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 huge influence in restricting plant growth and distribution worldwide (Harb et al., 2010; Shi et al., 2014; Zhou et al., 2017). Adaptability, the ability to change to fit unamiable surroundings, would confer plants with mixed strategies to respond to surroundings to get a superior survival rate (Sean et al., 2010; Takashi et al., 2010; Qin et al., 2011; Julia et al., 2012). Among these strategies, adjusting the expression level of related drought responsive genes can result in biochemical and physiological changes which might play an important role to tackle with drought stress. These related genes include early-response to dehydration (ERD) stress genes and resoponse to dehydration (RD) stress genes, which are closely related with plant senescence (Kiyosue et al., 1994; Rahma et al., 2014), and senescence-associated genes (SAG) which regulate expression of some genes related to chlorophyll degradation and cytoplasm damage (Gan et al.,1997). White clover (*Trifolium repens)* 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 it is planted in large quantities. However, white clover is susceptible to drought stress and exhibits significant changes in leaf senescence when confronts to dehydration stimulation (Zhao et al.2004). In terms of transcriptional factors (TFs) gene, a large number of documents have reported that the transcriptional activation of basic region/leucine-zipper motif (bZIP), dehydration responsive element binding (DREB), myeloblastosis (MYB) and WRKY,

can be influenced by drought. Thus, it is necessary to explore expression variation of some drought and senescence related genes to reveal the feasible adaptive mechanism in regulatory hierarchy.

Besides above, most of genes expression are regulated by plant hormones (Harb et al., 2010; Sean et al., 2010; Takashi et al., 2010; Qin et al., 2011; Du et al., 2013), including IAA, CTK, ABA, GA, JA and SA, together take as signal substances and play an important role in adaptation to environmental stresses. And the most important concern is the interaction between the TFs genes and plant hormones which is still unclear in white clover when exposed to dehydration.

Futher, IAA is one of the main naturally occurring auxins in plants, regulating many processes of plant growth and development (Yun et al., 2010). Recently, accumulating evidences also indicate possible linkages between auxin and abiotic stresses, even though there still exist cross talks between auxin and other major phytohormones, including CTK, GA, JA, SA and ABA (Junichi et al., 1994; Yoshiko et al., 1999; Yong et al., 2001; G.García-Martín et al., 2005; Kazan et al., 2009; Brian et al., 2010; Li et al., 2014). Thus, we intended to know the variations of these plant hormones in white clover after addition of IAA. Besides that, auxin stimulates RNA synthesis which depends on DNA (Maheshwari et al., 1966; Cherry, 1967), regulating gene expression response to auxin (Kim et al., 1997; Ulmasov et al., 1997; Dharmasiri et al., 2005; Dos et al., 2009; Vanneste et al., 2009). In previous studies, auxin response transcription factor (ARF) family (Allison et al., 2005) could mediate roles of IAA during plant growth. And TFs could be regulated by one and/or several of phytohormones (Eleonora et al., 2005; Yong et al., 2008; Christoph et al., 2014). Gretchen Hagen3 (GH3) gene family and IAA response gene, belonging to auxin response gene, probably induce some changes in white clover after IAA pretreatment. So, growth and development of white colver pre-treated with IAA under drought were also one of the main concerns of this study.

To sum up, in order to view strategies adopted by white clover to endure drought stress and changes caused by IAA addition, we extensively elucidated the role of IAA in plants under dehydration and investigated changes of several major phytohormones expression of ARF gene, GH3 family gene, IAA repsonse gene, TFs gene, ERD gene and RD gene, SAG gene in white clover under drought stress, provding some possible perspectives to comprehend the varied adaptations in white clover.

Materials And Methods

Plant material and growth condition

The seeds of 'Pixie' (*Trifolium repens cv.*) used in this experiment were purchased from Beijing Mammoth Seed Industry Company in China.Seeds were surface sterilized with 1% (W/V) sodium hypochlorite solution for 3 min and then rinsed with deionized 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 (16 h/23℃day and 8 h/19℃ night cycle, irradiance of about 500μmol quanta·m^{-2}·s^{-1} , 75% relative humidity). 25 mL of distilled water was applied each day. After germination, 25 mL of

Hoagland's nutrition solution (Hoagland et al., 1950) was used to nurture seedlings every day. In order to minimize the environmental effects, the position of the pots was rotated in the culture chamber daily.

IAA pre-treatment and drought stress

When the second true leaf of white clover fully unfolded, seedlings were subjected to four treatments: (1) Control check (labeled with CK), 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 following 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 remaining days. All plants were simultaneously treated with PEG-6000. Each treatment had 4 biological replicates. The seedlings were not less than 30 plants in each sample. Samples were collected at 0 d, 7 d and 14 d after PEG-6000 treatment.

Physiological measurements

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

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 and used as saturated weight (SW). To get the dry weight (DW), samples were placed in a kraft paper bag and kept in an oven at 105 ℃ for 30 min, then at 75 ℃ for 48 h and weighed them again. RWC was calculated using formula (Barrs et al., 1962): RWC(%)=(FW-DW)/(SW-DW)×100%。

Approximately 0.1 g of leaves (FW) were extracted with 10 mL of mixed solution of 80% acetone and 95% methanol (1:1，V/V) in glass tubes，kept in dark conditon till leaves became colorless (if it was necessary, pigment extract solution should be centrifuged for 5 min to make extract solution fully transparent).The extract solution was instantly assayed spectrophotometrically. Specific absorption wavelength 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 (Qi, 2000):

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

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

According to the method described previously with some minor modifications (Xiang et al, 2010) to quantify IAA, ABA, CTK (iPAs and ZRs),GA3, JA and SA , four replicates of leaf samples were ground to fine powder after having been frozen in liquid nitrogen. About 0.2 g (each replicate) of leaves were extracted twice with 800 μL of cold extraction buffer (2-propanol: ddH_2O: concentrated HCl, 1000/500/1, V/V/V) with internal standards. Quantification assay was performed in HPLC-ESI-MS (HPLC system, LC-10AD series, Shimadzu, Japan; ESI-MS system, ABI 4000 QTRAP). Applied Biosystems Analyst software

version 1.5.1 was used to control MS system and to perform data analysis and data management.Here, DW was calculated according to the average of interchange coefficients from FW to DW during estimation of RWC.

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 was evaluated by real-time quantitative PCR (qRT-PCR). Extraction of total RNA and reverse transcription of Trifolium repens leaves were performed through Plant RNA Kits (TianGen Biochemical Technology Co., Ltd) and iScriptTM cDNA Synthesis Kit (Bio-Rad company). The first strand of reverse transcription cDNA was used as template to do qRT-PCR by fluorescence quantitative PCR (Bio-Rad)。The 10 μL reaction system consisted of template cDNA 1 μL, upstream primer (10μM) 0.5 μL and downstream primer (10μM) 0.5μL, SYBR Green Super Mix 5 μL and dd H_2O 3 μL respectively. The reaction procedures were as followings: 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 ℃. After amplification, GAPDH was used as reference gene to correct numbers of template copy, then the relative expression of each gene was calculated according to the formula of $2^{-\Delta\Delta Ct}$ (Xia et al., 2009).

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

The first strand of reverse transcription cDNA was used as template to detect qRT-PCR by fluorescence quantitative PCR (Bio-Rad). The 10 μL reaction system consisted of template cDNA 1 μL, upstream primer (10μM) 0.5 μL and downstream primer (10μM) 0.5 μL, SYBR Green Super Mix 5 μL, dd H_2O 3 μL respectively. The reaction procedures were as follows: pre-denaturation 3 min at 95 ℃, denaturation 10 s at 95℃, annealing 15 s at 57.2 ℃(*bZIP37、bZIP107、MYB48、MYB112*), 58 ℃(*DREB2-5 、MYB14)*, 58.4℃ (*WRKY108715*), 61℃(*WRKY2、WRKY56、bZIP11)*, 56.4 ℃(*RD22)* and 59.5 ℃(*ERD*), 55.5 ℃ (*SAG101/SAG102*),extension 20 s at 72 ℃, 30 cycles, final extension 10 min at 72 ℃. GAPDH was used as reference gene to correct numbers of template copy and then the relative expression of each gene was calculated according to the formula of $2^{-\Delta\Delta Ct}$ (Xia et al., 2009).

Statistics and mapping

In this paper, Origin 8.5.1 was used to generate the histogram, SPSS 19.0 to analyze significance difference of data by using Fischer's least significance difference *(LSD)* at 0.05 probability level. Excel 2007 was utilized to record and alculate means and standard deviation.

Results

Effects of exogenous IAA *on drought tolerance of white clover*

Morphological appearance of white clover plants are shown as Figre 1A in all treatments during experiment. IAA pretreatment had an obvious facilitation to white clover plants under normal conditions, followed by a significant higher stem dry weight as compared to other treatments (Figure 1B), meanwhile, L-AOPP pretreatment (inhibiting auxin synthesis) remarkably reduced the total chlorophyll content（Chl） （Figure 1D）. In control treatment (CK), stem dry weight continuously increased (Figure 1B), relative water content (RWC and total Chl pretty remained unchanged throughout the experiment (Figure 1C-1D). As the stress period prolonged, white clover plants exposed to PEG-6000 treatment, stem dry weight, RWC and Chl content gradually decreased(Figure 1C-1D), However, with IAA pretreatment, stem dry weight, relative water content and total chlorophyll content were significantly higher than those without IAA pretreatment. Oppositely, L-AOPP pretreatment further diminished stem dry weight, relative water content (significantly) and total chlorophyll content compared to drought treatment (Figure 1B-1D).

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

Under normal condition, exogenous IAA pretreatment notably reduced CTK content, but enhanced GA3 and JA content, however, ABA and SA content remained unaffected. Contrary to this, L-AOPP pretreatment dramatically increased CTK and SA content and decreased GA3 and JA content with no impact on ABA content. In PEG-6000 treatments, ABA and JA content gradually elevated, accompanied by the gradual decline in CTK, GA3 and SA content during experimental period. Under drought stress, ABA, GA3 and JA contents were significantly higher, while SA content was significantly lower in IAA pretreatments than those without IAA pretreatment, however, L-AOPP pretreatment had opposite results (Figure 3A).

Endogenous IAA content and relative expression of auxin response genes

Exogenous IAA pretreatment prompted significant increase in endogenousIAA content but L-AOPP pretreatment exhibited significant decrease of endogenous IAA content in white clover plants under normal conditions. The content of endogenous IAA remained at stable level in control treatment but decreased dramatically in PEG-6000 treatment in all measuring time (Figure 2A). However, compared with PEG-6000 treatment, exogenous IAA pretreatment maintained a considerably higher endogenous IAA content in plants exposed to stress, inversely, the L-AOPP pretreatment showed a remarkable decreasing trend in endogenous IAA content (Figure 2A).

Under normal condition, exogenous IAA pretreatment induced higher expression of ARF (auxin response factors), GH3.9 and IAA8 genes , but lowered the expression of GH3.5 and IAA27 genes and remained the expression of GH3.1, GH3.3 and GH3.6 unchanged. L-AOPP mainly restrained the expression of GH3.1 and GH3.3 and had no prominent influence on other genes. PEG-6000 treatment inhibited the expression of ARF but up-regulated the expression of other genes. Under drought stress, significantly higher expression level of ARF, GH3.1, GH3.5, GH3.9 and IAA8, while significantly lower expression level of GH3.3 , GH3.6 and IAA27were detected in IAA pretreatment compared to without IAA pretreatment, moreover, L-AOPP down-regulated all genes expression besides GH3.5 (Figure 2B-2J) .

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

In this experiment, we took 3 genes as testing objects in bZIP, DREB, MYB and WRKY transcription factor family responsing to drought stress according to previous transcriptome sequencing in white clover respectively. Under no stress, IAA pretreatment significantly up-regulated the expression of bZIP107, MYB48, WRKY2 and WRKY56 genes, but down-regulated the expression of DREB5 and MYB48, and had not remarkably induced expression of bZIP11, bZIP37, DREB2, DREB4, MYB14 and WRKY108715, furthermore, L-AOPP significantly down-regulated the expression of bZIP107 but up-regulated the expression of DREB5. All detective transcription factor genes were signally induced by PEG-6000 treatment except a depressed expression of bZIP107 in present experiment. Under drought stress, addition of IAA further up-regulated the expression level of all the transcription factor genes, correspondingly L-AOPP treatment obviously lowered the expression of genes, exception for bZIP37 and bZIP107compared with PEG-6000 treatment (Figure 4).

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 pretreatment significantly induced the expression of RD22 gene. During water shortage, it enhanced expression of ERD and RD22 gene in large quantities, expression of ERD and RD22 gene was enormously higher in IAA+D treatment but lower in L-AOPP+D treatment than that in drought treatment (Figure 5). In addition, under normal conditions L-AOPP pretreatment upregulated the expression of SAG101 while drought stress extremely activated the expression of SAG101 and SAG102, but IAA pretreatment displayed a prominent suppression to the expression of SAG101 and SAG102 in white clover response to drought stress, contrarily L-AOPP further stimulated the expression of these two genes related to senescence .

Discussion

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 (Haitao et al., 2014). Under water stress condition, IAA application was effective in maintaining higher relative water content, enhancing photosynthetic efficiency and growth of barley. Application of IAA could alleviate the adverse effect brought by dehydration and succeeded in enhancing barley growth (M. Yasin et al., 2006). In accordance with these results, our results also showed that exogenous 1 μM IAA pretreatment on root mitigated the plant wilting and increased stem dry weight of white clover under drought stress, however, L-AOPP worsened wilting and decreased stem dry weight Meanwhile, IAA pretreatment significantly improved relative water content and total chlorophyll content in leaves. Undoubtedly, IAA made morphological and physiological parameters of white clover in IAA+D set much better as compared to PEG-6000 set (Fig. 1). In contrast to appearance of plants in L-AOPP+D, it could be concluded that a higher endogenous IAA concentration from exogenous IAA pretreatment showed positive effects on white clover exposed to drougt stress.

Under normal conditions, endogenous IAA content of leaves got the highest level among all sets. 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 2A). As an important plant hormone, IAA induced changes in other major phytohormones in white clover, altering expressions of multiple genes, including auxin response, transcriptional factors, drought resistance and leaf senescence. At last, morphological appearance and physiological adaptation demonstrated variations.

Changes of expression of genes responsing to IAA and their subsequent effects

Transcriptome data showed that rice AUX/IAA genes were induced by exogenous IAA and drought (Cheol et al., 2013). AUX/IAA1 in Sorghum was also up-regulated by drought (Wang et al., 2010). IAA8 and IAA27 gene relative expression got a homeostasis during all time when water was sufficient. Drought stress enorrmously prompted their expressions in 7 d and 14 d. Here, 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 (Andrew et al., 2003). Increased expression of IAA8 gene may improve vascular development in white clover to meliorate its growth. 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 (Carole et al., 2012). 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 was 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 a crucial role in varied metabolic pathways and some cellular processes in rice (Dekai et al., 2007). At present, there are few reports on the functional verification of ARF gene in other plants. It is necessary to further study the specific roles of ARF gene in plants responsing to IAA and drought stress.

GH3 family genes are also involved in plant responses to biotic and abiotic stresses. Here, our studies showed that expressions of GH3.1, GH3.3, GH3.6 and GH3.9 were immensely induced by drought stress (Figure 2C, 2D, 2F and 2G), denoting that these GH family genes could respond to drought stress. Besides, exogenous IAA pretreatment also prompted expressions of GH3.1 and GH3.9 genes (Figure 2C and 2G), 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 (Foyer et al., 1994). 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 (Du et al., 2012; Du et al., 2013). Activation of OsGH3.13 enhanced drought resistance in Rice (Shen et al., 2009). Moreover, exogenous auxin activated responsive gene GH3.9 and resulted in a strong drought resistance in plant (Khan et al., 2007). These results indicated that exogenous IAA could enhance drought resistance in white clover and GH3.1 and GH3.9 genes were involved indeed.

In this study, IAA drastically decreased expression of GH3.3 and GH3.6. OsGH3.6 plays a dual role in plant growth and defense by regulating auxin levels (Domingo et al., 2009). According to this, several of GH3 family genes may have multiple functions in white clover under dehydration. This result also showed that exogenous IAA up-regulated expression of some GH3 family genes and down-regulated expression of others.

Changes of content of Endogenous Phytohormones and expression of TF genes

The results of this study showed that drought stress induced increase in ABA and JA content, but decreased CTK, GA3 and SA content (Figure 3A-3E). IAA significantly increased ABA, GA3 and JA content (Figure 3A, 3C, 3D). These results implied that IAA might play an important role in synthesizing or accumulating ABA, GA3 and JA in white clover.

When ABA content increased, transcriptome data revealed that ABA induced and activated the expression of a large number of drought resistant genes (Yamaguchi et al., 2006). ABA regulates downstream response of dehydration stress gene RD29B by regulating bZIP gene (Uno et al., 2000). In this study, we also found that content of ABA and expression level of RD22 had a consistent correlation under stress (Figure 3A, Figure 5B), suggesting that there ABA probably regulates expression of RD22 gene in white clover.

In Arabidopsis and rice, the accumulation of ABA regulates the polar transport of auxin (Popko et al., 2010; Xu et al., 2013). It has been found that the interaction between IAA and ABA promotes the development of lateral roots in plants, and this pattern of root growth regulation is important for plants to respond to severe drought stress (Saini et al., 2013). Besides, exogenous ABA enhanced the recovery of photosynthetic rate in upland rice under PEG stress (Teng et al., 2014). Based on these experimental results and combined with Figure 1 and Figure 3 A, in this study we also could speculate that increase in ABA content promoted by exogenous IAA enhanced drought resistance through multiple ways, such as improved RD22 gene expression, more content of total chlorophyll and more stem dry weight. Meanwhile, L-AOPP had utterly opposite effects on these changes, further confirming that these changes were caused by IAA.

As far as IAA and GAs were concerned, some researchers proved that maintainning normal levels of bioactive GA1 required normal levels of IAA in elongating pea stems (John et al., 2000), and IAA promoted GA1 synthesis (John et al., 2001).It was found that GA induced the formation of porosity (Saibo et al., 2003). Under drought stress, stomatal densities and transpiration rate and leaf water status are closely related, suggesting that GA plays an important role in response to drought stress. 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. So, we could speculate that IAA promoted the accumulation of GA3 in white clover, and this accumulation was probably as a result of IAA's activation of enzemes ralated to GA3 synthesis, like IAA's promotion on GA1 synthesis (John et al, 2001).

Transgenic creeping bentgrass over-expressing prenyltransferase had a higher endogenous CTK content and improved photosynthesis and water use efficiency and further enhanced drought resistance (Merewitz et al., 2012). In the interaction between IAA and CTK, it was found that IAA down-regulated biosynthesis level of CTK (Nordström et al., 2004). The 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 in our experiment. It was found that CTK inhibited auxin transport protein PIN and reduced the accumulation of IAA, but inhibited lateral root growth (Moriwaki et al., 2011). However, the application of CTK resulted in a rapid increase in IAA in young parts (Jones et al., 2010). 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 (Fernández et al., 2012). 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 leads to accumulation of jasmonic acid isoleucine, which is a necessary condition for ABA synthesis under drought stress (Ollas et al., 2015). Like these results, improved content of JA could spur content of ABA to a high level. The exact mechanisms on these results deservingly need to be further studied.

Exogenous application of SA could improve photosynthetic activity, leaf water content and membrane permeability, thus enhanced tolerance of tomato to drought (Hayat et al., 2008). This study 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, exogenous application of GA could increase content of SA, and alleviated environmental stress (Alonso et al., 2009). 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.

Transcription factors (TFs) play an important regulatory role 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 can be divided into bZIP, DREB, MYB and WRKY family group etc, according to differences of DNA binding domain. Due to their abilities to regulate a number of genes associated with stress, TFs activated by specialized phytohormone are important regulators to improve the stress resistance of plants.

bZIP family transcription factors regulate plant development and growth in response to stress, and as a big family, only a small part of them was identified to play a role in plant growth and development, abiotic stress and hormone signal transduction, however its potential molecular mechanism is still unknown, and

needs further exploration (Golldack et al., 2011). Previous studies have shown that OsbZIP23 in maize is involved in ABA signalings and actively regulates drought and salt stress (Xiang et al., 2008). Other researchers found that bZIP11 transcription factor in Arabidopsis interacted with one adapter proteins via an amino-terminal activation domain to recruit histone acetylation system to specific auxin-responsive genes (Christoph et al., 2014). bZIP37 expressed highly in the salt-stressed plants, which might effectively activated downstream of ABA-inducible gene expression (Guo et al., 2016). However, functions of bZIP genes were rarely reported in white clover, and, here, in this study, we found that expression of bZIP11 gene was also induced by exogenous IAA, and that of bZIP37 gene induced by PEG-6000 stress. So, we could speculate that bZIP11 gene responded to IAA and bZIP37 responded to drought stimulus, to triger other successive reactions. Thinking about bZIP37 and ABA content (Figure 3A), we could infer that bZIP37 in white clover probably played an important role in activating downstream of ABA-inducible gene expression influenced by drought but not IAA.

DREB (dehydration-responsive element-binding protein) transcription factors play an important role in plant response to drought stress and were found to be activated in a way dependent on ABA or not (Todaka et al., 2015). This study showed that exogenous IAA always positively enhanced the expression of DREB2 and DREB4 and L-AOPP always negatively inhibited the expression of DREB2 and DREB4 (Figure 4D, 4E). Studies have shown that DREB transcription factors regulate expression of many downstream genes of drought resistance, and over-expression of DREB gene can enhance drought resistance in plants (Liu et al., 1998). This shows that the improved drought resistance of white clover by exogenous IAA could be associated with the role of DREB2 and DREB4 expression.

MYB transcription factors also play an important role in regulating plant growth, development, metabolism and stress response, and almost all eukaryotes have MYB transcription factors. The response mechanism of MYB in stress environment is not very clear. This study found that both exogenous IAA and drought stress positively regulated expression of MYB14 and MYB48, however, L-AOPP, decreased their expression (Figure 4G, 4H). Xiong et al found that over-expression of MYB48-1 promoted biosynthesis of ABA and improved drought resistance of transgenic rice (Xiong et al., 2014). AtMYB60 regulates stomatal movement and promotes Arabidopsis thaliana to respond to drought stress (Eleonora et al., 2005). 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 is found that cold, heat, salt, drought and hormone induced WRKY gene expression quickly. WRKY transcription factors also play an important role in plant drought stress, and regulate plant responses to abiotic stress through interaction with the hormone and protein kinases (Eulgem et al., 2007), but the current understanding of the molecular mechanism of its regulation is still limited. The other study found that WRKY transcription factors were involved in plant stress regulatory network and WRKY protein expression was induced by drought stress (Tripathi et al., 2014). WRKY transcription factor ABO3 induces expression of drought resistance genes, such as RD29A and COR47, and positively regulates drought resistance (Ren et al., 2010). In terms of WRKY2, WRKY56, and WRKY108715 genes,

here, we found that drought also induced their expression levels. 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 senescene –associated gene

In term of ERD and RD22 gene, our results suggested that drought stress up-regulated expression levels of them, furthermore, exogenous IAA also prompted their expressions level, however, L-AOPP drastically decreased their expressions level. Other studies also showed that high expression level of ERD and RD22 subserved plant resistance to drought (Almazroue, 2014; Shinozaki et al., 1998). ERD11 and ERD13 genes belonging to ERD family could encode some polypeptides which were homologous to glutathione S-transferases in tobacco and maize. Besides, the expression of ERD11 and ERD13 gene was induced by dehydration, but not influenced by GA, ABA, 6-BA and 2,4-D (Tomohiro et al., 1993). Some studies also found that RD22 gene was double regulated by both ABA and MYB proteins (Abe et al., 1997).

As far as SAG101 and SAG102 genes were concerned, this study found that drought extremely improved their expression levels, however, IAA significantly decreased them, and L-AOPP simultaneously enhanced expression levels of SAG101 and SAG102 genes. SAG101 in Arabidopsis encoded an Acyl Hydrolase involved in leaf senescence (Yuehui et al., 2002). It was found that exogenous IAA inhibited transcription level of SAG12 (Noh et al., 1999) 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 (Im et al., 2011). Therefore, the depressed expression of SAG101 and SAG102 by IAA could play a part in delaying senescence resulted from drought stress in white clover.

Conclusion

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

Based on the previous correlated findings and the current results, a hypothesis working model for IAA regulating the feasible drought response pathyway in white clover under drought stress is shown in Figure 7, although further research on control strategy of IAA in improving drought resistance should be conducted.

Declarations

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Youzhi Zhang, Yaping Li, Zhou Li and Yan Peng conceived the project and designed the experiments; Yaping Li performed the experiments; Youzhi Zhang analyzed the data; Youzhi Zhang finalized the manuscript; Yaping Li, Zhou Li,Yan Peng and Muhammad Jawad Hassan discussed the results and reviewed the manuscript.

co: These authors contributed equally.

All authors have given approval to the final version of the manuscript.

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References

Abe H, Yamaguchi-Shinozaki K, Urao T, et al (1997). Role of Arabidopsis MYC and MYB homologs in drought-and abscisic acid-regulated gene expression. *The Plant Cell,* 9(10): 1859–1868. DOI: https://doi.org/10.1105/tpc.9.10.1859.

Allison C. Mallory, David P. Bartel, et al (2005). MicroRNA-Directed Regulation of Arabidopsis auxin repsonse factor17 Is Essential for Proper Development and Modulates Expression of Early Auxin Response Genes. *The plant cell,* 17 (5): 1360–1375. DOI: https://doi.org/10.1105/tpc.105.031716.

Alonso-Ramírez A, Rodríguez D, Reyes D, et al (2009). Evidence for a role of gibberellins in salicylic acid-modulated early plant responses to abiotic stress in Arabidopsis seeds. *Plant Physiol,* 150(3):1335–1344.

Almazroue H A (2014). Identification, Cloning, and Expression of Tobacco Responsive to Dehydration like Protein (RD22), SBIP–355 and Its Role in SABP2 Mediated SA Pathway in Plant Defense.

Andrew T., Groover, Amy Pattishall et al (2003). IAA8 expression during vascular cell differentiation. *Plant Molecular Biology,* 51(3):427–435. DOI: 10.1023/A: 1022039815537.

Barrs H D, Weatherley P E (1962). A re-examination of the relative turgidity techniques for estimating water deficits in leaves. *Australian Journal of Biological Sciences,* 15:413–428. DOI: https://doi.org/10.1071/BI9620413.

Brian Jones, Sara Andersson Gunnera, Sara V Petersson, et al (2010). Cytokinin Regulation of Auxin Synthesis in Arabidopsis Involves a Homeostatic Feedback Loop Regulated via Auxin and Cytokinin Signal Transduction. *The Plant Cell,* 22: 2956–2969. DOI: https://doi.org/10.1105/tpc.110.074856.

Carole Bassa, Isabelle Mila, Mondher Bouzayen et al (2012). Phenotypes Associated with Down-Regulation of Sl-IAA27 Support Functional Diversity Among Aux/IAA Family Members in Tomato. *Plant and Cell Physiology,* 53(9): 1583–1595. DOI: https://doi.org/10.1093/pcp/pcs101.

Cheol Park H, Cha J Y, Yun D J (2013). Roles of YUCCAs in auxin biosynthesis and drought stress responses in plant. *Plant signaling & behavior,* 8(6): 337–349. DOI:https://doi.org/10.4161/psb.24495.

Cherry J. H (1967). Nucleic acid biosynthesis in seed germination: influences of auxin and growth regulating substances. *Ann. N. Y. Acad. Sci.,* 144: 154–168. DOI: 10.1111/j.1749–6632.1967.tb34010.x.

Christoph Weiste, Wolfgang Dröge-Laser (2014). The Arabidopsis transcription factor bZIP11 activates auxin-mediated transcription by recruiting the histone acetylation machinery. *Nature communications,* 5: 1–12. DOI:10.1038/ncomms 4883.

Dekai Wang, Kemei Pei,Yaping Fu (2007). Genome-wide analysis of the auxin response factors (ARF) gene family in rice (Oryza sativa).*Gene,* 394 (1–2):13–24. DOI: https://doi.org/10.1016/j.gene.2007.01.006.

Dharmasiri N, Dharmasiri S, Estelle M (2005). The F-box protein TIR1 is an auxin receptor. *Nature,* 435: 441–445.DOI:10.1038/nature03543.

Dos Santos Maraschin F, Memelink J, Offringa R (2009). Auxin-induced, SCFTIR1-mediated poly-ubiquitination marks AUX/IAA proteins for degradation. *The Plant Journal,* 59(1): 100–109. DOI: 10.1111/j.1365–313X.2009.03854.x.

Du H, Wu N, Fu J, et al (2012). A GH3 family member, OsGH3–2, modulates auxin and abscisic acid levels and differentially affects drought and cold tolerance in rice. *Journal of experimental botany,* 63(18): 6467–6480. DOI: https://doi. org/10. 1093/jxb/ers300.

Eleonora Cominelli, Galbiati M, Vavasseur A, et al (2005). A guard-cell-specific MYB transcription factor regulates stomatal movements and plant drought tolerance. *Current biology,* 15(13): 1196–1200. DOI: https://doi.org/10.1016/j. cub.2005.05. 048.

Eulgem T, Somssich I E (2007). Networks of WRKY transcription factors in defense signaling. *Current opinion in plant biology,* 10(4): 366–371. DOI: https://doi.org/10.1016/j.pbi.2007.04.020.

Fernández-Arbaizar A, Regalado J J, Lorenzo O (2012). Isolation and characterization of novel mutant loci suppressing the ABA hypersensitivity of the Arabidopsis coronatine insensitive 1–16 (coi1–16) mutant during germination and seedling growth. *Plant and Cell Physiology,* 53(1): 53–63. DOI: https://doi.org/10. 1093/pcp/pcr174.

Foyer C H, Descourvieres P, Kunert K J (1994). Protection against oxygen radicals: an important defence mechanism studied in transgenic plants. *Plant, Cell & Environment,* 17(5): 507–523. DOI: 10.1111/j.1365–3040.1994.tb00146.x.

Gan S, Amasino R M(1997). Making sense of senescence (molecular genetic regulation and manipulation of leaf senescence). *Plant physiology,* 1997, 113(2): 313. DOI: https://doi.org/10.1104/pp.113.2.313.

G.García-Martín, J. A.ManzaneraEmail, M. E. González-Benito(2005). Effect of exogenous ABA on embryo maturation and quantification of endogenous levels of ABA and IAA in Quercus suber somatic embryos. *Plant Cell, Tissue and Organ Culture,* 80(2): 171–177. DOI: 10.1007/s11240–004–1056-y.

Golldack D, Lüking I, Yang O (2011). Plant tolerance to drought and salinity: stress regulating transcription factors and their functional significance in the cellular transcriptional network. *Plant cell reports,* 30(8): 1383–1391. DOI: 10.1007/ s00299–011–1068–0.

Guoqiang Fan, Limin Wang, Minjie Deng, et al(2016). Changes in Transcript Related to Osmosis and Intracellular Ion Homeostasis in Paulownia tomentosa under Salt Stress. *Front Plant Sci.* 7: 384. DOI: 10.3389/fpls.2016.00384

Haitao Shi, Li Chen, TiantianYe, Xiaodong Liu, et al (2014). Modulation of auxin content in Arabidopsis confers improved drought stress resistance. *Plant Physiology and Biochemistry,* 82: 209–217. DOI: https://doi.org/10.1016/j.plaphy. 2014.06.008.

Harb, A., Krishnan, A., Ambavaram, et al (2010). Molecular and physiological analysis of drought stress in Arabidopsis reveals early responses leading to acclimation in plant growth. *Plant Physiol.* 154, 1254–1271. DOI: https://doi.org/10.1104/pp.110.161752.

Hayat S, Hasan S A, Fariduddin Q, et al (2008). Growth of tomato (Lycopersicon esculentum) in response to salicylic acid under water stress. *Journal of Plant Interactions,* 3 (4): 297–304.DOI: https://doi.org/10.1080/17429140802320797.

Hoagland, D. R., and Arnon, D. I. (1950). The water culture method for growing plants without soil. *California Agricultural Experiment Station Circular.* 347, 357–359. DOI: https://doi.org/citeulike-article-id:9455435.

Im Kim J, Murphy A S, Baek D, et al (2011). YUCCA6 over-expression demonstrates auxin function in delaying leaf senescence in Arabidopsis thaliana. *Journal of experimental botany,* 62(11): 3981–3992. DOI: https://doi.org/10.1093/jxb/err094

Jones B, Gunnerås S A, Petersson S V, et al (2010). Cytokinin regulation of auxin synthesis in Arabidopsis involves a homeostatic feedback loop regulated via auxin and cytokinin signal transduction. *The Plant Cell,* 22(9): 2956–2969. DOI: https://doi.org/10.1105/tpc.110.074856.

John J. Ross, Damian P. O'Neill, Jennifer J. Smith et al (2000). Evidence that auxin promotes gibberellin A1 biosynthesis in pea. *The plant journal,* 21(6):547–552. DOI:https://doi.org/10.1046/j.1365–313x.2000.00702.x

John Ross, Damian O'Neill (2001). New interactions between classical plant hormones. *TRENDS in Plant Science* 6(1):1–3. DOI: https://doi.org/10.1016 /S1360–1385(00)01795–7.

Julia Krasensky, Claudia Jonak (2012). Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. *Journal of Experimental Botany,* 63(4): 1593–1608. DOI: https://doi.org/10.1093/jxb/err460.

Junichi Ueda, Kensuke Miyamoto, Miyako Aoki (1994).Jasmonic Acid Inhibits the IAA-Induced Elongation of Oat Coleoptile Segments: a Possible Mechanism Involving the Metabolism of Cell Wall Polysaccharides. *Plant and Cell Physiology,* 35(7): 1065–1070. DOI:https://doi.org/10.1093/oxfordjournals.pcp. a078695.

Kazuko Yamaguchi-ShinozakiKazuo Shinozak (1993).The plant hormone abscisic acid mediates the drought-induced expression but not the seed-specific expression of rd22, a gene responsive to dehydration stress in Arabidopsis thaliana. *Molecular and General Genetics,* 238 (1–2): 17–25. PMID: 8479424.

Kazan K., Manners J.M(2009). Linking development to defense: auxin in plant pathogen interactions. *Trends Plant Sci.* 14, 1360–1385. DOI: https://doi.org/10.1016/j.tplants.2009.04.005.

Khan S, Stone J M (2007). Arabidopsis thaliana GH3.9 influences primary root growth. *Planta,* 226(1): 21–34. DOI 10.1007/s00425–006–0462–2.

Kim J, Harter K, Theologis A (1997). Protein–protein interactions among the Aux/IAA proteins. *Proceedings of the National Academy of Sciences,* 94(22): 11786–11791. DOI: 10.1073/pnas.0402504101.

Kiyosue T, Yamaguchi-Shinozaki K, Shinozaki K (1994).Cloning of cDNAs for genes that are early-responsive to dehydration stress (ERDs) in Arabidopsis thaliana L.: identification of three ERDs as HSP cognate genes. *Plant Molecular Biology,* 25(5): 791–798. DOI: 10.1007/BF00028874.

Li Mengsha, Yan Xiufeng (2014). Jasmonic acid signaling in plants and its biological functions in relation to environment. *Acta Ecologica Sinica,* 34 (23): 6779–6788. DOI: http://dx.doi.org/10.5846/stxb201303090383.

Liu Q, Kasuga M, Sakuma Y, et al (1998). Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought-and low-temperature-responsive gene expression, respectively, in Arabidopsis. *The Plant Cell,* 10(8): 1391–1406. DOI: https://doi.org/10.1105/tpc.10.8.1391.

Maheshwari, S. C., S. Guha, and S. Gupta(1966). Effect of indoleacetic acid on the

incorporation of orthophosphate and adenine into plant nuclei in vitro. *Biochim. Biophys.* Acta, 117: 470–472. DOI:https://doi.org/10.1016/0304–4165(66)90097–3

Merewitz E B, Du H, Yu W, et al (2012). Elevated cytokinin content in ipt transgenic creeping bentgrass promotes drought tolerance through regulating metabolite accumulation. *Journal of Experimental Botany,* 63 (3): 1315–1328. DOI: https://doi.org/10.1093/jxb/err372.

Miyawaki K, Matsumoto-Kitano M, Kakimoto T(2004). Expression of cytokinin biosynthetic isopentenyltransferase genes in Arabidopsis: tissue specificity and regulation by auxin, cytokinin, and nitrate. *The Plant Journal,* 37(1): 128–138. DOI: 10.1046/j.1365–313X.2003.01945.x.

Moriwaki T, Miyazawa Y, Kobayashi A, et al (2011). Hormonal regulation of lateral root development in Arabidopsis modulated by MIZ1 and requirement of GNOM activity for MIZ1 function. *Plant Physiology,* 157(3): 1209–1220. DOI: https://doi.org/10.1104/pp.111.186270.

M. Yasin Ashraf, Nazila Azhar, M Hussain (2006). HussainIndole acetic acid (IAA) induced changes in growth, relative water contents and gas exchange attributes of barley (Hordeum vulgare L.) grown under water stress conditions. Plant Growth Regul, 50:85–90. DOI 10.1007/s10725–006–9130–6.

Nordström A, Tarkowski P, Tarkowska D, et al (2004). Auxin regulation of cytokinin biosynthesis in Arabidopsis thaliana: a factor of potential importance for auxin-cytokinin-regulated development. *Proceedings of the National Academy of Sciences of the United States of America,* 101(21): 8039–8044. DOI: 10.1073/pnas.0402504101.

Noh Y S, Amasino R M (1999). Identification of a promoter region responsible for the senescence-specific expression of SAG12. *Plant molecular biology,* 41(2): 181–194. DOI: 10.1023/A:1006342412688.

Ollas C, Arbona V, GóMez-Cadenas A (2015). Jasmonoyl isoleucine accumulation is needed for abscisic acid build-up in roots of Arabidopsis under water stress conditions. *Plant, cell & environment,* 38(10): 2157–2170. DOI: 10.1111/pce. 12536.

Popko J, Hänsch R, Mendel R R, et al (2010). The role of abscisic acid and auxin in the response of poplar to abiotic stress. *Plant Biology,* 12(2): 242–258. DOI: 10.1111/j.1438–8677.2009.00305.x.

Qin F., Shinozaki K., Yamaguchi-Shinozaki K (2011). Achievements and challenges

in understanding plant abiotic stress responses and tolerance. *Plant Cell Physiol,* 52, 1569–1582. DOI: https://doi.org/10.1093/pcp/pcr106.

Rahma Jardak Jamoussi, Mohamed Malek Elabbassi et al (2014). Physiological responses of transgenic tobacco plants expressing the dehydration responsive RD22 gene of Vitis vinifera to salt stress. *Turkish Journal of Botany ,* 38: 268–280. DOI:10.3906/bot–1301–53.

Ren X, Chen Z, Liu Y, et al (2010). ABO3, a WRKY transcription factor, mediates plant responses to abscisic acid and drought tolerance in Arabidopsis. *The Plant Journal,* 63(3): 417–429. DOI: 10.1111/j.1365–313X.2010.04248.x.

Saibo N J, Vriezen W H, Beemster G T, van Der Straeten D (2003). Growth and stomata development of Arabidopsis hypocotyls are controlled by gibberellins and modulated by ethylene and auxins. *Plant J.,* 33 (6):989–1000. DOI: 10.1046/j.1365–313X.2003.01684.x.

Saini S, Sharma I, Kaur N, et al (2013). Auxin: a master regulator in plant root development. *Plant cell reports,* 32(6): 741–757. DOI:10.1007/s00299–013- 1430–5.

Sean R. Cutler, Pedro L. Rodriguez, Ruth R. Finkelstein et al (2010). Abscisic Acid: Emergence of a Core Signaling Network. *Annual Review of Plant Biology,* 2010, 61: 651–679. DOI: https://doi.org/10.1146/annurev-arplant–042809–112122

Shimizu-Sato S, Tanaka M, Mori H (2009). Auxin–cytokinin interactions in the control of shoot branching. *Plant molecular biology* 69(4): 429. DOI: 10.1007/s 00299–015–1815–8.

Shinozaki K, Yamaguchi-Shinozaki K (1998). Molecular responses to drought stress [M]. Amsterdam, Elsevier Science BV.

Shenwei Zhang, Chen Hui Li, Cao J, et al (2009). Altered architecture and enhanced drought tolerance in rice via the down-regulation of indole–3-acetic acid by TLD1/OsGH3. 13 activation. *Plant physiology,* 151(4): 1889–1901. DOI: https://doi.org/10.1104/pp.109.146803.

Shi H, Ye T, Chan Z (2014). Comparative proteomic responses of two bermudagrass (Cynodon dactylon (L). Pers.) varieties contrasting in drought stress resistance. *Plant Physiology and Biochemistry,* 82: 218–228. DOI: https://doi.org/10.1016/j. plaphy. 2014.06.006.

Takashi Hirayama, Kazuo Shinozaki (2010). Research on plant abiotic stress responses in the post-genome era: past, present and future. *The plant Journal,* 61 (6): 1041–1052. DOI: 10.1111/j.1365–313X.2010.04124.x.

Teng K, Li J, L iu L, et al (2014). Exogenous ABA induces drought tolerance in upland rice: the role of chloroplast and ABA biosynthesis-related gene expression on photosystem II during PEG stress. *Acta physiologiae plantarum,* 36(8): 2219–2227. DOI: 10.1007/s11738–014–1599–4.

Tripathi P, Rabara R C, Rushton P J (2014). A systems biology perspective on the role of WRKY transcription factors in drought responses in plants. *Planta,* 239(2): 255–266. DOI 10.1007/s00425–013–1985-y.

Todaka D, Shinozaki K, Yamaguchi-Shinozaki K (2015). Recent advances in the dissection of drought-stress regulatory networks and strategies for development of drought-tolerant transgenic rice plants. *Frontiers in plant science,* 6: 84. DOI: 10.3389/fpls.2015.00084.

Tomohiro Kiyosue, KazukoYamaguchi-Shinozaki, Kazuo Shinozaki (1993). Characterization of two cDNAs (ERD11 and ERD13) for dehydration-inducible genes that encode putative glutathione S-transferases in Arabidopsis thaliana L. *Federation of European Biochemical Societies,* 335(2): 189–192. DOI: https://doi.org/10.1016/0014–5793(93)80727-C.

Ulmasov T, Murfett J, Hagen G, et al (1997). Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *The Plant Cell,* 9(11): 1963–1971. DOI: https://doi.org/10.1105/tpc. 9.11. 1963

Uno Y, Furihata T, Abe H, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K (2000). Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. *Proc Natl Acad Sci USA,* 97:11632–11637. DOI: https://doi.org/10. 1104/pp. 110.159111.

Vanneste S, Friml J (2009). Auxin: a trigger for change in plant development. *Cell,* 136(6): 1005–1016. DOI: https://doi.org/10.1016/j.cell.2009.03.001.

Wang S K, Bai Y H, Shen C J, et al (2010). Auxin-related gene families in abiotic stress response in Sorghum bicolor. Functional & integrative genomics, 10(4): 533–546. DOI 10.1007/s10142–010–0174–3.

Xia X J, Wang Y J, Zhou Y H, et al (2009). Reactive oxygen species are involved in brassinosteroid-induced stress tolerance in cucumber. *Plant Physiology,* 150(2): 801–814. DOI: https://doi.org/10.1104/pp.109.138230.

Xiang Y, Tang N, Du H, et al (2008). Characterization of OsbZIP23 as a key player of the basic leucine zipper transcription factor family for conferring abscisic acid sensitivity and salinity and drought tolerance in rice. *Plant physiology,* 148(4): 1938–1952. DOI: https://doi.org/10.1104/pp.108.128199.

Xiangqing Pan, Ruth Welti, Xuemin Wang (2010). Quantitative analysis of major plant hormones in crude plant extracts by high-performance liquid chromatography–mass spectrometry. *Nature Protocols,* 5 (6):985–991. DOI:10.1038/nprot.2010.37.

Xiong H, Li J, Liu P, et al (2014). Overexpression of OsMYB48–1, a novel MYB-related transcription factor, enhances drought and salinity tolerance in rice. *PLoS One,* 9(3): e92913. DOI: https://doi.org/10.1371/journal.pone.0092913.

Xu W, Jia L, Shi W, et al (2013). Abscisic acid accumulation modulates auxin transport in the root tip to enhance proton secretion for maintaining root growth under moderate water stress. *New Phytologist,* 197(1): 139–150. DOI: 10.1111/nph.12004.

Yamaguchi-Shinozaki K, Shinozaki K(2006). Transcriptional regulatory networks in cellular responses and toletance to dehydrationand cold stresses. *Annual Review of Plant Biology,* 57:781–803. DOI:

10.1146/annurev.arplant.57.032905.105444

Yong Xiang, Ning Tang, Hao Du et al (2008). Characterization of OsbZIP23 as a Key Player of the Basic Leucine Zipper Transcription Factor Family for Conferring Abscisic Acid Sensitivity and Salinity and Drought Tolerance in Rice. *Plant Physiology,* 138: 1938–1952. DOI: 10.1104/pp.108.128199.

Yongyin Wang, Susan, MopperKarl et al (2001). Effects of Salinity on Endogenous Aba, Iaa, Ja, and Sa in Iris hexagona. *Journal of Chemical Ecology,* 27(2):327–342. DOI: 10.1023/A:1005632506230.

Yoshiko Koshitaa, ToshioTakaharaa, Tatsushi Ogataa (1999). Involvement of endogenous plant hormones (IAA, ABA, GAs) in leaves and flower bud formation of satsuma mandarin (Citrus unshiu Marc.). *Scientia Horticulturae,* 79 (3–4): 185–194. DOI: https://doi.org/10.1016/S0304–4238(98)00209-X.

Yuehui He, Susheng Gan (2002). A Gene Encoding an Acyl Hydrolase Is Involved in Leaf Senescence in Arabidopsis. *The plant cell,* 14(4):805–815. DOI: https://doi.org/10.1105/tpc.010422.

Zhao Gui-Qin, Wang Suo-Min, Ren Ji-Zhou(2004). Research progress on genetic transformation and ecological adaptability in white clover. *Acta Ecologica Sinica,* 24(3): 592–598. DOI: 10.3321/j.issn:1000–0933.2004.03.029

Zhou Li, Yan Zhang, Xinquan Zhang, et al (2017). Metabolic Pathways Regulated by Chitosan Contributing to Drought Resistance in White Clover. *Journal of proteome of research,*, 16, 3039–3052. DOI: 10.1021/acs.jproteome. 7b00334

Qi Zhou. Experimental guidance of Plant Physiology [M]. Beijing: China Agriculture Press, 2000:161–174.

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

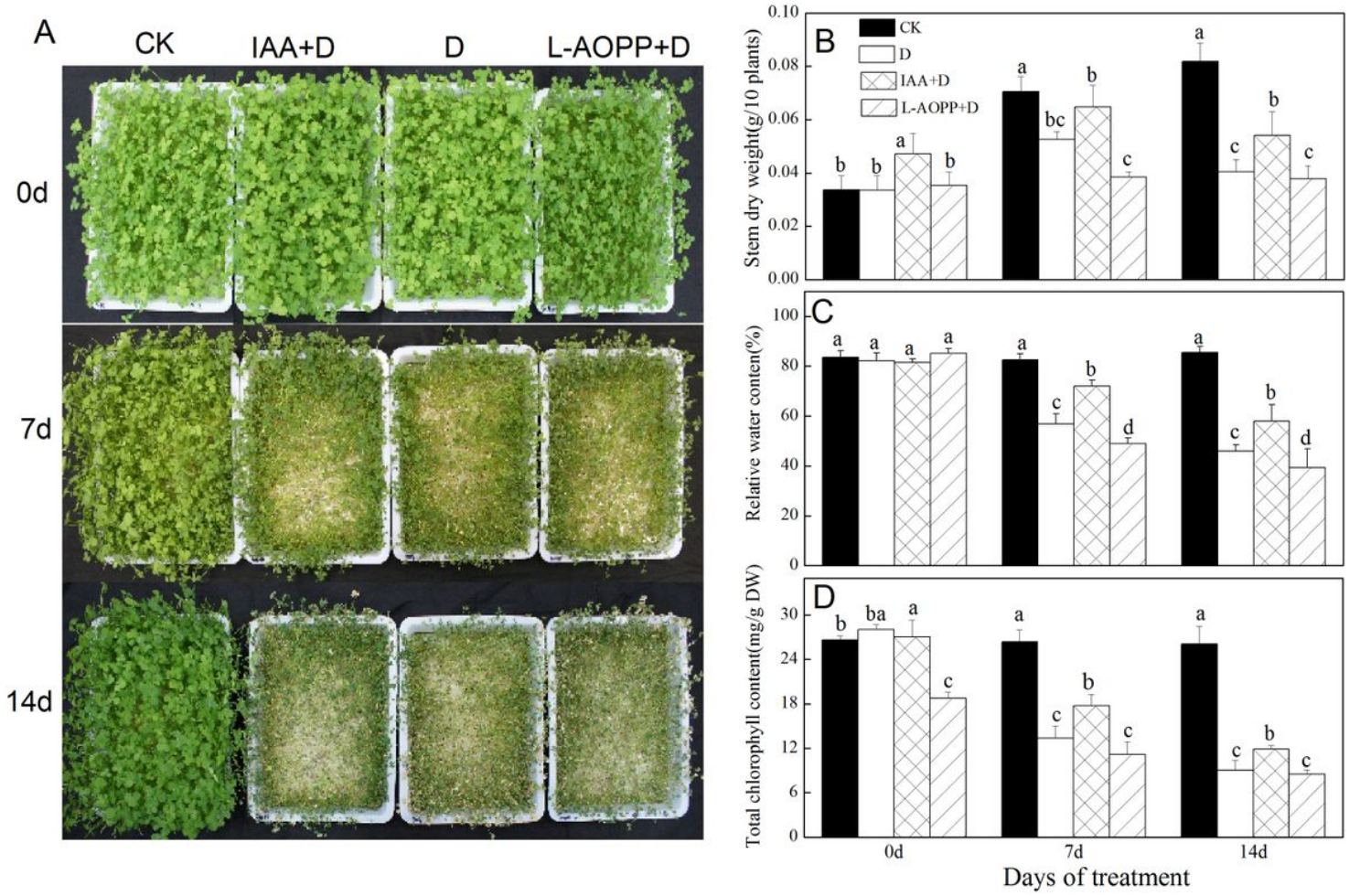

Figure 1

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).

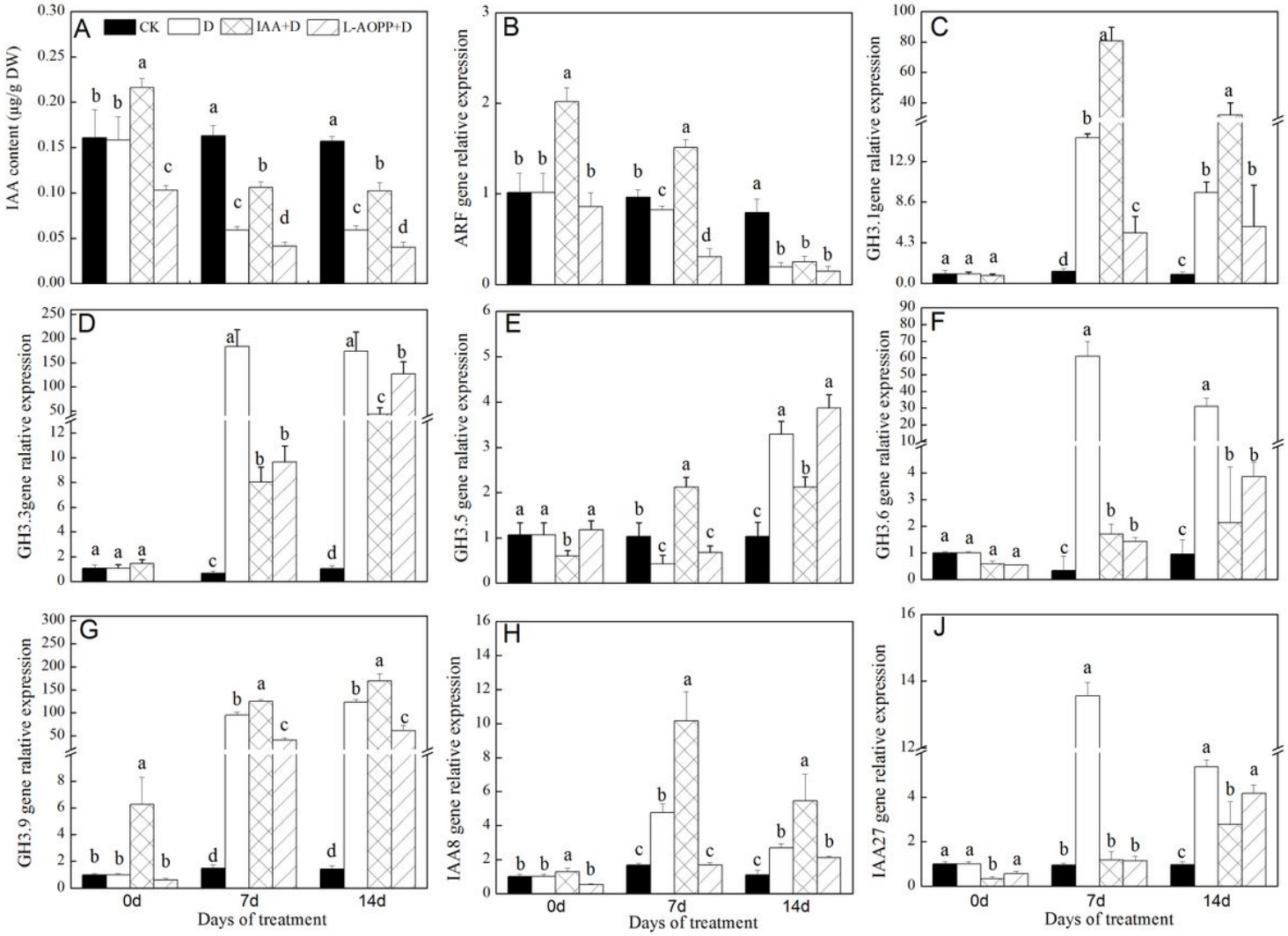

Figure 2

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 ($P< 0.05$).

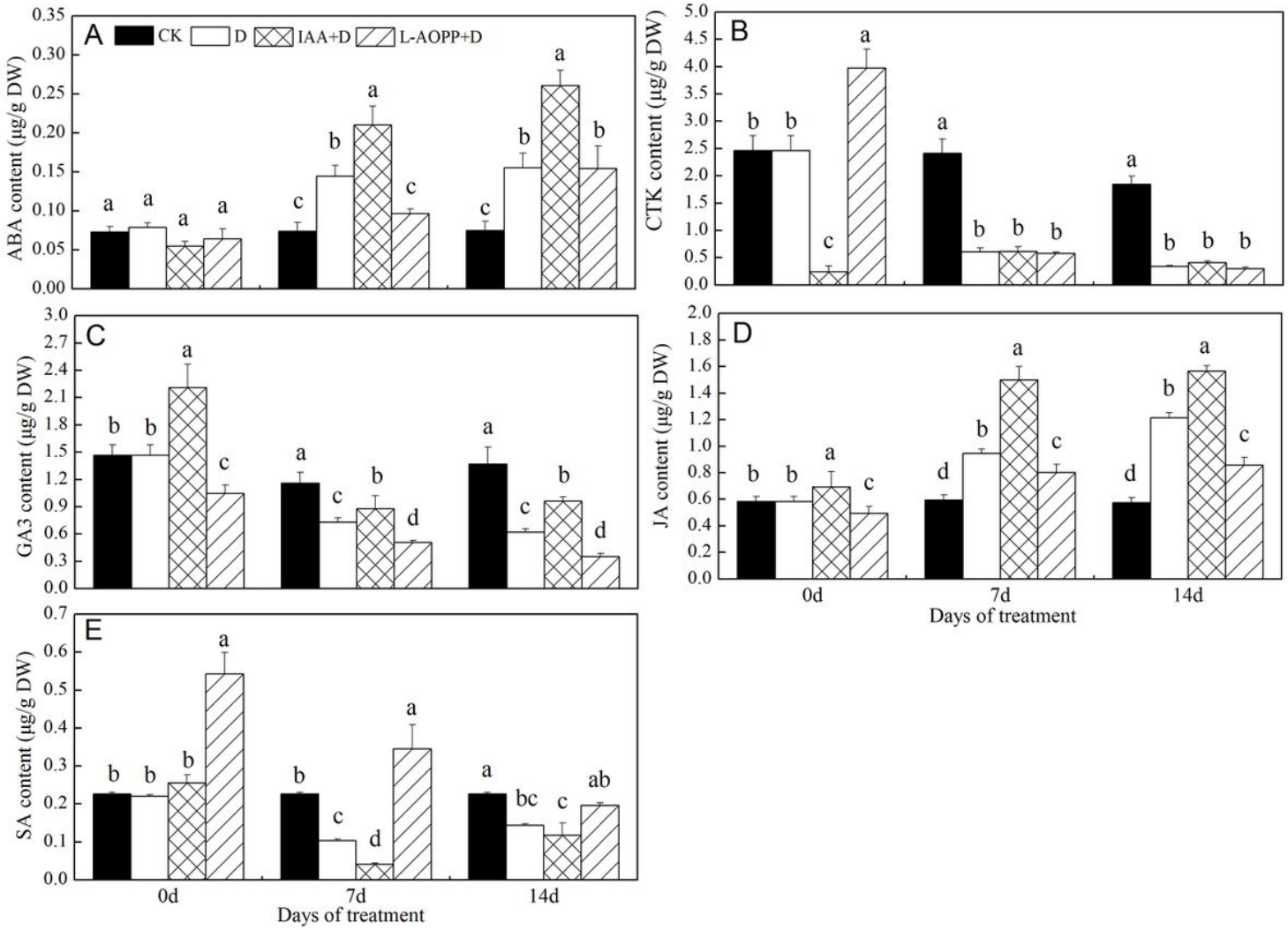

Figure 3

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).

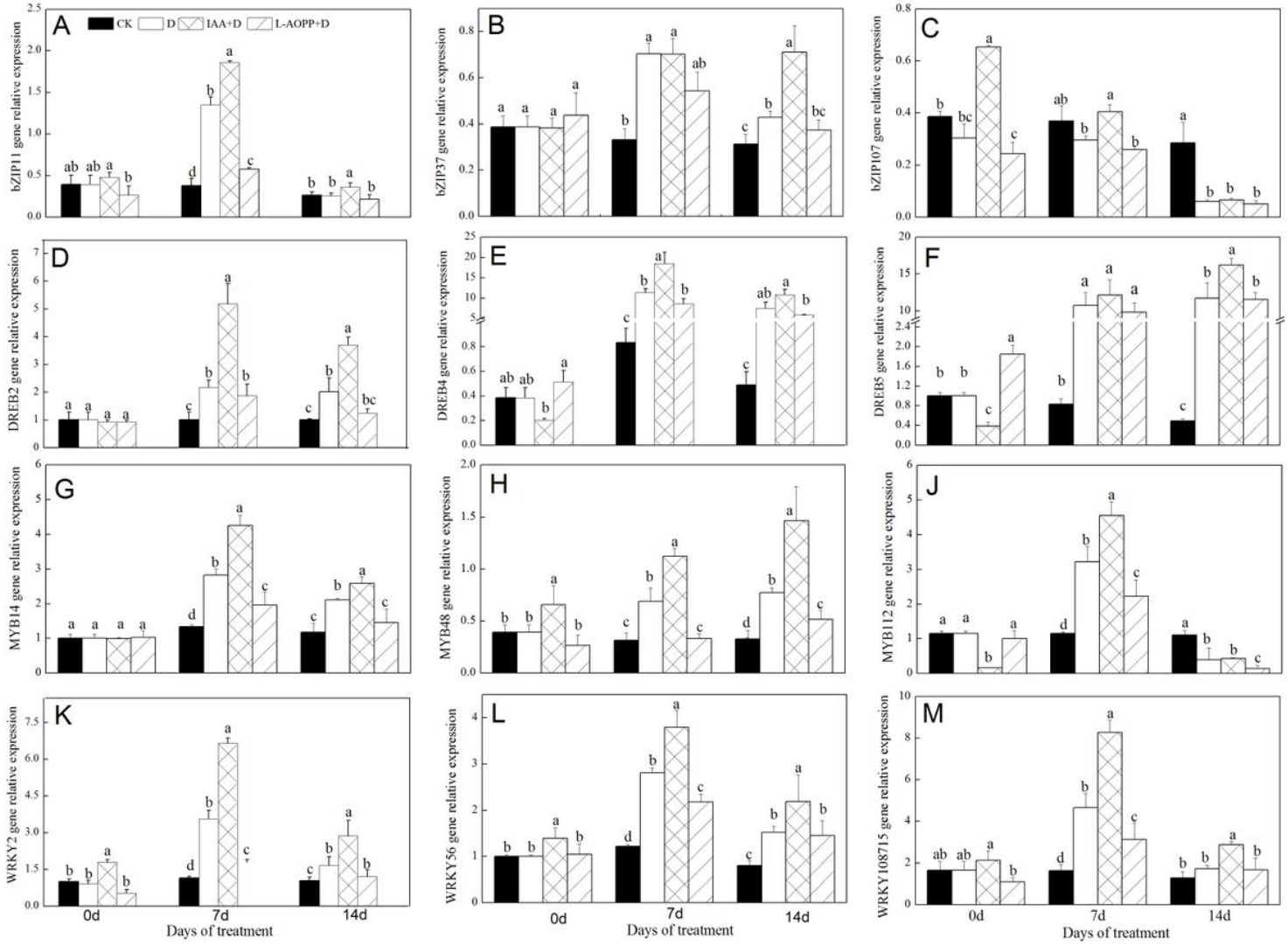

Figure 4

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 ($P < 0.05$).

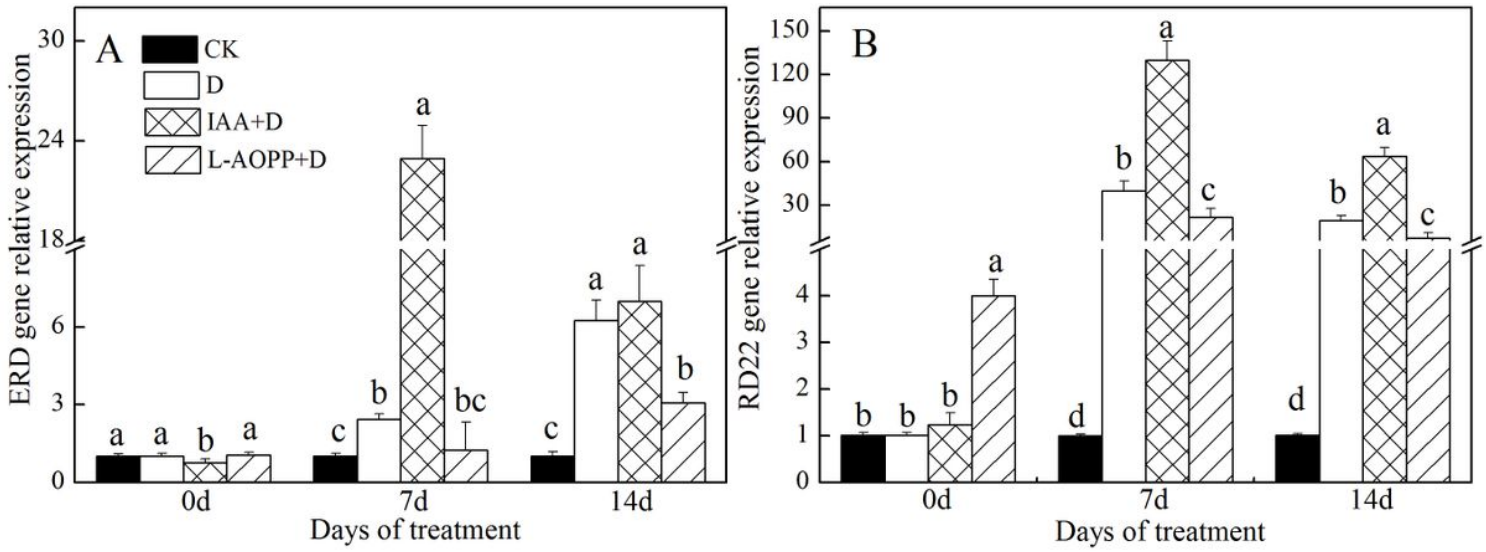

Figure 5

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).

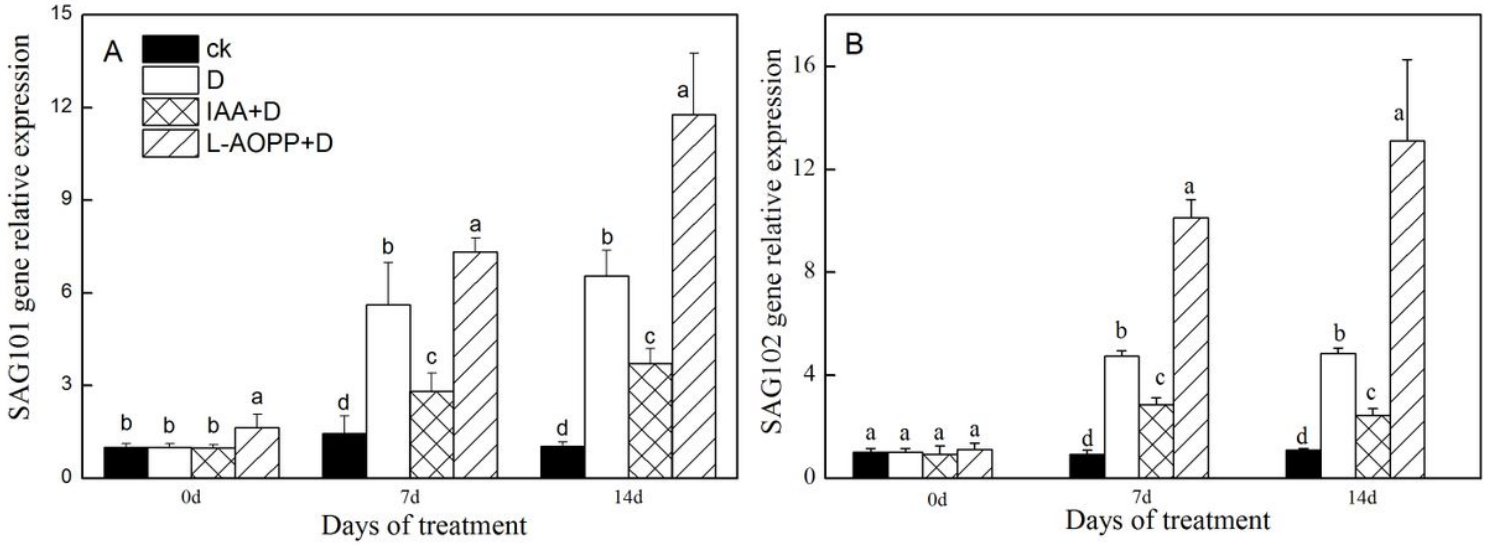

Figure 6

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).

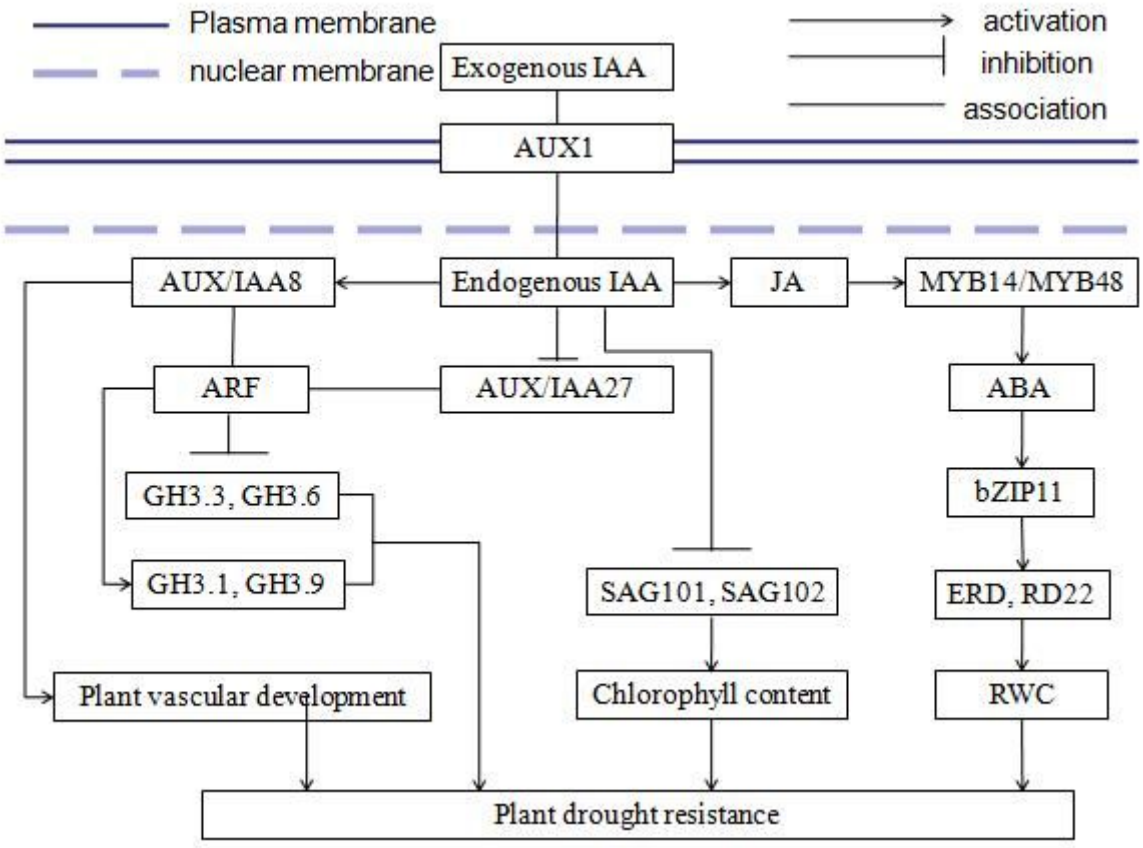

Figure 7

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

Supplementary Files

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- keymessage.doc