Indole -3-acetic acid improves drought tolerance of white clover associated with activating auxin-related genes, abscisic acid and jasmonic acid-induced stress responsive transcription factors, and inhibiting senescence genes

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Abstract

Auxin plays an important role in regulating plant development. To aquire feasible regulation effects and mechanism of IAA on drought tolerance in white clover, relative water content, chlorophyll content, several major phytohormone level, the expression of gene-related to IAA signal perception and synthesis, as well as drought-resistance related transcription factor genes and senescence genes were investigated in white clover plants under 15% polyethylene glycol-6000 (PEG-6000) after 1μM exogenous IAA pre-treatment and 100μM L-AOPP (inhibitor of IAA synthesis) pre-treatment. Compared to control, drought stress significantly diminished stem dry weight (g/10 plants), relative water content (RWC, %) and total chlorophyll content (Chl, mg/g). Exogenous IAA significantly increased RWC and Chl, however, L-AOPP, drastically decreased stem dry weight (g/10 plants), RWC and Chl compared with direct drought treatment. Besides that, compared with direct drought treatment, exogenous IAA significantly improved ABA content and JA content, up-regulated expression level of auxin-responsing genes (GH3.1, GH3.9, IAA8), drought-resistance related transcription factor genes (bZIP11, DREB2, MYB14, MYB48, WRKY2, WRKY56, WRKY108715) and drought resistance gene (RD22), meanwhile, down-regulated expression level of auxin-responsing genes (GH3.3, GH3.6, IAA27) and leaf senescence genes (SAG101 and SAG102). L-AOPP, contrarily, compared with direct drought treatment, significantly reduced ABA, GA3 and JA content, down-regulated expression level of GH3.1, GH3.9, IAA8, bZIP11, DREB2, MYB14, MYB48, WRKY2, WRKY56, WRKY108715, ERD and RD22, meanwhile, up-regulated SAG101 and SAG102. Collectively, these data suggested a positive role of exogenous IAA in alleviating drought stress damage in white clover.

Introduction

Drought continuously attracted researchers' concern partly because of its influence in restricting plant growth and distribution worldwide [1, 2, 3]. Adaptability, capability of being or becoming adapted, would give rise to some biochemical and physiological changes to confer plants with improved survival rate in arid environments [4, 5, 6, 7]. In essence, these changes are results of expression of direct or indirect related drought responsive genes, including early-response to dehydration (*ERD*) stress genes and resoponse to dehydration (*RD*) stress genes which are closely related with plant senescence[8, 9] and senescence-associated genes (*SAG*) which regulate expression of some genes related to chlorophyll degradation and cytoplasm damage[10]. Also, it was reported that transcriptional activation of transcriptional factors (*TFs*), such as basic region/leucine-zipper motif (*bZIP*), dehydration responsive element binding (*DREB*), myeloblastosis (*MYB*) and *WRKY*, could be influenced by drought and regulate plant physiological state.

Futhermore, genes expression are also regulated by hormones, including IAA, CTK, ABA, GA, JA and SA. Among them, IAA, regulating many processes of plant growth and development [1, 4, 5, 6, 11], is one of the main naturally occurring phytohormones in plants. Recently, accumulating evidences also indicated possible linkages between auxin and abiotic stresses, even though crosstalks between auxin and other major phytohormones [12, 13, 14, 15, 16, 17, 18].

Besides that, auxin stimulates RNA synthesis, regulating gene expression response to auxin [19, 20, 21, 22, 23], which depends on DNA [24, 25]. In previous studies, auxin response transcription factor (*ARF*) family could mediate roles of IAA during plant growth [26]. Similarly, TFs could be regulated by one and/or several of phytohormones [27, 28, 29].

White clover (*Trifolium repens*), one legume plant, is one of important forages for many domesticated animals in the world due to its high yield, good quality and rich nutritional value, and thus is planted in large quantities. However, white clover is susceptible to drought stress and exhibits significant changes in leaf senescence when confronting to dehydration stimulation [30]. However, there are few detailed studies about IAA's effects on dehydration in white clover, from phytohormones to TFs, from IAA responsive genes to senescence genes. In order to explore biochemical and physiological changes of white clover in drought stress and changes caused by IAA addition, we investigated contents of six major phytohormones (including IAA itself)、expression of ARF gene, *GH3* family genes (belonging to auxin-responsive gene), IAA repsonse gene, *TFs* genes, *ERD* gene and *RD* gene, *SAG* genes in white clover under drought stress, with the purpose of provding some possible perspectives of the varied adaptations of white clover in dehydration. And we formulate hypotheses that exogenous IAA improved drouhgt resistance in white clover through several biochemical and physiological changes, ranging from contents of several phytohormones, to expressions of auxin-responsive genes, some *TFs* genes and senescence genes. These changes confer a better appreance of white clover and reinforce adaptability of it in drought.

Materials And Methods

Plant material and growth condition

The seeds of 'Pixie' (*Trifolium repens cv.*) were purchased from Beijing Mammoth Seed Industry Company in China. The seeds were sterilized with 1% (W/V) sodium hypochlorite solution for 3 min and then rinsed with sterile water for 3 times, ~0.8 g of seeds were evenly spread in culture pot (9 cm depth, 18 cm width, 24 cm length) containing sterilized quartz sand, then placed in the growth chamber (23℃/16 h day and 19℃/ 8 h night cycle, irradiance of about 300μmol quanta·m^{-2}·s^{-1} , 75% relative humidity). 25 mL of deionized water was added to each pot in every day. After germination, instead of deionized water, Hoagland's nutrition solution was used. In order to minimize the environmental effects, the positions of the pots in the culture chamber were also daily replaced.

Experimental design

When the second leaf of white clover fully unfolded, seedlings were subjected to four sets: (1) Control, Hoagland's nutrition solution was used as previously; (2) PEG-6000 treatment (labeled with D), Hoagland's nutrition solution with 15% PEG-6000 (W/V, -0.3Mpa); (3) IAA pre-treatment and PEG-6000 treatment (labeled with IAA+D), Hoagland's nutrition solution with 1 μM IAA for the first 7-day, and then Hoagland's nutrition solution with 15% PEG-6000 for the subsequent days; (4) L-AOPP pre-treatment and

PEG-6000 treatment (labeled with L-AOPP+D), Hoagland's nutrition solution with 100 μM L-AOPP for the first 7-day, and then Hoagland's nutrition solution with 15% PEG-6000 for the last days. All plants were simultaneously treated with PEG-6000. Each treatment had 4 biological replicates. Samples were collected on 0 d, 7 d and 14 d after adding PEG-6000.

Physiological measurements

Relative water content (RWC), stem dry weight and Total Chlorophyll content (Chl)

For RWC, approximately 0.2 g of leaves (FW) were wrapped with clean gauze and then dipped into distilled water. After 24 hours at 4 ℃, weighed them as saturated weight (SW). Samples were placed in kraft paper bags, kept in an oven at 105 ℃ for 30 min, then at 75 ℃ for 48 h, and weigh them as dry weight (DW). RWC was calculated through formula [31]: RWC(%)=(FW-DW)/(SW-DW)×100%

For total chlorophyll content, approximately 0.1 g of leaves (FW) were extracted with 10 mL of mixed solution (80% acetone/95% methanol, 1:1 V/V) in glass tubes in dark till leaves became colorless.The extract solution was instantly assayed spectrophotometrically. Specific absorption of Chl a and Chl b was recorded at 645 nm and 663 nm, respectively. Total chlorophyll content was calculated according to the following formula [32]:

Chl (a+b)(mg/g) =$(20.2\times OD_{645}+8.02\times OD_{663})/(DW\times 1000)$

Quantification of IAA, ABA, CTK (iPAs and ZRs), GA3, JA and SA

HPLC–ESI–MS method [33] with minor modification was used to quantify IAA, ABA, CTK (iPAs and ZRs), GA3, JA and SA. Leaves of white clover were weighted (~0.2g) and transferred to a 5 ml screw-cap tube with one steel ball whose diameter was less than 5mm. Kept tubes in liquid nitrogen for 10 min to frezze plant samples and ground them into powder through a Plant Tissue Breaker. Add 200 μl of the working solution of internal standards [33] to each tube. Add 2 ml extraction solvent [33]. Shake tubes at a speed of 100 rpm for 30 min at 4 °C. Add 2 dichloromethane to each tube and shake for 30 min in a 4 °C room. Centrifuge at a speed of 12,000 rpm for 5 min at 4 °C. Transfer ~1.8 ml of the solvent from the lower phase into a screw-cap vial and concentrate it by using a nitrogen evaporator. The samples were redissolved in 0.2 ml methanol. Inject 20 μl of the last methanol solution into the C18 (reverse-phase) HPLC column for analysis. The HPLC–ESI–MS (HPLC system, LC-10AD series, Shimadzu, Japan; ESI-MS system, ABI 4000 QTRAP) conditions and settings were used. Applied Biosystems Analyst software version 1.5.1 was used to control MS system and to perform data analysis and data management.

Expression analysis of genes related to IAA

The expression of *GH3.1*, *GH3.3*, *GH3.5*, *GH3.6*, *GH3.9*, *IAA8*, *IAA27* and *ARF* in Trifolium repens leaves were evaluated through real-time quantitative PCR (qRT-PCR). Total RNAs extraction from *Trifolium repens* leaves were performed through Plant RNA Kits (TianGen Biochemical Technology Co., Ltd) and their reverse transcriptions through iScriptTM cDNA Synthesis Kit (Bio-Rad company). cDNAs were used

as templates to do qRT-PCR。The 10 μL reaction system consisted of template cDNA 1 μL, upstream primer (10μM) 0.5 μL, downstream primer (10μM) 0.5μL, SYBR Green Super Mix 5 μL and ddH_2O 3 μL. The reaction procedures were: pre-denaturation 3 min at 95 ℃, denaturation 10 s at 95 ℃, annealing 15 s at 59.3 ℃ (*GH3.1*), 61 ℃ (*GH3.5*), 60 ℃ (*GH3.3*, *GH3.6*, *GH3.9, IAA8*, *IAA27*) and 58.9 ℃(*ARF*), extension 20 s at 72 ℃, 30 cycles, final extension 10 min at 72 ℃. *GAPDH* was used as reference gene to correct number of template copy, then the relative expression of each gene was calculated according to the formula of $2^{-\Delta\Delta Ct}$ [34]. Primer sequences of the genes related to IAA and their corresponding GeneBank accession numbers were listed in Table 1 in Appendixes.

Expression of drought-induced transcription factor (TF) genes, response to drought (ERD/RD22) and senescence-associated genes(SAG101/SAG102)

cDNAs were used as templates in qRT-PCR. The 10 μL reaction system consisted of template cDNA 1 μL, upstream primer (10μM) 0.5 μL, downstream primer (10μM) 0.5 μL, SYBR Green Super Mix 5 μL, ddH_2O 3 μL. The reaction procedures were: pre-denaturation 3 min at 95 ℃, denaturation 10 s at 95℃, annealing 15 s at 57.2 ℃(*bZIP37*、*bZIP107*、*MYB48*、*MYB112*), 58 ℃(*DREB2*、*DREB4*、*DREB5* 、*MYB14)*, 58.4℃ (*WRKY108715*), 61℃(*WRKY2*、*WRKY56*、*bZIP11)*, 56.4 ℃(*RD22)* and 59.5 ℃(*ERD*), 55.5 ℃ (*SAG101/SAG102*), extension 20s at 72 ℃, 30 cycles, final extension 10 min at 72 ℃. *GAPDH* was used as reference gene to correct number of template copy and then the relative expression of each gene was calculated according to the formula of $2^{-\Delta\Delta Ct}$ [34]. Primer sequences of drought-induced transcription factors and drought-induced genes and their corresponding GeneBank accession numbers are listed in Table 2 in Appendixes.

Statistics and mapping

In this paper, Origin 8.5.1 was used to generate the histogram, SPSS 19.0 to analysis of variance (ANOVA) at 0.05 probability level. Data were transformed to meet normality and homogeneity of variance. Fisher's LSD was used to determine differences among sets.

Results

Effects of exogenous IAA on drought tolerance of white clover

Morphological appearance of white clover plants in all experiment sets were shown as Figre 1A. IAA pretreatment had an obvious facilitation to white clover under normal condition, followed by a significant higher stem dry weight as compared to other treatments (Figure 1B). However, L-AOPP (inhibitor of auxin synthesis) pretreatment remarkably reduced total chlorophyll content（Chl）（Figure 1D）. In control, stem dry weight increased (Figure 1B) by days, RWC and Chl remained stable throughout the experiment (Figure1C-1D). When drought stress went on, stem dry weight, RWC and Chl content gradually decreased in white clover in all sets (Figure1C-1D). However, stem dry weight, RWC and Chl were significantly higher in IAA pretreatment than those without IAA pretreatment. Furthermore, L-AOPP significantly diminished them when compared to drought sets (Figure 1B-1D).

Content of ABA, CTK (iPAs and ZRs), GA3, JA and SA

On 0 day, IAA reduced CTK content by 90.2% and improved GA3 and JA content by 45.2% and 18.4% (Figure 2B-2D), respectively. And, it had no influence on ABA and SA content (Figure 2A-2E). However, L-AOPP increased CTK content by 61.4% and SA content by 130% (Figure 2B, 2E), decreased GA3 and JA content by 28.8% and 13.8% (Figure 2C, 2D), respectively. And it had no impact on ABA content (Figure 2A). In PEG-6000 treatment, ABA and JA content gradually got rose, accompanied by the gradual decline in CTK, GA3 and SA content during experiment period. On 7 day and 14 day, ABA, GA3 and JA contents were significantly higher in IAA set than those without IAA pretreatment (Figure 2A, 2C, 2D), while SA content was significantly lower in IAA set than it in others. L-AOPP nearly had the opposite appearances when compared with IAA (Figure 2A, 2C, 2D).

Endogenous IAA content and relative expression of auxin response genes

IAA set showed a significant increase in endogenous IAA content while L-AOPP set exhibited a significant decrease of endogenous IAA content in white clover under normal condition. The content of endogenous IAA remained at a stable level in control but decreased much in PEG-6000 treatment from start to end (Figure 3A). However, compared with PEG-6000 treatment, exogenous IAA pretreatment maintained a considerably higher endogenous IAA content in white clover exposed to stress, inversely, the L-AOPP pretreatment showed a remarkable decreased trendancy in endogenous IAA content (Figure 3A).

Under normal condition, exogenous IAA improved the expression of *ARF* (auxin response factors), *GH3.9* and *IAA8*, lowered the expression of *GH3.5* and *IAA27*, and had no effects on the expression of *GH3.1*, *GH3.3* and *GH3.6*. L-AOPP mainly restrained the expression of *GH3.1* and *GH3.3* and had no prominent influence on other genes. PEG-6000 inhibited the expression of *ARF*, up-regulated the expression of other genes. Under drought stress, significantly higher expression level of *ARF*, *GH3.1*, *GH3.5*, *GH3.9* and *IAA8* and lower expression level of *GH3.3* , *GH3.6* and *IAA27* were detected in IAA set compared to IAA-absence set. However, L-AOPP down-regulated all genes expression except *GH3.5* (Figure 3B-3J) .

Relative expression of transcription factor (TF) genes responsed to drought stress in white clover

In this experiment, we chose 3 genes in *bZIP*, *DREB*, *MYB* and *WRKY* transcription factor family, respectively. Under no stress, IAA significantly up-regulated expression of *bZIP107*, *MYB48*, *WRKY2* and *WRKY56*, down-regulated expression of *DREB5* and *MYB48*, and had no remarkable influence on expression of *bZIP11*, *bZIP37*, *DREB2*, *DREB4*, *MYB14* and *WRKY108715*. L-AOPP significantly down-regulated *bZIP107* and up-regulated *DREB5*. All concerned transcription factor genes were induced by PEG-6000 except that expression of *bZIP107* was depressed in present experiment. Under drought stress, addition of IAA further up-regulated the expression level of all the transcription factor genes, L-AOPP lowered the expression of these genes, exception for *bZIP37* and *bZIP107*compared with PEG-6000 set (Figure 4B, 4C).

Expression of drought-response genes and senescence-associated genes

Under no stress, exogenous IAA showed a conspicuous inhibition to the relative expression of *ERD*. However, L-AOPP significantly induced expression of *RD22* gene. Drought enhanced expression of *ERD* and *RD22* in large. IAA also improved expression of *ERD* and *RD22* and L-AOPP lowered their expressions (Figure 5). In addition, drought stress extremely activated expression of *SAG101* and *SAG102*, L-AOPP up-regulated expression of *SAG101*, while IAA displayed a prominent suppression to expression of *SAG101* and *SAG102* in white clover.

Discussion

Improved growth and physioloies in white clover

Under drought stress, our results showed that exogenous 1 μM IAA pretreatment on root mitigated plant wilt whereas L-AOPP worsened it (Fig. 1A). Meanwhile, IAA pretreatment significantly improved stem dry weight, relative water content and total chlorophyll content in leaves, however, L-AOPP decreased all of them (Fig. 1B-1D). Studies have shown that IAA is closely related to drought tolerance in plants, and wild type Arabidopsis plants pre-treated with IAA exhibited enhanced drought resistance [35]. Under water deficiency, IAA effectively maintained relative water content and enhanced photosynthetic efficiency and growth of barley. Application of IAA could alleviate the adverse effects brought by dehydration and succeed in enhancing barley growth [36]. Apparently, IAA made morphological and physiological state of white clover in IAA+D set much better than in PEG-6000 set (Figure1). In contrast to appearance of plants in L-AOPP+D, it could be concluded that IAA pretreatment had a positive effect in improving drought tolerance in white clover.

Content Variations of Endogenous Phytohormones

It was showed that drought stress induced an increase in ABA and JA content, but a decrease in CTK, GA3 and SA content (Figure 2A-2E). IAA significantly increased ABA, GA3 and JA content (Figure 3A, 3C, 3D). These results implied that IAA played an important role in synthesizing or accumulating ABA, GA3 and JA in white clover.

Transcriptome data revealed that increase of ABA content induced and activated the expression of a large number of drought resistant genes [37]. ABA regulated downstream response of *RD29B* (dehydration stress gene) by regulating *bZIP* gene [38]. In our studies, we also found that there was a consistent correlation between content of ABA and expression of *RD22* under stress (Figure 3A, Figure 5B), suggesting that ABA also probably regulated expression of *RD22* gene in white clover.

In Arabidopsis and rice, the accumulation of ABA regulated the polar transport of auxin [39, 40]. It has been found that the interaction between IAA and ABA promoted the development of lateral roots in plants, and this pattern of root growth regulation was important for plants to respond to severe drought stress [41]. Besides, exogenous ABA enhanced the recovery of photosynthetic rate in upland rice under PEG stress [42]. Based on these experimental results and combined with Figure 1 and Figure 3 A, we also could speculate that an increase in ABA content enhanced drought resistance through multiple ways,

such as improved *RD22* gene expression, more content of total chlorophyll and more stem dry weight. However, L-AOPP had opposite effects on them, further confirming that these changes were caused by IAA.

As far as IAA and GAs were concerned, some researchers proved that normal level of bioactive GA1 required normal level of IAA in elongating pea stems [43] and IAA promoted GA1 synthesis [44]. It was found that GA induced the formation of porosity [45]. Here, our results showed that content of exgenous IAA significantly increased the content of GA3 (Figure 3C), and IAA content and GA3 content had consistency in change. And this consistency probably was a result of IAA's activation in enzemes ralated to GA3 synthesis, like IAA's promotion on GA1 synthesis [44].

Transgenic creeping bentgrass over-expressing prenyltransferase had a higher endogenous CTK content and improved photosynthesis and water use efficiency and further enhanced drought resistance [46]. In the interaction between IAA and CTK, it was found that IAA down-regulated biosynthesis level of CTK [47]. Our results showed that the increase in endogenous IAA content significantly decreased content of CTK under water sufficiency, but had no significant effect on the level of CTK under drought stress. It was found that CTK inhibited auxin transport protein PIN and reduced the accumulation of IAA, but inhibited lateral root growth [48]. However, the application of CTK resulted in a rapid increase in IAA in young parts [49]. According to these results, interaction between CTK and IAA possibly could be complicated and dependent on different tissues.

JA content increased rapidly under drought stress, and a strong interaction between JA and ABA signaling pathway was observed [50]. Our results indicated that exogenous IAA also increased content of JA (Figure 3D). Some studies have shown that JA is in the upstream of ABA biosynthesis, and the accumulation of JA at early stage led to accumulation of jasmonic acid isoleucine, which is one necessary condition for ABA synthesis under drought stress [51]. Like these results, improved content of JA could spur content of ABA to an improved level.

Exogenous application of SA could improve photosynthetic activity, leaf water content and membrane permeability, thus enhanced tolerance of tomato to drought [52]. Our studies found that content of SA was decreased by drought stress but not regularly influenced by exogenous IAA. Additionally, Alonso-Ramírez A found that GA positively regulated SA and exogenous application of GA could increase content of SA and alleviated environmental stress [53]. Here, GA3 probably also alleviated drought stress through some certain regulatory ways except its influence on variation of SA content.

Taken together, we speculated that the altered phytohormones tend to reach to a new homeostasis after application of exogenous IAA under drought stress. These variations of major phytohormones could contribute to drought resistance for plants through certain signal transduction and gene regulation pathways.

Changes of expression of genes responsing to IAA and TF genes

Transcriptome data showed that rice *AUX/IAA* genes were induced by exogenous IAA and drought [54]. *AUX/IAA1* in Sorghum was also up-regulated by drought [55]. *IAA8* and *IAA27* gene relative expression got a homeostasis during all time when water was sufficient. Drought stress enorrmously prompted their expressions on 7 d and 14 d. We found that exogenous IAA up-regulated expression of *IAA8* and down - regulated expression of *IAA27*. In zinnia, transcript level of *IAA8* was particularly induced by auxin and was expressed in plant vascular development [56]. Tomato transgenic plants with under-expression of *Sl-IAA27* gene showed multiple phenotypes interrelated to vegetative growth. Silencing of it resulted in higher auxin sensitivity, with change of root development and diminished Chl content in leaves [57]. Here, down-regulation of *IAA27* also probably had multiple effects on growth and root development in white clover.

High content *ARF* gene expression nearly corresponded to high content of endogenous IAA, meaning that ARF genes expression were largely modulated by endogenous IAA. One study also found that auxin treatment could affect transcript abundance of seveal *OsARF* genes, and these *ARF* genes might play crucial roles in varied metabolic pathways and some cellular processes in rice [58]. At present, there are few reports on the functional verification of ARF gene in other plants. It is necessary to further study the specific role of *ARF* gene in plants responsing to IAA and drought stress.

GH3 family genes were also involved in plant responsing to biotic and abiotic stresses. Our studies showed that expressions of *GH3.1*, *GH3.3*, *GH3.6* and *GH3.9* were induced by drought stress (Figure 3C, 3D, 3F and 3G), denoting that these *GH* family genes could respond to drought stress. Besides, exogenous IAA also prompted expressions of *GH3.1* and *GH3.9* genes (Figure 3C and 3G), indicating that these two genes may have relation to endogenous IAA content. Arabidopsis thaliana seedlings pretreated with IAA showed strong drought tolerance, and other studies showed that exogenous IAA regulated a variety of gene expression related to stress [59]. It was found that decreased endogenous IAA content in rice mutants accompanied with deficiency in carotenoid and transgenic plants overexpressing *OsGH3.2* showed the sensitivity to drought [11]. Activation of *OsGH3.13* enhanced drought resistance in Rice [60]. Moreover, exogenous IAA activated responsive gene *GH3.9* and resulted in the strong drought resistance in plant [61]. These results indicated that exogenous IAA could enhance drought resistance in white clover and *GH3.1* and *GH3.9* gene was involved in drought tolerance indeed.

Transcription factors (TFs) play important regulatory roles in growth, development, morphogenesis and response to external environments. At present, hundreds of transcription factors regulating plant resistance to drought, low temperature, high salt and disease have been found in higher plants. They were divided into *bZIP*, *DREB*, *MYB* and *WRKY* family groups etc, according to differences of DNA binding domains. Due to their abilities to regulate a number of genes associated with stress, TFs activations by special phytohormone are important to improve stress resistance of plants.

For *bZIPs*, only a small part of them were identified to play roles in plant growth and development, abiotic stress and hormone signal transduction, however their potential molecular mechanisms are still unknown, and need further exploration [62]. Previous study has shown that *OsbZIP23* in maize is

involved in ABA signalings and actively regulates drought and salt stress [63]. Other researchers found that *bZIP11* in Arabidopsis interacted with one adapter proteins via an amino-terminal activation domain to recruit histone acetylation system to specific auxin-responsive genes [29]. *bZIP37* expressed highly in the salt-stressed plant, which might effectively activated downstream of ABA-inducible gene expression [64]. We found that expression of *bZIP11* was also induced by exogenous IAA (Figure 4A), and that of *bZIP37* was induced by PEG-6000 stress (Figure 4B). So, we speculated that *bZIP11* gene responded to IAA and *bZIP37* responded to drought stimulus.

DREBs (dehydration-responsive element-binding proteins) play important roles in plant response to drought stress and were found to be activated in ways dependent on ABA or not [65]. It was showed that exogenous IAA positively enhanced expression of *DREB2* and *DREB4*, and L-AOPP negatively regulated expression of *DREB2* and *DREB4* (Figure 4D, 4E) in our studies. Other study has shown that *DREBs* regulate expression of many downstream genes of drought resistance and over-expression of DREB gene can enhance drought resistance in plants [66]. Our results showed that the improved drought resistance of white clover by exogenous IAA could be associated with the role of *DREB2* and *DREB4* expression.

MYBs also are important in regulating plant growth, development, metabolism and stress response, and almost all eukaryotes have *MYB* transcription factors. The response mechanisms of *MYBs* in stress environment are not very clear. Our studies found that both exogenous IAA and drought stress positively regulated expression of *MYB14* and *MYB48* and L-AOPP decreased their expression (Figure 4G, 4H). Xiong found that over-expression of *MYB48-1* promoted biosynthesis of ABA and improved drought resistance of transgenic rice [67]. *AtMYB60* regulated stomatal movement and promoted Arabidopsis thaliana to respond to drought stress [27]. Different *MYBs* showed varied functions in progress of responding to drought and improved drought resistance directly or indirectly.

At present, the research progress of *WRKY* transcription factors in abiotic stress has been gradually developed. It was found that cold, heat, salt, drought and hormone induced *WRKY* gene expression quickly. *WRKYs* also played important roles in plant drought stress and regulated plant response to abiotic stress through interaction with hormones and protein kinases [68], However the molecular mechanism of its regulation were still limited. It was found that *WRKYs* were involved in plant stress regulatory networks and *WRKY* proteins expressions were induced by drought stress [69]. *WRKY* transcription factor *ABO3* induced expression of drought resistance genes, such as *RD29A* and *COR47*, and positively regulated drought resistance [70]. In terms of *WRKY2*, *WRKY56*, and *WRKY108715* genes, we found that drought also induced their expressions. Moreover, exogenous IAA also significantly up-regulated expression levels of *WRKY* family genes, comparing with direct drought treatment. It seemed that these *WRKYs* played considerably important role in white clover response to drought and IAA improved capability of this response.

Expression of stress gene and senescence -associated gene

In term of *ERD* and *RD22* genes, our results suggested that drought up-regulated expression levels of them. Furthermore, exogenous IAA also prompted their expression levels. L-AOPP decreased their

expression levels. Several studies also showed that high expression level of *ERD* and *RD22* subserved plant resistance to drought [71, 72]. *ERD11* and *ERD13* genes could encode some polypeptides which were homologous to glutathione S-transferases in tobacco and maize. Besides, expressions of *ERD11* and *ERD13* genes were induced by dehydration, but not influenced by GA, ABA, 6-BA and 2,4-D [73]. Other studies also found that *RD22* gene was double improved by both ABA and MYB proteins [74].

As far as *SAG101* and *SAG102* genes were concerned, our studies found that drought extremely improved their expression levels, IAA significantly decreased expression levels of them and L-AOPP enhanced expression levels of them. *SAG101* in Arabidopsis encoded an Acyl Hydrolase involved in leaf senescence [75]. It was found that exogenous IAA inhibited transcription level of *SAG12* [76] and retarded senescence of leaves. The plant with over-expression of *YUCCA6* gene improved content of endogenous IAA and hindered senescence of plant through down-regulated expression of *SAG12* [77]. Similarily, the depressed expression of *SAG101* and *SAG102* by IAA could play a part in delaying senescence resulted from drought stress in white clover.

Conclusion

Under normal condition, endogenous IAA content of leaves in IAA+D was the highest (Figure 3A). Under PEG-6000 treatment, IAA pretreament on roots of white clover improved endogenous IAA level while L-AOPP pretreatment decreased IAA content of leaves (Figure 3A). As an important plant hormone, IAA induced changes in other major phytohormones in white clover, altering expressions of multiple genes which included auxin response, transcriptional factors, drought resistance and leaf senescence. At last, morphological appearance and physiological adaptations demonstrated variations.

This study demonstrated a positively protective role of exogenous IAA on drought resistance in white clover.

Declarations

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Youzhi Zhang, Yaping Li, Zhou Li and Yan Peng conceived the research and designed the experiments; Yaping Li performed the experiments; Youzhi Zhang analyzed the data; Youzhi Zhang worte the

manuscript; Yaping Li, Zhou Li, Yan Peng and Muhammad Jawad Hassan discussed the results and reviewed the manuscript. All authors have given approval to the final version of the manuscript.

co: These authors contributed equally.

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Tables

Table 1 Primer sequences of the genes related to IAA and their corresponding GeneBank accession numbers

Target gene	Accession No.	Forward primer (5'-3')	Reverse primer (5'-3')
GH3.1	MF099746	TCGTCAACTTCTATACAGCCTTCT	CACTTGGTGTCCTTGTTTCTGA
GH3.3	MF099747	TGACTCGGACAAAACAGACG	CTTCATCACTAGGTGGATTAGAAG
GH3.5	MF099748	GATGCTGAGAATGTTCAAAAGG	AGAAACATCACCACCAACCA
GH3.6	MF099749	GAAGAAGAGTTAGGGAGGAGAAG	CCAGGTGTTTTAGCCTCAGAT
GH3.9	MF099750	CATTGAAGCAGTGGTTACAGG	CACCAAAGTAACACTCAGAAGAAG
IAA8	MF099751	ATGCTATCGCCTAGACCTGTT	TGCCTTAGATGCTGGCTGTG
IAA27	MF099752	CCTCAAAGCTACTGAACTGAGAC	ACCCATTTACCAGAACCTCC
ARF	MF099753	TCTGCTGAGTTTACGAGGGTTC	GGTTTTGTTGCTTGCTGATGC
GAPDH	F968420.1	TTACAGAAAGGCACAGGGATGAC	CGGGAGACTAAGGAGGAACTAT

Table 2 Primer sequences of drought-induced transcription factors and drought-induced genes and their corresponding GeneBank accession numbers

Target gene	Accession No.	Forward primer (5'-3')	Reverse primer (5'-3')
DREB2	EU846194.1	CAAGAACAAGATGATGATGGTGAAC	AAGAAGAAGAATTGGAGGAGTCATG
DREB3	EU846196.1	GCTCAATAGGACTCAACCAACTCAC	TGACGTTGTCTAACTCCACGGTAA
DREB4	EU846198.1	CTTGGTTGTGGAGATAATGGAGC	AAGTTGCAATCTGAATTCTGAGGAC
DREB5	EU846200.1	GCGATAGGTTCAAAGAAAGGGTG	AGAGCAGCATCTTGAGCAGTAGG
bZIP11	MF099755	TTCCTTGCCTCCACTTAGTCC	GATCGTCTGTGCCCTTTACG
bZIP 37	MF099754	GAACCCGTCTGAACATAACTGAA	AGCGACTTTGGAGCCATCAT
bZIP 107	MF099756	AGACCCACCAATAACCAAACTG	CATAAAAGGAAGAAGAAGGAGGAG
MYB14	JN117923.1	GACGAAGAGAAAGAACTATCCGCA	TTGATCCGAACAAGGCGACA
MYB48	MF099757	CGAGAAAGGTCATACAAACAAAGG	TGAGGTCAGGGCGGAGATAG
MYB112	MF099758	GCCAGGAAGAACCGACAATG	GCCAGGAAGAACCGACAATG
WRKY2	MF099759	GGCACATAACCACCCGAAAC	AAATTAGCCCAGCCACGATC
WRKY56	MF099760	GCTCTTTTGCTCCAAGCTGTC	AATTGAGGCTCACGCTACGG
WRKY108715	MF099761	GAACAGACCAACTCCAAACAGC	GCAAATCAGGATGGAAAGGAC
ERF019	MF099762	GATATTGCTATGGATGTCGATGC	AAGTCCTCTTGTTGGCTAGAAACT
ERF098	MF099763	TGCGGCGGAGATACGAGAT	GGAAGAAGTGGGCTTAGAAGGA
ERF110	MF099764	TTCGCCATCGCTTTCTTTGT	TCCGCTACGAGATTGATCTTCC
ERD	XM_003612152.2	CCATCGCTGTCTATGCTCGTA	TTCTTCCTCGTCTGAATCGGTA
RD22	XM_003588503.2	GTCCAAACTTCCCACAACTCA	CCTCCTTTTCCTACAGCTACTG
GAPDH	JF968420.1	TTACAGAAAGGCACAGGGATGAC	CGGGAGACTAAGGAGGAACTAT
SAG101	XM_004489275.2	CATTTCGTACTCGCTGGCTCT	CACGTAATCCTTACCACCGTCT
SAG102	XM_003590568.2	ATCATTGGACTTGGTCTTGTTGG	GAAGTGGGCAAGGGAGGAAT

Figures

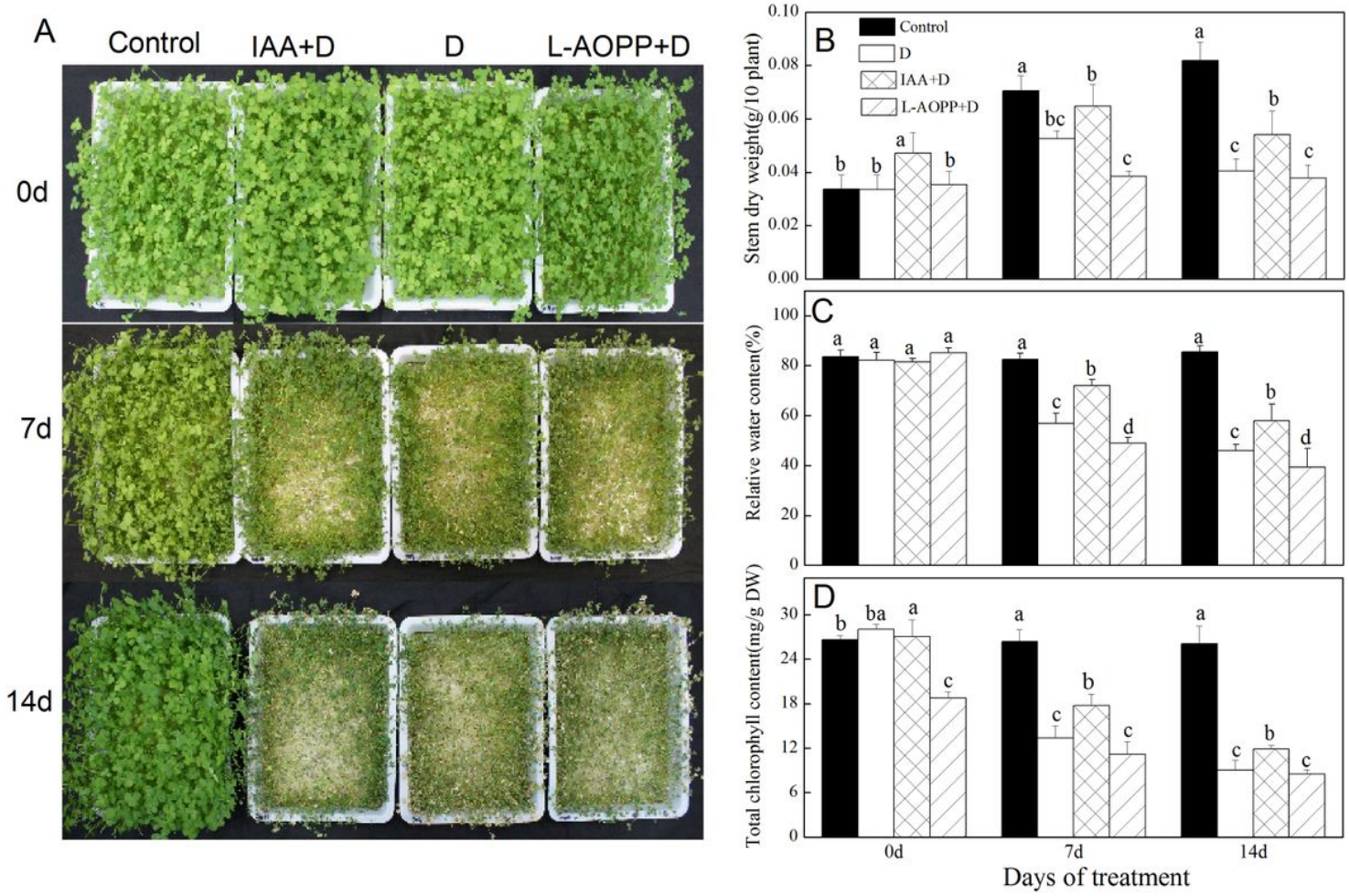

Figure 1

IAA /L-AOPP pretreatment, drought stress and sampling time. Figure 1. Morphological appearance(A) , Stem dry weight (B), relative water content (C) and total chlorophyll content (D) of white clover in different treatments. Vertical columns represent Mean+STD (n=4). The same letter indicates no significant difference and the different letters indicate significant difference in a particular day of treatment (P< 0.05).

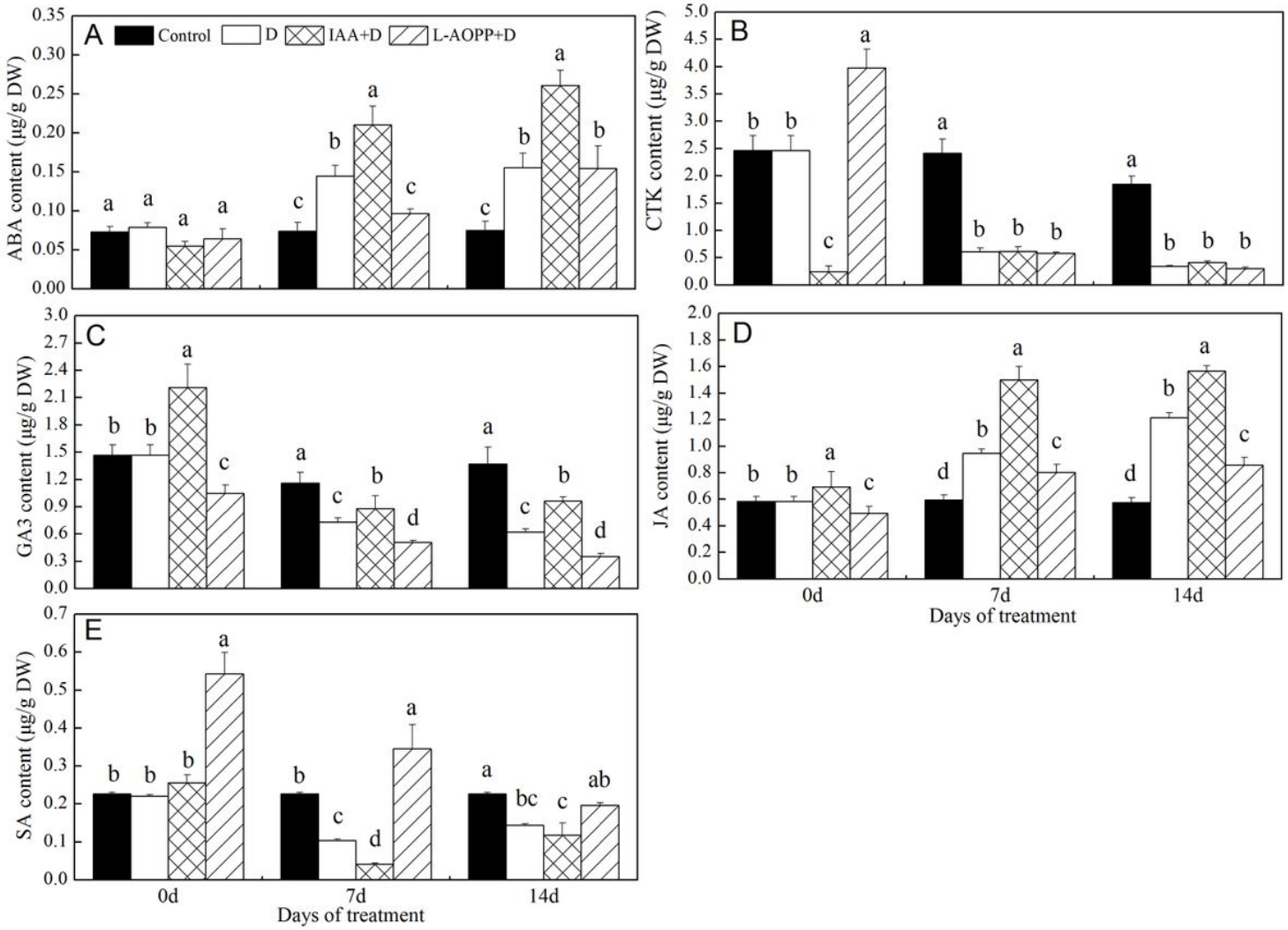

Figure 2

Endogenous IAA content (A) and relative Expression of auxin response genes of white clover leaves in different treatments. ARF (B). GH3.1 (C), GH3.3 (D), GH3.5 (E), GH3.6 (F), GH3.9 (G), IAA8 (H), IAA27 (J) and Vertical columns represent Mean+STD (n=4). The same letter indicates no significant difference and the differenet letters indicate significant difference in a particular day of treatment (P< 0.05).

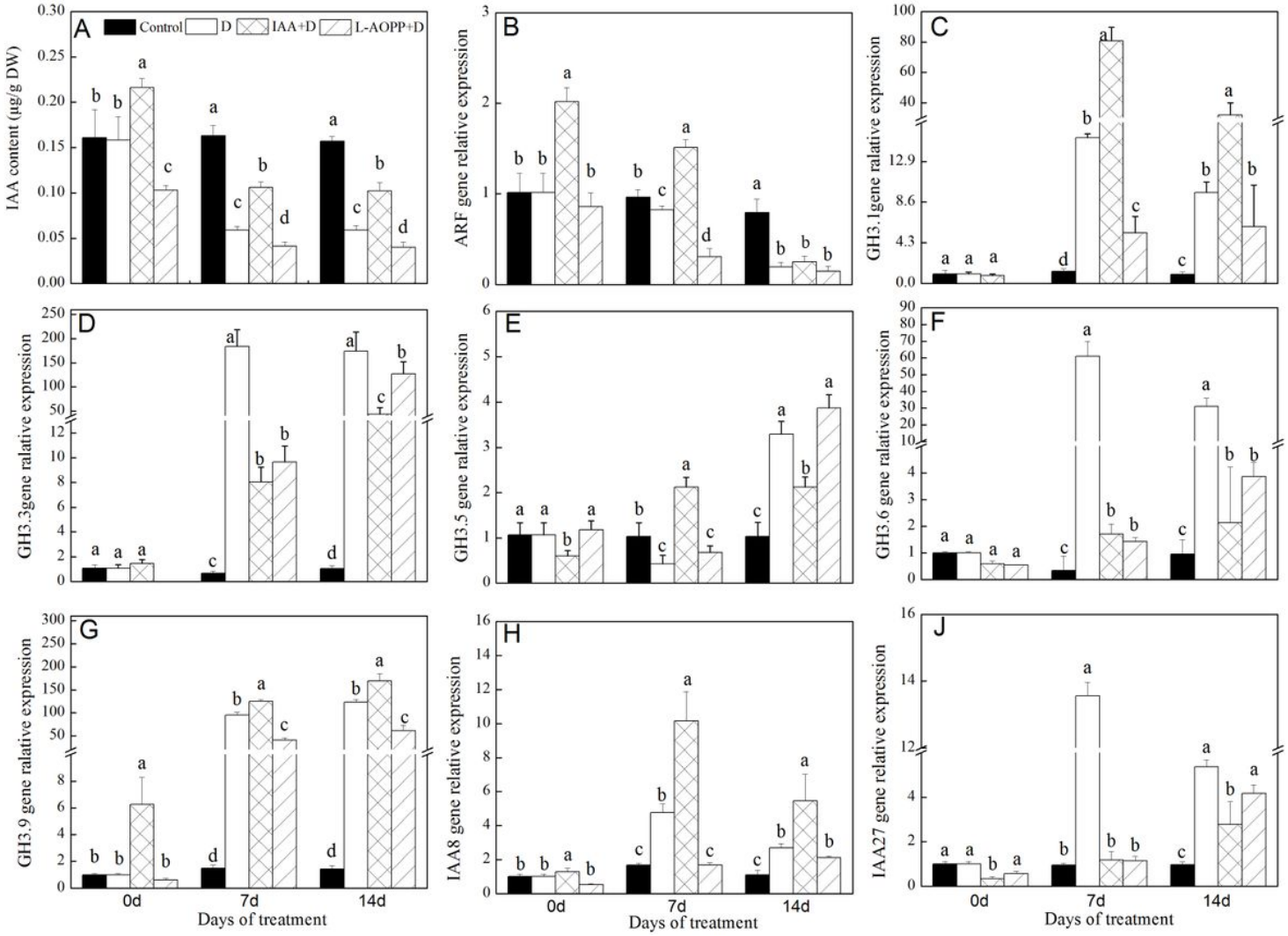

Figure 3

Contents of other major phytohormones of white clover leaves in different treatments. ABA content (A), CTK content (B), GA content (C), JA content (D) and SA content (E).Vertical columns represent Mean+/- std (n=4). The same letter indicates no significant difference and the different letters indicate significant difference in a particular day of treatment. (P< 0.05).

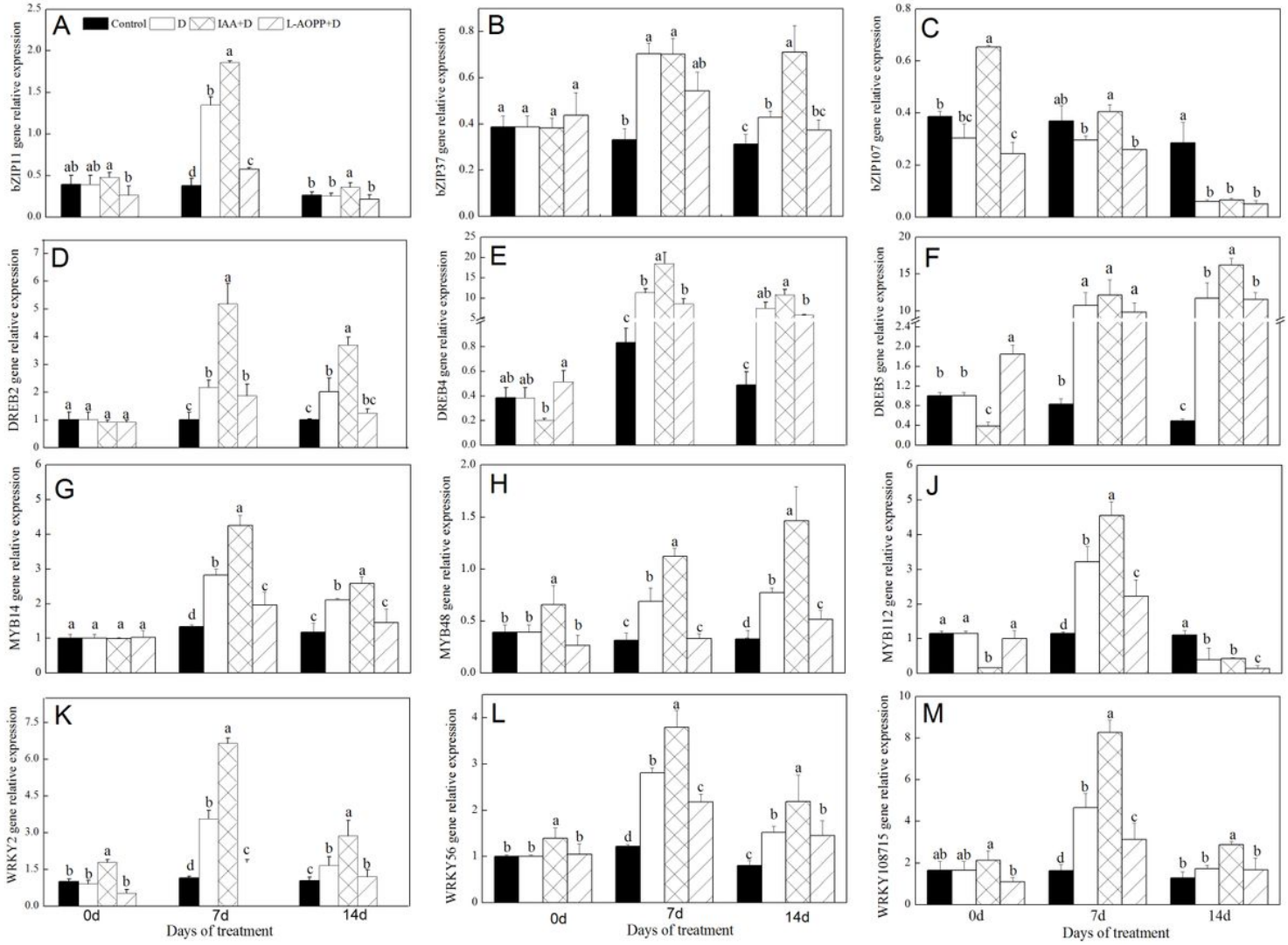

Figure 4

Expression of drought-induced transcriptional factors of white clover leaves in different treatments. bZIP11 (A), bZIP 37 (B), bZIP 107 (C), DREB2 (D), DREB4 (E), DREB5 (F), MYB14 (G), MYB48 (H), MYB112 (J),WRKY2 (K), WRKY56 (L) and WRKY108715 (M).Vertical columns represent Mean+STD (n=4). The same letter indicates no significant difference and the different letters indicate significant difference under a particular day of treatment ($P < 0.05$).

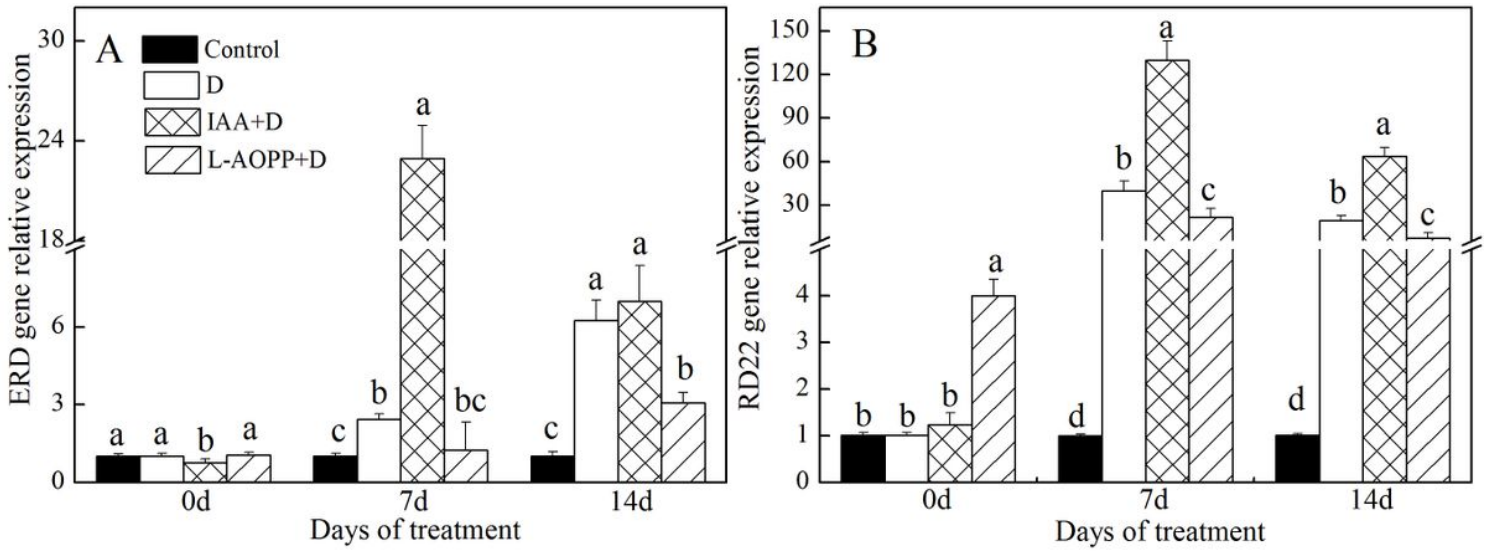

Figure 5

Expression of drought-induced genes of white clover leaves in different treatments. ERD (A) and RD22 (B). Vertical columns represent Mean+STD (n=4). The same letter indicates no significant difference and the different letters indicate significant difference in a particular day of treatment (P< 0.05).

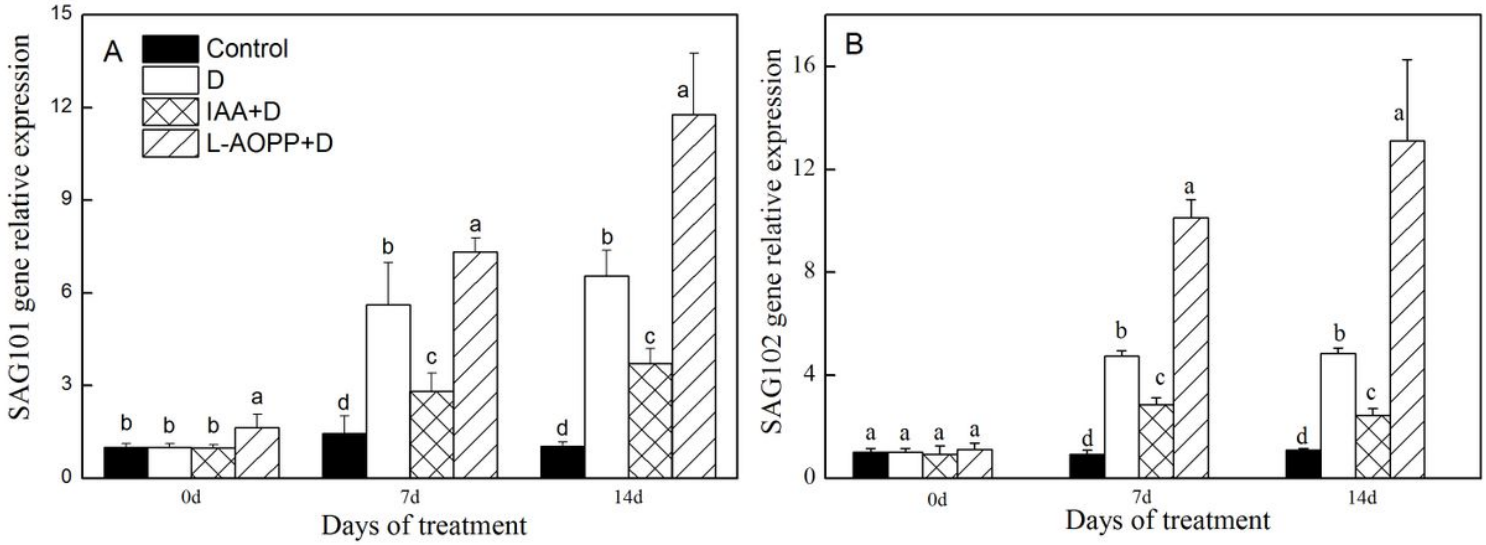

Figure 6

Expression of senescence-associated genes of white clover leaves in different treatments. SAG101 (A) and SAG102 (B). Vertical columns represent Mean+STD (n=4). The same letter indicates no significant difference and the different letters indicate significant difference in a particular day of treatment (P< 0.05).

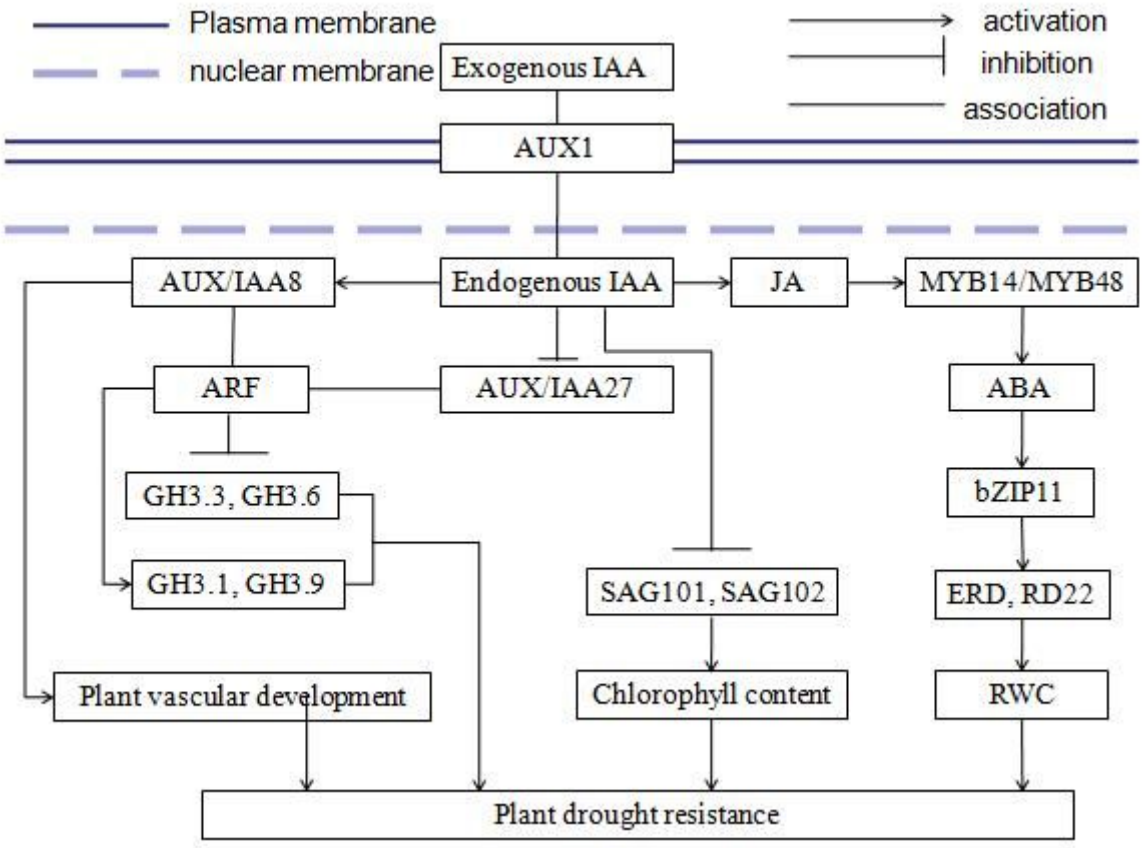

Figure 7

Hypothesis working model for IAA's improving droght resistance in white colver.

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