA) participates in growth and development, regulates plant resistance under adversity conditions (Finkelstein et al., 2002; Ton et al. 2009). Therefore, understanding ABA signaling is necessary to improve plant performance in the future. ABA signaling pathway core components include ABA receptors PYLs (Pyrabactin resistance1/PYR1-like/regulatory components of ABA receptor), PP2Cs (Clade A type 2C protein phosphatases), and SnRK2s (Sucrose nonfermenting-1 related protein kinase 2) (Ma et al., 2009; Park et al., 2009; Raghavendra et al., 2010). In the absence of ABA, PP2Cs stably bind to SnRK2s and inhibit SnRK2s activity (Melcher et al., 2009; Nishimura et al., 2009), in the presence of ABA, PP2Cs forms a stable complex with ABA and PYLs, relieves the inhibition of SnRK2s by PP2Cs, activated SnRK2s (mainly SnRK2.2, SnRK2.3, and SnRK2.6) phosphorylate downstream target genes, trigger the physiological response that depends on the ABA pathway (Fujita et al. 2013).

PYLs, sensing and binding ABA, belongs to the START (star-related lipid transfer) domain/Bet v 1-fold proteins superfamily, the proteins of this family have a ligand-binding pocket formed by the four conserved loops of CL1-CL4 that contribute to ABA signaling (Melcher et al., 2009; Park et al., 2009). In *Arabidopsis*, there are 14 AtPYLs. According to the oligomeric nature of the apo receptors, 14 AtPYLs can be divided into monomeric: AtPYL4-AtPYL12, dimeric: AtPYR1/AtPYL1/AtPYL2, AtPYL3 has two forms: monomeric or dimeric (Hao et al., 2011). Dimeric receptors are strictly ABA-dependent for their function, and monomeric receptors have different binding characteristics with ABA, and selectively interact with PP2Cs (Ma et al., 2009). 14 AtPYLs can also be divided into three subfamilies based on sequence similarity (Tischer et al. 2017). Since the discovery of the AtPYLs family in *Arabidopsis*, homologous genes of PYLs in other plants have been identified at genome-wide levels, including 6 PYLs in *sweet orange* (Romero et al. 2012), 8 PYLs in *grape* (Boneh et al. 2012), 13 PYLs in *rice* (He et al. 2014), 14

PYLs in *tomato* (González-Guzmán et al., 2014), 14 PYLs in *rubbertree* (Guo et al. 2017), 27 PYLs in *cotton* (Zhang et al. 2017), these PYLs are highly conserved in terrestrial plants.

Research on the PYLs gene family has been prevalent since their members have been demonstrated to participate in multiple physiological processes, such as seed dormancy and germination, seedling growth, stomata movement, fruit maturation, and so on (Hsu et al. 2021; Huang et al. 2019; Kim *et al.* 2004). In recent years, PYLs respond to abiotic stress, especially drought stress has been reported. In the liverwort *Marchantia polymorpha*, overexpression of *MpPYL1*, the transgenic plant showed ABA-hypersensitive growth with enhanced desiccation tolerance, while *Mppyl1* was generated by CRISPR-Cas9-mediated genome editing showed the opposite phenotype (Jahan et al., 2019). In *tomatoes*, overexpression of monomeric ABA receptors enhances drought resistance of transgenic *Arabidopsis* (González-Guzmán et al., 2014). However, how the performance of the PYLs family in wood plants are poorly understood.

Populus euphratica, as a large tree species with excellent adaptability to extreme temperature, drought, and salinity in the desert areas, has become a model woody plant to explore drought resistance mechanisms (Ma et al. 2013). In this study, we analyzed the 12 PePYLs, the cis-acting elements of PePYLs promoter showed more clearly the physiological processes that the PePYLs family may participate in, furthermore, we generated *PePYL6* and *PePYL9* transgenic *Arabidopsis*, by evaluating the performance of overexpression of *PePYL6* and *PePYL9* seedlings under drought stress, some valuable clues are provided for further investigation of the role of other PePYLs.

Materials And Methods

Plant materials and growth conditions

One-year-old seedlings of *P. euphratica* were transplanted in individual pots (10 L) containing sandy soil (approximately sand: soil = 7:3) in the nursery of Beijing Forestry University, Beijing, China (40°00'N, 116°20'E) was used in this study.

The *Arabidopsis thaliana* Col-0 was selected as the control. *pyl6* (SAIL_1179_D01) and *pyl9* (SALK_083621) were ordered from the Arabidopsis Biological Resource Center (<http://abrc.osu.edu/>). *Arabidopsis* seeds were sterilized with 75% ethanol for 10 min, and washed 5 times with distilled water. Seeds were sown on 1/2 Murashige and Skoog (MS) medium containing 3% sucrose and 0.6% agar, cultured at 4°C for 2 d, then transferred to a 22°C culture room under 16/8 light/dark cycle.

Identification of the PYLs gene family in *P. euphratica* genome

According to the amino acid sequence of 14 AtPYLs in the Arabidopsis genome, the PePYLs in the *P. euphratica* genome were found in the NCBI database (<https://www.ncbi.nlm.nih.gov/>). Using ExPASy website to analyze the amino acid number, isoelectric point (pI), and molecular weight (MW). The exon-

intron structure of PePYLs gene family was analyzed by GSDS software (GSDS 2.0: an upgraded gene feature visualization server).

Phylogenetic analysis of the PePYLs gene family

The PYLs amino acid sequences of *Arabidopsis* and *P. euphratica* were used to construct a phylogenetic tree. Multiple alignments of all protein sequences were analyzed by the neighbor-join (NJ) algorithm using MEGA 7 software. Accession numbers in Table S2.

Analysis of cis-acting elements in the promoter of the PePYLs gene family

The cis-elements of 2000 base pairs (bp) upstream of *PePYLs* start code (ATG) were analyzed using PlantCare Online (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>). The cis-acting elements in Table S3.

Subcellular localization

For subcellular localization of PePYLs in plants, GFP fusion proteins were observed using a laser confocal fluorescence microscopy (Leica TCS SP8).

Quantitative Real-Time qRT-PCR Analysis

Gene expression analysis in different tissues. *P. euphratica* seedlings about 50 cm high. Collect young leaves, adult leaves, old leaves, stems, and roots, and immediately immerse them in liquid nitrogen for tissue expression analysis. The total RNA was extracted by the CTAB method described in this article (Springer, 2010). Use ABI StepOnePlus Real-Time PCR System (ABI, Foster City, CA) according to the manufacturer's specifications to perform Quantitative Real-Time RT-PCR (qRT-PCR). *PeActin* and *AtActin* as internal control, to quantify the relative expression level of genes in samples.

GUS staining and activity assay

Histochemical GUS staining. Transgenic *Arabidopsis* were incubated in GUS staining solution at 37°C, and then 75% ethanol was used to remove chlorophyll. The activity of GUS was detected by the fluorescence of 4-methylumbelliferone (4-186 MU) produced from the β -glucuronidase substrate 4-methylumbelliferyl β -D187 glucuronide (Jefferson et al., 1987), and the protein concentration was quantified according to the previous protocol (Bradford, 1976).

Cloning of PePYLs gene and transformation of *Arabidopsis*

The cDNA of *PePYL6* and *PePYL9* were amplified by PCR using primers *PePYL6* F/R and *PePYL9* F/R. The primer sequences in Table S4.

To obtain *35S:PePYL6*, *35S:PePYL9*, *Pro_{PePYL6}::GUS*, and *Pro_{PePYL9}::GUS* transgenic plants, the 2000 bp promoter sequence of *PePYL6* and *PePYL9* was cloned into pCAMBIA-1391 vector, the cDNA of *PePYL6* and *PePYL9* were cloned into pBI121 vector, transformed into Col-0 plants by the floral dip method using *Agrobacterium tumefaciens* GV3101 (Bechtold et al. 2003). 1/2 MS medium supplemented with 30 mg/L hygromycin was used to identify the transgenic lines.

Germination and root length analysis

100 seeds of different lines were sown on 1/2 MS medium containing 0, 0.5, and 1.0 μM ABA each time, and the germination rate was counted once every 12 h to confirm the germination rate. The Col-0, transgenic, and mutant plants germinated were transplanted into 1/2 MS medium containing 0, 5, and 10 μM ABA was grown vertically for 10 d, and then the primary root length was measured.

Drought experiments

Drought stress experiments were conducted by controlling plant water in the greenhouse. The seedlings of the Col-0, transgenic and mutant plants were transplanted into the soil and watered for 15 d. One seedling was planted in each pot. After 4 weeks of growth, the Arabidopsis plants with the same growth were selected to carry out the water-withheld experiment for 8 d.

Physiological analysis

The net photosynthetic rate (P_n), transpiration rate (T_r) of the Col-0, transgenic, and mutant plants under the same conditions were measured by Li-Cor portable photosynthesis meter (LI-COR 6400) at an ambient CO_2 concentration of $500 \mu\text{mol mol}^{-1}$, the photosynthetic photon flux density of $800 \mu\text{mol m}^{-2} \text{s}^{-1}$, and a chamber temperature of 22°C . Instantaneous water use efficiency (iWUE) ($\text{iWUE} = P_n / T_r$).

Water loss analysis. The Col-0, transgenic, and mutant plants' detached rosette leaves were weighed immediately and incubated at room temperature. Losses of fresh weight in rosette leaves were weighted every half an hour until the weight remains constant. The percentage of initial fresh weight represents the water loss rate.

After 20 min of dark adaptation, the photosynthetic activity of leaves before and after the drought was monitored by PAM Chlorophyll Fluorometer (PAM100), and the maximum PSII quantum yield (F_v/F_m) value (reflecting the potential maximum light energy conversion efficiency) (Lian et al., 2018).

Remove one leaf from different genotypes each time, weigh the leaves' fresh weight, put it in distilled water for 3 h, wipe the surface water of the leaf, weigh it and record it as the leaf turgid weight (TW), then the leaves were dried to a constant weight at 65°C , reweighed to obtain the leaf dry weight (DW). The leaf $\text{RWC} = [(\text{FW}-\text{DW})/(\text{TW}-\text{DW})]\times 100\%$ (Wang et al., 2016).

The Proline content was measured with customized kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Statistical analysis

All data were analyzed with SPSS software, and Student's *t*-test ($*P \leq 0.05$; $**P \leq 0.01$) was used to test whether there was a significant difference between the measured dataset and the control dataset.

Results

Genome-wide identification and characterization of PePYLs in poplar

We identified 12 putative PYLs from the NCBI *P. euphratica* genome based on 14 AtPYLs amino acid sequences and named PePYL1-PePYL12 according to sequence similarity. The characteristics of PePYLs, including gene name, gene ID, coding DNA sequence (CDS) length, theoretical isoelectric point (pI), molecular weight (MW), and protein length are presented in (Table S1).

To investigate the phylogenetic relationship between PePYLs and AtPYLs, the Neighbor-Joining method was used and the tree was constructed using MEGA 7 (Fig. 1a). The Gene Structure Display Server (GSDS v2.0) further analyzed the exon-intron structure of the *PePYLs* and *AtPYLs* gene (Fig. 1b). The conserved domain of the PePYLs was analyzed by DNAMAN software. The results showed that 12 PePYLs proteins can be divided into three subfamilies, among them, PePYL7 to PePYL11 belong to subfamily I; PePYL3 to PePYL6 belong to subfamily II; PePYL1, PePYL2, and PePYL12 belong to subfamily III. The amino acid sequences of 12 PePYLs all contained four highly conserved surface loops CL1-CL4 (Fig. 1c).

The promoter cis-acting elements analysis of PePYLs in poplar

To study the potential functions of PePYLs, the cis-acting elements on the *PePYLs* promoter sequence were analyzed by PlantCARE online (Table S3). The results showed that the 12 *PePYLs* promoter sequences mainly include five hormone response-related elements (Fig. 2): abscisic acid-responsive element, MeJA-responsive element, salicylic acid-responsive element, auxin-responsive elements and, gibberellin-responsive elements; three abiotic stress-related elements: defense and stress-responsive elements, low-temperature-responsive element and MYB binding site involved in drought-inducibility. Some *PePYLs* promoters contain elements related to growth and development, such as, circadian elements, which are involved in circadian control; MBSI elements, which are MYB binding sites involved in the regulation of flavonoid biosynthetic genes; HD-Zip 1 elements, which regulate the differentiation of the palisade mesophyll cells. In summary, *PePYLs* with different cis-acting elements may be involved in a variety of physiological processes.

Subcellular localization of PePYLs

To clarify the localization of PePYLs in cells, 35S:PePYLs-GFP (green fluorescent protein) fusion protein was transiently transfected into tobacco leaves. The results showed that: PePYL2, PePYL4, PePYL5, PePYL6, and PePYL9 are located in the cytoplasm and nucleus, the fluorescence signals of PePYL10 and PePYL12 were weak, among which PePYL10 is more obvious in the nucleus, while PePYL1, PePYL3,

PePYL7, PePYL8, and PePYL11 did not detect the fluorescence signal, and its localization in cells cannot be determined (Fig. 3).

The expression patterns of PePYL6 and PePYL9

The expression of *PePYL6* and *PePYL9* was detected in different tissues (Fig. 4a, d). Previous studies showed that the transcription levels of *PePYL6* and *PePYL9* were up-regulated under ABA and mannitol treatment. To further determine the expression patterns of *PePYL6* and *PePYL9*, we generated *Arabidopsis* plants with GUS driven by *PePYL6* and *PePYL9* promoter. The results showed that ABA and mannitol treatment enhanced β -glucuronidase (GUS) staining (Fig. 4b, e), GUS activity further confirmed GUS staining (Fig. 4c, f), *PePYL6* and *PePYL9* were induced by ABA and mannitol.

To further investigate the performance of the PYLs gene in response to drought stress, *PePYL6* and *PePYL9* were stably transformed into *Arabidopsis*. Col-0, *pyl6* and *pyl9* were used in this study. Transgenic *Arabidopsis* was verified by PCR and qRT-PCR, the high expression levels plants were selected for experiments (Fig. 4g-i) (Czechowski et al., 2004).

Overexpression of PePYLs increases ABA sensitivity of seed germination and root growth

As a potential ortholog of *AtPYLs*, the function of *PePYLs* in the process of seed germination and root growth regulated by ABA has been studied. The inhibition of seed germination by exogenous ABA changed with the concentration of ABA, and the higher ABA concentration, the lower seed germination rate (Fig. 5a). On the 1/2 MS medium, the Col-0, *pyl6*, and *pyl9* plants germinated normally, while OEPePYLs plants showed delayed germination (Fig. 5b). On the medium supplemented with 0.5 μ M ABA, the germination rates of Col-0, *pyl6*, and *pyl9* plants were comparable, OEPePYLs plants were lower than those of Col-0 and mutant plants (Fig. 5c). After 24 h of growth on the medium supplemented with 1.0 μ M ABA, the germination of OEPePYLs plants was about 15.35%, while the germination of Col-0 plants was 55.95%, and the difference of germination rate between Col-0 and OEPePYLs plants was significant with time (Fig. 5d). Therefore, overexpression of *PePYL6* and *PePYL9* increased the ABA sensitivity during seed germination, even without ABA treatment.

Root length is another phenotype for assessing ABA sensitivity under exogenous ABA treatment (Fig. 6a). The results showed the average root length of Col-0 plants decreased from 4.56 cm to 2.15 cm when grown on the 1/2 MS medium supplemented with 0, 5 and 10 μ M ABA, while OEPePYLs plants root length decreased overall more than Col-0 plants, such as OEPePYL6 plants root length decreased from 4.21 cm to 1.23 cm, the root length of OEPePYL9 plants decreased as much as that of OEPePYL6 plants, and when OEPePYL9 plants on the medium supplemented with 10 μ M ABA, the average root length of OEPePYL9 plants was only 0.95 cm. In conclusion, overexpression of *PePYL6* and *PePYL9* increased the ABA sensitivity during root growth (Fig. 6b-d).

Overexpression of PePYLs improves the drought resistance of transgenic Arabidopsis

It has been reported that increasing ABA sensitivity enhances the drought resistance of plants (He et al., 2018). To investigate whether overexpression of *PePYL6* and *PePYL9* increases ABA sensitivity affects drought resistance of transgenic *Arabidopsis*. Col-0, OE*PePYLs*, and mutant plants were transplanted into the soil for 8 d without water. Before the drought stress, the phenotypes of Col-0, OE*PePYLs*, and mutant plants were not significantly different. After the drought stress, Col-0 and mutant plants showed more severe wilt, however, leaves of OE*PePYLs* plants did not wither, remained green (Fig. 7a). The physiological analysis showed that OE*PePYLs* plants had a higher net photosynthetic rate (P_n) than the Col-0 and mutant plants, mutant plants had the lowest net photosynthetic rate (Fig. 7b), the transpiration rate (T_r) of OE*PePYLs* transgenic plants were lower than that of Col-0 and mutant plants (Fig. 7c), which resulted in higher instantaneous water use efficiency (iWUE) ($iWUE = P_n / T_r$) of OE*PePYLs* transgenic plants under the same conditions (Fig. 7d).

The survive rate, water loss, leaf RWC, and Maximal PSII quantum yield (F_v/F_m) of all lines before and after the drought were measured. The survive rate of all lines was 95%-97% under the control condition, however, the survive rate of OE*PePYLs* plants was 70%-85%, higher than the Col-0 and mutant plants (Fig. 7e). The data of water loss of OE*PePYLs* plants showed that transgenic plants were lower than that of Col-0 and mutant plants (Fig. 7f), and the RWC and F_v/F_m values were also higher than other plants (Fig. 7g, h) under the drought stress. The RWC of Col-0 plants decreased from 82.38–53.24%, the RWC of *pyl6* and *pyl9* mutant plants was similar to that of Col-0 plants, while RWC of OE*PePYLs* plants decreased slightly. Drought-induced proline accumulation can stabilize the metabolic process of the plant under adverse conditions (Szabados and Saviouré, 2010). After the drought stress, OE*PePYLs* plants had higher proline content than that of Col-0 and mutant plants (Fig. 7i). Therefore, overexpression of *PePYL6* and *PePYL9* enhances drought resistance of transgenic plants.

Overexpression *PePYL6* and *PePYL9* altered the expression of downstream genes

To study whether overexpression of *PePYL6* and *PePYL9* enhanced the drought tolerance of transgenic *Arabidopsis* by regulating the expression of downstream stress-related genes, we compared the expression of *ABF2*, *RAB18*, *P5CS1*, *RD29A*, and *RD29B* genes (Cutler et al., 2010) previously reported to be induced by drought and ABA in Col-0 and OE*PePYLs* plants under water and withholding water conditions. The data showed that the expression of all these genes in the transgenic plants was significantly higher than those of the Col-0 plants (Fig. 8), which suggested that *PePYLs* overexpressed altered the expression patterns of downstream stress-related genes and thus contributed to the enhancement of drought tolerance of transgenic plants.

Discussion

ABA regulates plant growth development and stress response. PYLs, as the ABA signaling pathway core regulatory component, play a major role in ABA perception and signal transduction (Cutler et al., 2010). In this study, a total of 12 *PePYLs* were identified in poplar. Amino acid sequence analysis showed that 12 *PePYLs* contained the conserved loops of the PYLs family, among them, CL2 and CL3 represent

conserved gate and latch domains (Fig. 1c), which are important for ABA signal transduction (Melcher et al., 2009). The structure of exon-intron in *PePYLs* gene was similar to *AtPYLs* (Fig. 1b), and phylogenetic analysis further confirmed that the *PePYLs* could be grouped into three subfamilies (Fig. 1a) (Tischer et al., 2017). Thus, the function of *PYLs* gene in the same subfamily in *Arabidopsis* and *P. euphratica* may be conserved. Given that many *AtPYLs*' functions have been reported in *Arabidopsis*, the potential function of *PePYLs* in poplars can be better investigated.

The cis-acting elements of *PePYLs* promoters have been analyzed. ABA, MeJA, SA, GA, and auxin five hormone-responsive elements were found in the 12 *PePYLs* promoter regions, and most *PePYLs* promoter regions existed one or more hormone-related elements (Fig. 2), suggesting that *PePYLs* may be involved in the hormone crosstalk pathway. Previous studies reported that *NtPYL4* (*Nicotiana tabacum*) is involved in jasmonate signaling transduction and regulates metabolic reprogramming in *Arabidopsis* and *tobacco* to balance growth and defense processes (Lackman et al., 2011), *AtPYL8* directly interact with *MYB77* to enhance the *MYB77*-dependent transcription of auxin-responsive genes (Zhao et al., 2014). Therefore, understanding the existence of cis-acting elements of the *PYLs* gene promoter will be helpful to further study the functional characteristics of the *PYLs* gene in *poplar* or other species.

The expression of *PePYL6* and *PePYL9* were different in leaves, stems, and roots contribute to understanding the characteristics of these genes in depth. *PePYL6* was expressed abundantly in mature leaves, *PePYL9* was mainly expressed in leaves and roots (Fig. 4a, d), the tissue expression of the gene may be closely related to its function. It has been reported that the *pRD29A::PYL9* transgenic *Arabidopsis* and rice promote the senescence of old leaves by inducing the expression of senescence-related genes through ABA-responsive element-binding factors (ABFs) and Related to ABA-Insensitive 3/VP1 (RAV1) transcription factors (Zhao et al., 2016), the *pyl8-pyl9* double mutant significantly reduced ABA sensitivity to primary root growth and lateral root formation, while the overexpression of *PYL8* and *PYL9* restore lateral roots by directly interacting with *MYB77* and *MYB44* under ABA treatment (Xing et al., 2016). Therefore, the tissue expression of genes helps to explore genes' function accurately.

We generated *PePYL6* and *PePYL9* transgenic *Arabidopsis* to gain insight into the performance of the *PYLs* gene. Our study showed that overexpression of *PePYL6* and *PePYL9* increased ABA sensitivity during seed germination and root length (Fig. 5, 6). Previous reports have shown that the *PYLs* gene plays an important role in the perception of ABA during seed germination and root growth. The sextuple mutant *pyr1/pyl1/pyl2/pyl4/pyl5/pyl8* exhibited ABA-insensitive phenotype and reduced seed yield (Gonzalez-Guzman et al., 2012), 35S:*PYL4*^{A194T} transgenic plants were more sensitive to ABA during seed germination and seedling growth than 35S:*PYL4* plants (Pizzio et al., 2013). Our results were consistent with those reported, further suggested that the *PYLs* are a positive regulator of ABA signaling.

Since ABA perception and signaling pathways also regulate the response to abiotic stress, so many *PYLs* gene overexpressed has been reported to enhance plant tolerance to abiotic stress. Such as overexpression of *rice OsPYL5* (Kim et al., 2014) and *Artemisia annua* L *AaPYL9* enhanced drought resistance of transgenic plants (Zhang et al., 2013), ectopic expression of *OsPYL3* enhanced cold

tolerance in *Arabidopsis* (Lenka et al., 2018). Transgenic *Arabidopsis* with *PePYL6* and *PePYL9* promoter-driven GUS expression showed deeper GUS staining after ABA and mannitol treatment, supported that *PePYL6* and *PePYL9* may be involved in drought stress (Fig. 4b, e). Our data further confirmed that overexpression of *PePYL6* and *PePYL9* enhanced drought resistance in transgenic *Arabidopsis* by improving WUE. Under the same conditions, the phenotypes of OE*PePYLs*, Col-0, and mutant plants were not significantly different (Fig. 7a), the CO₂ assimilation rates of OE*PePYLs* plants were higher than Col-0 and mutant plants (Fig. 7b), the transpiration of OE*PePYLs* plants were lower than other plants (Fig. 7c), which led to higher WUE of transgenic *Arabidopsis* (Fig. 7d). It is well known that the WUE of C₃ photosynthetic plants varies with water availability, and WUE increases when water is deficient (Medrano et al., 2002). Therefore, after the drought stress, OE*PePYLs* plants remained green and turgid, Col-0 and mutant lines wilting, transgenic plants were more drought-resistant (Fig. 7). The same conclusions of improving WUE confer drought stress have been reported in the overexpression of TaPYL4 in wheat (Usman et al., 2020).

Generally, the drought resistance of plants was negatively correlated with the water loss of detached leaves (Gupta et al., 2020), the lower water loss rate in OE*PePYLs* plants, and the more drought resistance in transgenic plants confirmed this view (Fig. 7f). The maximum PSII quantum yield (F_v/F_m), indicated the potential maximum light energy conversion efficiency, which was between 0.8 and 0.85 without stress, when plants were subjected to environmental stress, the value decreased (Zhou et al., 2019), OE*PePYLs* plants showed higher F_v/F_m values, suggesting that transgenic plants were less damaged under drought stress (Fig. 7h). The RWC and proline content of the OE*PePYLs* plants were also higher than those of Col-0 plants (Fig. 7g), and the high RWC ensured that the photosystem II could still transport electrons properly and maintain normal CO₂ absorption in the transgenic *Arabidopsis* under severe water stress, the high proline content has a positive effect on regulating protein and antioxidant enzyme stability, ROS scavenging, and the balance of intracellular redox homeostasis (Fig. 7i) (Szabados and Savouré, 2010). More importantly, the up-regulation expression of downstream stress-related genes by *PePYL6* and *PePYL9* also ensured that the transgenic plants suffered less damage under drought stress. (Fig. 8), Therefore, OE*PePYLs* plants were better adaptable to drought stress.

In conclusion, a total of 12 *PePYLs* homologous genes were identified in poplar, and overexpression of *PePYL6* and *PePYL9* increased ABA sensitivity, improved WUE, and enhanced drought resistance of transgenic *Arabidopsis*, which laid a foundation for further study on biological functions of other *PePYLs* genes in poplar.

Declarations

Acknowledgments

We are very grateful for the support of grants from the National Natural Science Foundation of China (32071734 and 31770649).

Funding

This research was supported by grants from the National Natural Science Foundation of China (32071734 and 31770649).

Authors' contributions

QL, QQT, WLY, and XLX conceived and designed the research. QL and QQT performed the research. YZ, MXN, XQY and LCL participated in the experiments. MXN, XQY and LCL analyzed the experimental data. QL wrote the manuscript. QQT, and CL contributed to writing the manuscript. All authors discussed the results and approved the final manuscript.

Conflicts of interest

Authors declare no competing financial interests.

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Figures

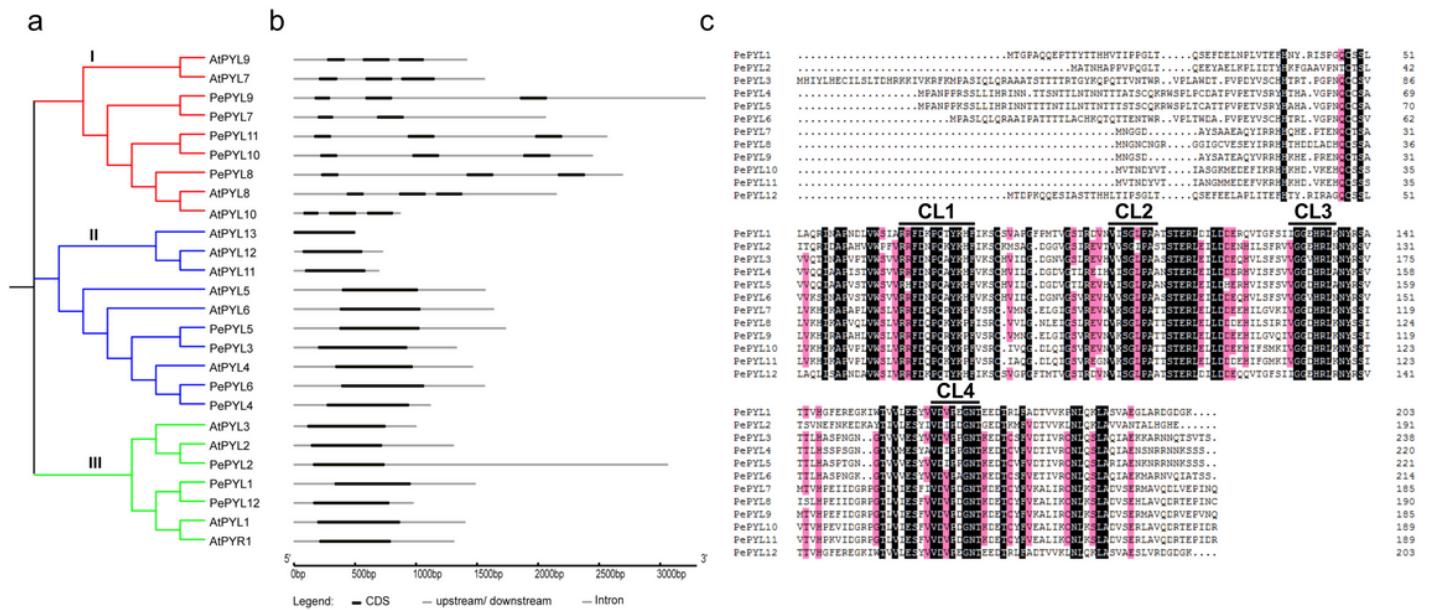


Figure 1

Molecular characterization of PePYLs in *P. euphratica*. (a) Phylogenetic analysis of 12 PePYLs and 14 AtPYLs. The PYLs protein is classified into three subfamilies: I, II, and III. (b) The exon-intron structure of PePYLs and AtPYLs. The lengths of the exons and introns for each PYLs gene can be calculated following the scale at the bottom. (c) Four conserved loops sequence of 12 PePYLs.

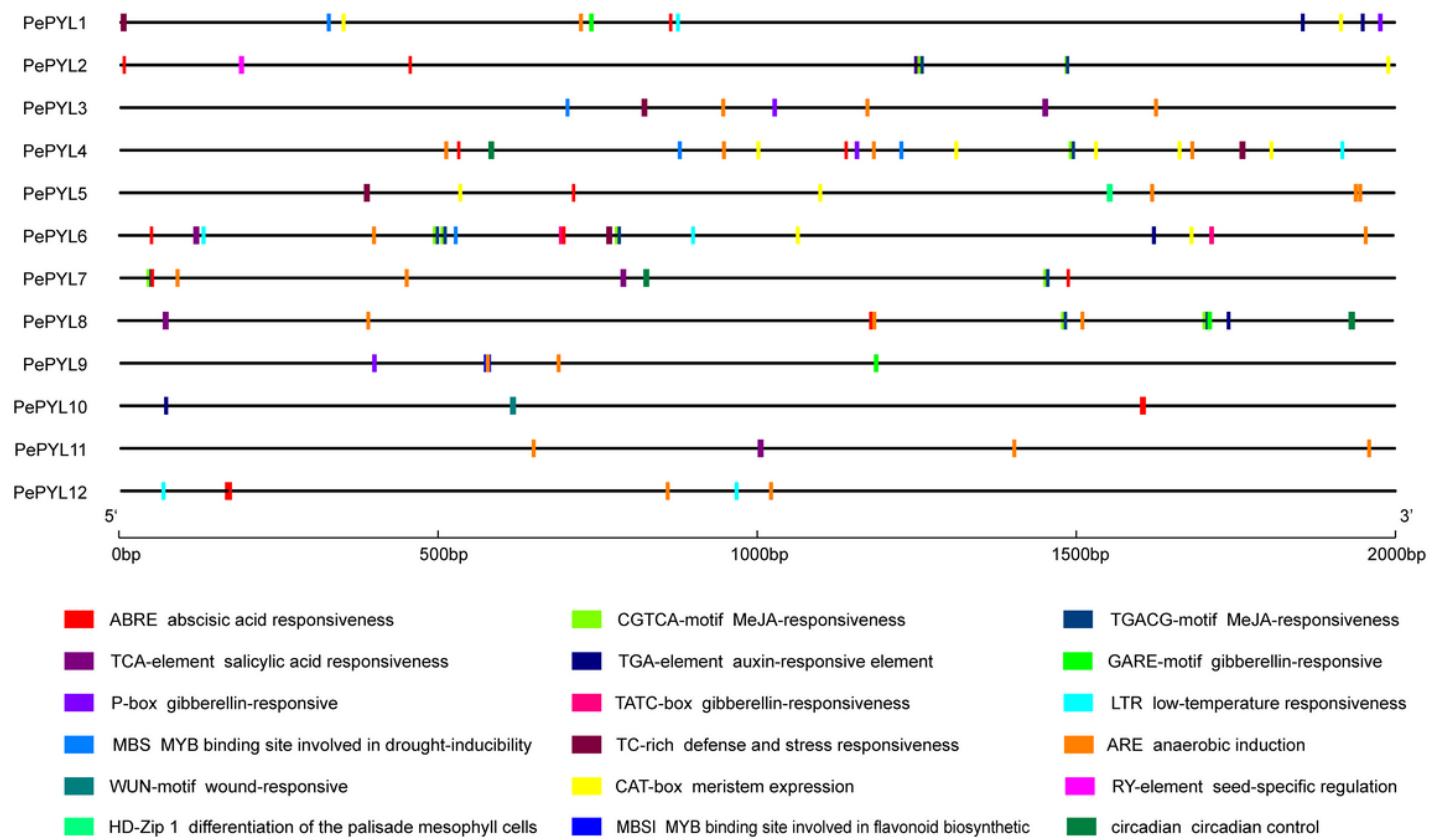


Figure 2

The cis-acting elements analysis of PePYLs promoter. The 2000 bp promoter sequences of 12 PePYLs were analyzed by PlantCARE. Positional distribution of predicted cis-acting elements are shown as vertical bars, each color represents an individual cis-acting element.

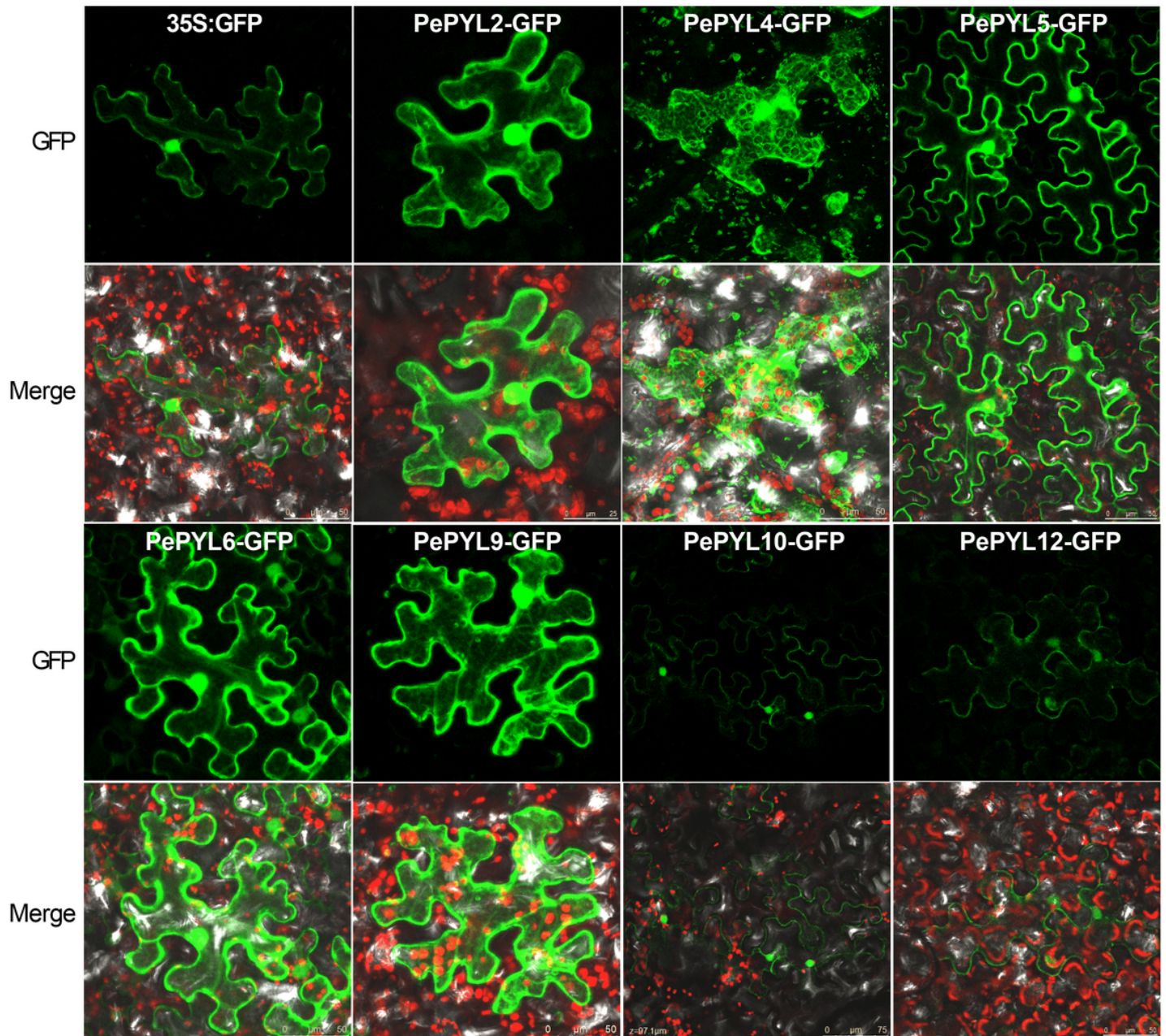


Figure 3

The subcellular localization of 35S:PePYLs-GFP and control 35S:GFP expression in transiently expressed tobacco leaves. GFP: GFP Field, Merge: overlap image of chloroplast field and GFP field. Bars = 75 μ m.

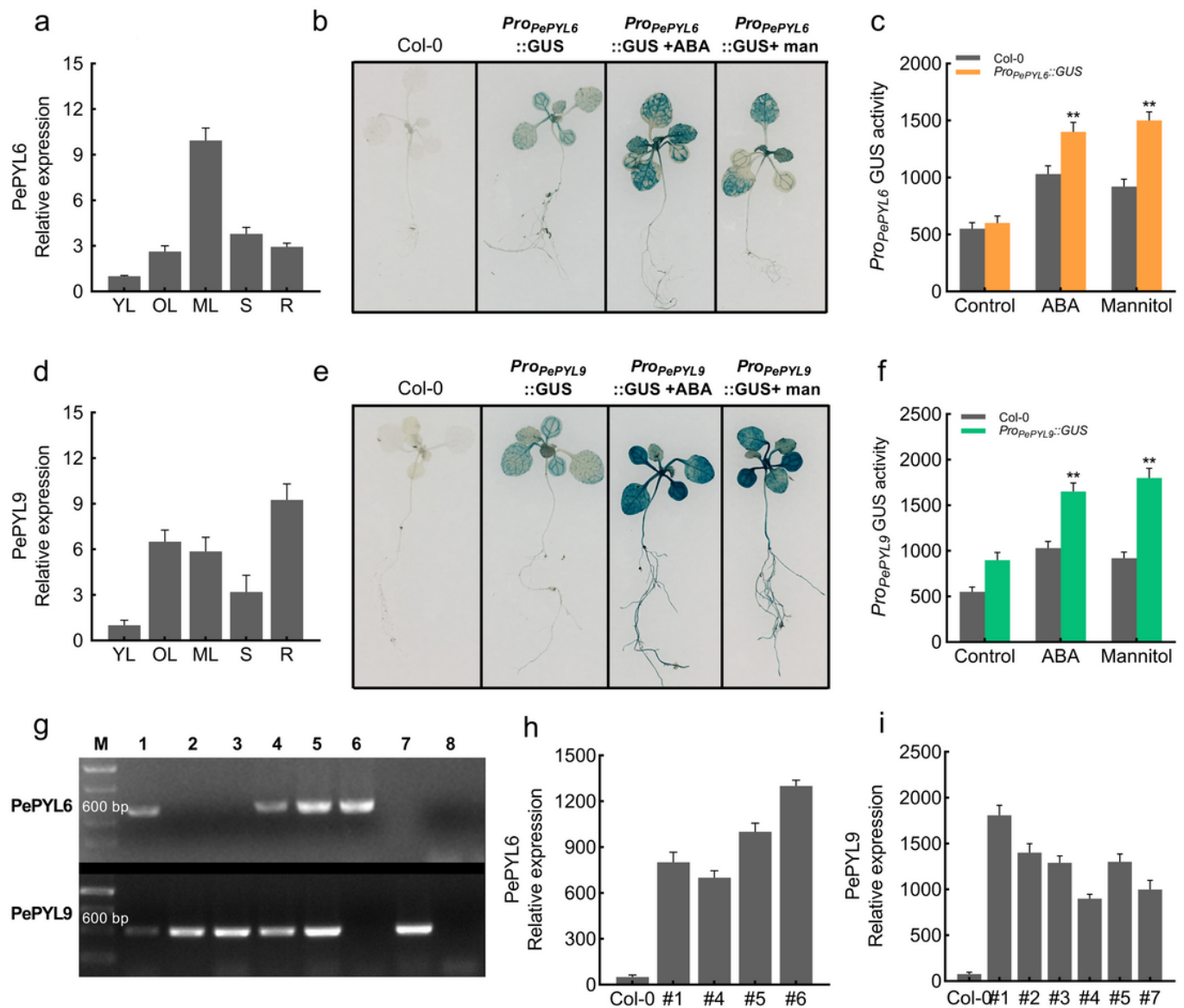


Figure 4

PePYL6 and *PePYL9* expression patterns in different tissues, ABA, and mannitol treatments. (a) The expression of the *PePYL6* in different tissues, YL: young leaves, AL: adult leaves, OL: old leaves, S: stems, R: roots. (b) β -glucuronidase (GUS) staining of *ProPePYL6::GUS* transgenic Arabidopsis seedling under normal, ABA, and mannitol treatment. (c) GUS activity of *ProPePYL6*. (d) The expression of the *PePYL9* in different tissues. (e) GUS staining of *ProPePYL9::GUS* transgenic Arabidopsis seedling under normal, ABA, and mannitol treatments. (f) GUS activity of *ProPePYL9*. (g) PCR confirmation of transgenic plants. M, λ -EcoT14 I digest DNA markers; 1-8: OEPePYL6 and OEPePYL9 plants obtained by transformed *PePYL6* and *PePYL9* into Col-0 plants, respectively. (h) qRT-PCR analysis expression levels of *PePYL6*, (i) and *PePYL9* in different transgenic lines. Error bars are means $\pm$ SE ($n = 5$). Asterisks denote significant differences: * $P \leq 0.05$, ** $P \leq 0.01$.

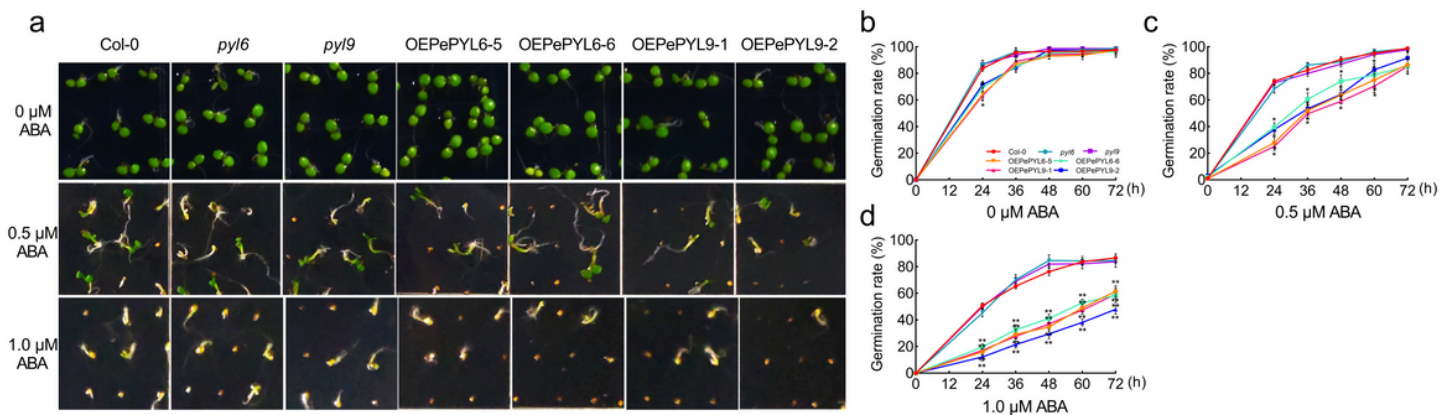


Figure 5

Overexpression of PePYL6 and PePYL9 in Arabidopsis increased the ABA sensitivity during seed germination. (a) The phenotype of seed germination on 1/2 MS medium supplemented with 0, 0.5, 1.0 μ M ABA. (b) Time course of seed germination with 0 μ M ABA, (c) 0.5 μ M ABA, (d) 1.0 μ M ABA. Error bars are means $\pm$ SE (n = 50). Asterisks denote significant differences: *P $\leq$ 0.05, **P $\leq$ 0.01.

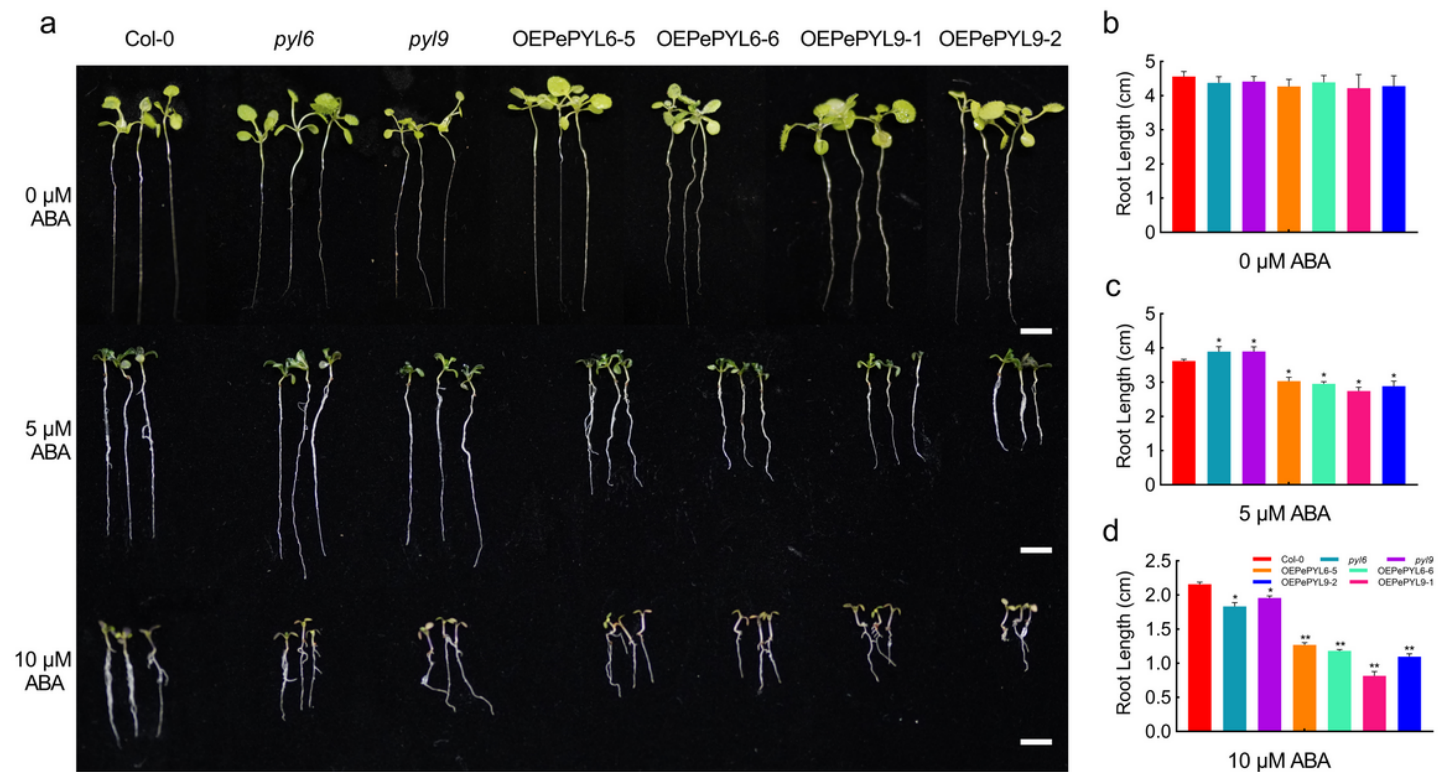


Figure 6

Overexpression of PePYL6 and PePYL9 in Arabidopsis increased the ABA sensitivity during root length. (a) Morphological comparisons of the primary root length on 1/2 MS medium supplemented with 0, 5, 10 μ M ABA for 10 d. (b) The difference in the primary root length with 0 μ M ABA, (c) 5 μ M ABA, (d) 10 μ M

ABA. Bar = 0.5 cm. Error bars are means $\pm$ SE (n = 50). Asterisks denote significant differences: *P $\leq$ 0.05, **P $\leq$ 0.01.

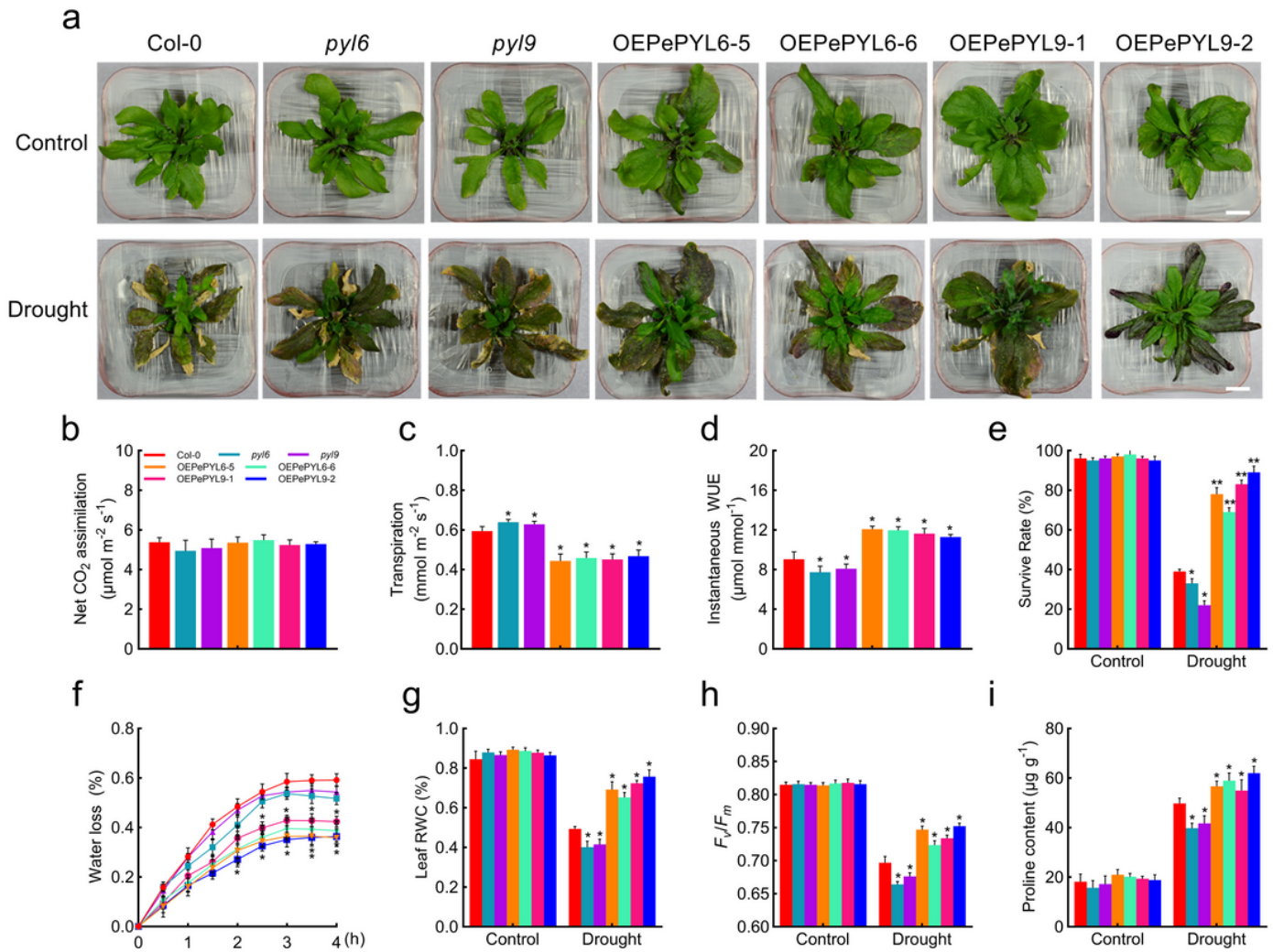


Figure 7

Overexpression of PePYL6 and PePYL9 confers drought resistance in Arabidopsis. (a) Morphological differences in drought experiments. The seedlings were grown in soil for 15 d under well-watered conditions, thereafter, water was withheld for 8 d. (b) Net photosynthetic rate (Pn), (c) transpiration rate (Tr), (d) instantaneous WUE (iWUE) of seedlings under the same conditions. (e) Survive rate, (f) water loss, (g) leaf relative water content (RWC), (h) maximal PSII quantum yield (F_v/F_m), (i) the content of Proline of all plants before and after the drought stress. Error bars are means $\pm$ SE (n = 50). Bar = 1.0 cm. Asterisks denote significant differences: *P $\leq$ 0.05, **P $\leq$ 0.01.

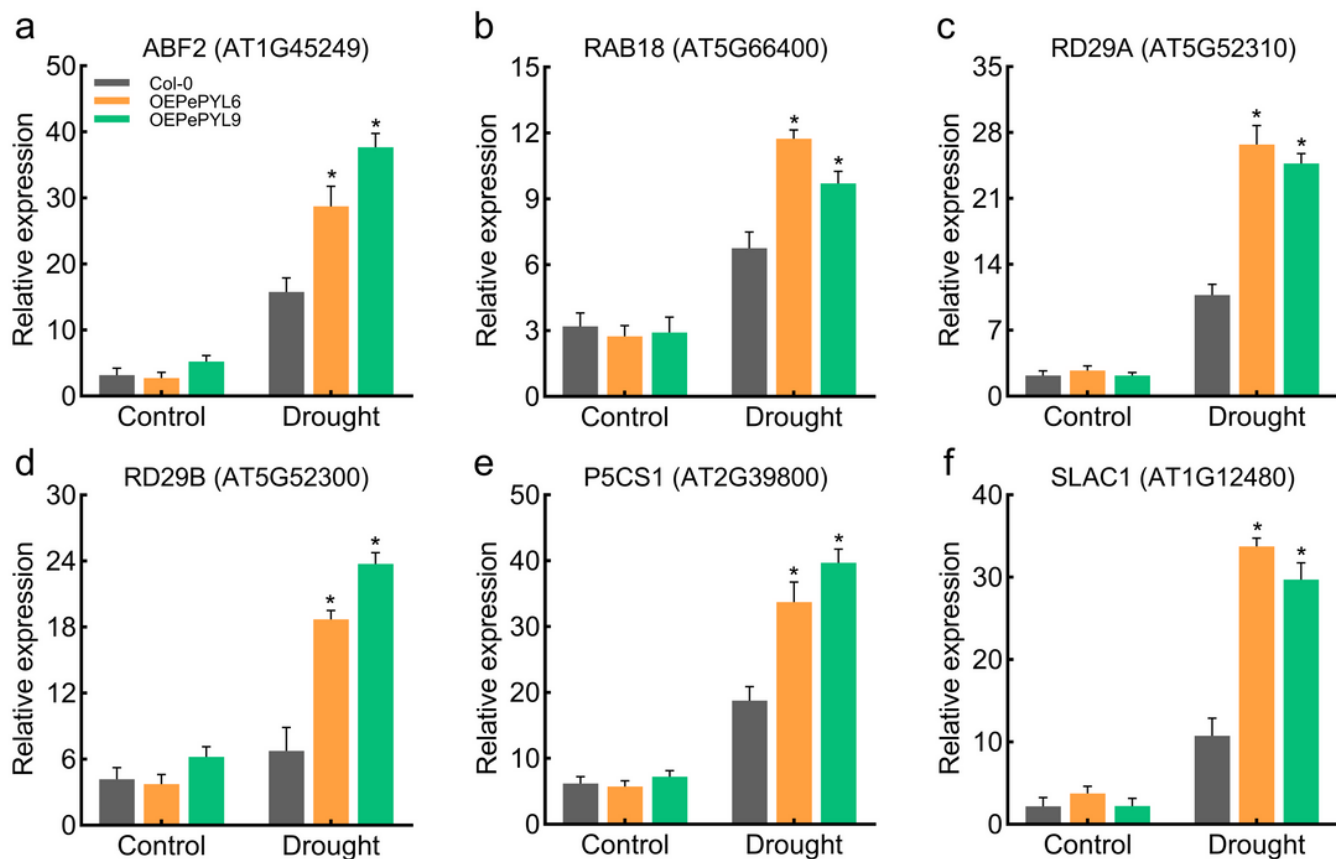


Figure 8

Stress-responsive genes expressed in Col-0 and OEPePYLs plants under water and withholding water conditions. Relative expression levels of (a) ABF2, (b) RAB18, (c) RD29A, (d) RD29B, (e) P5CS1, and (f) SLAC1 in Col-0 and OEPePYLs plants under water and withholding water conditions. Error bars are means $\pm$ SE ($n = 5$). Asterisks denote significant differences: * $P \leq 0.05$, ** $P \leq 0.01$.

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