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EFFECTS OF SOIL DROUGHT STRESS ON PLANT REGENERATION EFFICIENCY AND ENDOGENOUS HORMONE LEVELS OF IMMATURE EMBRYOS IN WHEAT (*TRITICUM AESTIVUM* L.)

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Abstract

In this study, the water supply in soil for wheat mother donor plants was controlled, leading to drought stress conditions, and the relative soil water content (RSWC) was measured in different soil depths. The immature embryos of common wheat (*Triticum aestivum* L.) 13 days post anthesis (DPA) were used to test regeneration capacity. The accumulation of the plant growth regulators (PGRs) including abscisic acid (ABA), indole-3-acetic acid (IAA), and hydrogen peroxide (H_2O_2) in the wheat embryos grown under the two conditions was measured. The results indicated that RSWC difference between the drought treatment and the irrigated control was more than 13% at the various soil depths, with the maximum difference was observed at 40 cm depth. Tissue culture evaluation showed that the plant regeneration efficiency of the immature embryos grown under drought stress treatment was significantly higher than that of the tissues grown under the control condition. Assay for PGR found that the drought stress caused obviously increased concentration of endogenous ABA and H_2O_2, and slightly decreased level of IAA in the target tissues. Therefore, it seems that the concentration of endogenous ABA, IAA, and H_2O_2 in immature wheat embryos is very important in regeneration capacity. Drought stress can improve the regeneration capacity by changing the levels of ABA, IAA, and H_2O_2. Our results would be helpful to efficient development of genetically modified wheat plants through improvement of regeneration via manipulating the endogenous PGRs.

Key words: Wheat, Drought stress, Immature embryos, Plant regeneration, Plant growth regulators.

Introduction

Wheat (*Triticum aestivum* L.) is one of the world's most important crops, but the technology for its genetic engineering and transgenic breeding has lagged behind that of the other major cereal crops. This is largely due to the low efficiency of wheat genetic transformation, which impedes the functional analysis of candidate genes. Immature wheat embryos are one of the most popularly used explants tissues for wheat biotechnology as they have high efficiency of somatic embryogenesis compared to other tissues. Many factors affect wheat plant regeneration frequency; these include genotype (Nadolska-Orczyk & Malepszy, 1989; Pellegrineschi *et al.*, 2002; Rashid *et al.*, 2002; Zhao *et al.*, 2006); explant tissue used (Tang *et al.*, 2006); and environmental factors, for example, the growth temperature of donor plants (Kiyosue *et al.*, 1993; Kamada *et al.*, 1994; Wang *et al.*, 2014).

Environmental stress has a significant impact on the induction of somatic embryogenesis in plants. Many studies have shown that stress conditions enhance the regeneration capacity of plant tissues (Khanna &Daggard, 2001; Papadakis *et al.*, 2001; Blokhina *et al.*, 2003; Szechyńska-Hebda *et al.*, 2007; Obert *et al.*, 2005; Karami & Saidi 2010). Though it must be noted that excessive stress leads to cell death, it is also known that light stress does not increase regeneration efficiency (Lichtenthaler *et al.*, 1998). Pasternak *et al.* (2002) found that stress not only promoted dedifferentiationof cultured cells *In vitro*, but also impacted the somatic embryogenesis. Jain *et al.* (1996) discovered that partial desiccation of aromatic *indian* rice calli resulted in an up to threefold increase in the shoot regeneration frequency. It is also known that drought stress treatment can increase the plant regeneration rate in soybean (*Glycine max* L.) (Hammatt & Davey, 1987), *japonica* rice (Tsukahara & Hirosawa, 1992), and *indica* rice (Rancé *et al.*, 1994). Related studies have indicated that drought stress can simultaneously alter the accumulation of endogenous plant growth regulators (PGRs) such as abscisic acid (ABA), indole-3-acetic acid (IAA), and hydrogen peroxide (H_2O_2) in plants (Zhu, 2002; Farooqi *et al.*, 2005; Luna *et al.*, 2005).

ABA as a plant hormone is known to be very important in plant somatic embryogenesis (Nishiwaki *et al.*, 2000). When ABA biosynthesis mutant tobacco (*Nicotiana plumbaginifolia*) lines *aba1* and *aba2* and the wild type tobacco treated with an ABA biosynthesis inhibitor (fluridone) were cultured on ABA-free medium, their somatic embryogenesis development was disturbed at the early stages because ABA biosynthesis pathway was blocked. However, when ABA was complemented in the culture medium, somatic embryo development was restored in the *In vitro* cell cultures (Senger *et al.*, 2001). The application of 2.5 to 5 mM ABA to coconut (*Cocos nucifera* L.) cultures for 5 weeks was found to be effective for inducing somatic embryogenesis. Compared with medium that contained low levels of 2,4-D, a large number of somatic embryos were produced on the

medium containing ABA, and these developed normal shoots and complete plants (Fernando & Gamage, 2000). Ivanova *et al*. (1994) found that rapid induction of embryogenesis in *Medicago falcata* was correlated with high IAA and low ABA levels in initial explants, and that high endogenous ABA concentrations inhibited the response of plant cells to stress or exogenous ABA signal. In the process of sunflower somatic embryogenesis, ABA was shown to increase the synthesis of endogenous IAA and to influence plant regeneration (Charriére *et al.*, 1999). Huang *et al*. (2012) used high levels of ABA and IAA in the culture medium for rice andobtained high increased shoot organogenesis rates.

High concentrations of IAA are supposed to be closely related to embryonic development in the tissue culture of cherry rootstock (Michalczuk & Druart, 1999). Application of exogenous 2, 4-D can promote the accumulation of endogenous IAA in carrot, and increase embryogenesis capacity, which is likely to be closely related to the rapid increase of endogenous IAA that is induced by exogenous 2, 4-D application (Michalczuk *et al.*, 1992). Pasternak *et al.* (2002) found that exogenous 2, 4-D application could induce mass formation of endogenous IAA in the calli derived from alfalfa leaf protoplasts (*Medicago sativa* subsp. *varia* A2) during the first 2-3 days of culturing. IAA content was elevated by different level in both embryonic and non-embryonic calli of alfalfa under the above situation.

It is known that a lot of oxidative stress reactions occur during plant tissue culture (Papadakis *et al.*, 2001; Obert *et al.*, 2005; Pasternak *et al.*, 2005; Szechyńska-Hebda *et al.*, 2007; Zhang *et al.*, 2010). Oxidative stress resulting from the low capacity of cereal crops to remove reactive oxygen species (ROS) is thought to be one of the main reasons that these species exhibit low regeneration efficiencies for protoplast cultures (Cutler *et al.*, 1991). Studies have shown that many physiological, growth, and development processes in plants are regulated by H_2O_2, which include stomatal closure, geotropism, phototropism, programmed cell death, system adaptability obtaining, morphologic development shifting and growth controlling (Neill *et al.*, 2002). In *Larixleptolepis*, higher H_2O_2 concentrations induced by ABA are known to boost callus growth and to mediate the expression of genes including *APX*, *CAT*, and *SOD* in somatic embryogenesis (Zhang *et al.*, 2010). Increased H_2O_2 concentrations typically suggest that a destructive oxidative balance may promote the expression of the genes involved in morphogenesis (Libik *et al.*, 2005). Researchers also found that H_2O_2 can be used in the culture medium for *Gladiolus* (*Gladiolus hybridus* Hort.) to improve somatic embryogenesis (Gupta & Datta, 2003). Szechyńska-Hebda *et al.* (2007) suggested that immature wheat embryos that derived calli with optimal regeneration ability had higher antioxidant enzyme activities. She *et al.* (2013) also found that wheat calli with higher regeneration capacity had higher levels of H_2O_2 accumulation.

In summary, drought stress has been demonstrated to have a positive effect on plant regeneration and to influence endogenous hormones in some model plants. But, very little research directly addressing the effect of drought stress on somatic embryogenesis of immature wheat embryos has been conducted to date. In this study, we observed that drought stress caused changes in the concentrations of ABA, IAA, and H_2O_2 in immature wheat embryos and that these changes positively influenced the regeneration efficiency of these materials for the first time.

Materials and Methods

Plant materials and growth conditions: Three wheat varieties, CB037, Kenong199 (KN199), and Yangmai158 (YM158), were used in this study for our investigation of the regeneration of immature embryos growing under different conditions. CB037 and KN199 were kindly provided, respectively, by Prof. Xiao Chen and Prof. Yang Zhou at the Institute of Crop Science (ICS) of the Chinese Academy of Agricultural Sciences (CAAS) in Beijing, China. YM158 was kindly provided by Prof. Shunhe Cheng of the Lixiahe Institute of Agricultural Sciences of the Jiangsu Academy of Agricultural Sciences (JAAS) in Yangzhou, China. The materials were grown in the autumn of 2011 inside an auto-anti-canopy at ICS-CAAS, in which the water supply was controlled during the wheat growth period. Before wheat sowing, the field was well irrigated. Subsequently, for the duration of the experiment, no water was applied for the drought condition treatment; the control was irrigated before and after the winter period as well as the jointing and booting stages. The immature wheat seeds of the three wheat genotypes, 13 days post anthesis, were collected from the treatment and control plots in May of 2012 for the *In vitro* regeneration experiments and the analysis of endogenous hormone content.

Culture medium: MSS medium (MS basic medium + 3% sucrose + 1.0 mg L^{-1} VB1 + 150.0 mg L^{-1} asparagine + 2.4 g L^{-1} phytagel + 2.0 mg L^{-1} 2, 4-D, pH 5.8) was used to induce embryonic calli from the immature wheat embryos. IEFH medium (1/2 MS basic medium + MS vitamins + 2% sucrose + 2.4 g L^{-1} phytagel, pH 5.8) was used to induce shoot production from the embryonic calli. Media was autoclaved at 121°C and 1.1 Mpa for 20 min.

Plant regeneration: Immature wheat seeds were surface sterilized in 70% ethyl alcohol for 1 min and in 15% sodium hypochlorite for 10 min. Seeds were then washed four times with sterile water. The immature embryos of around 1.0 mm width were isolated from the sterilized seeds and cultured on MSS medium, with the scutellum facing up. About 450 immature embryos were used for each treatment of every genotype in three replications. Embryonic calli were counted when the immature embryos were inoculated for 21 d on MSS medium in darkness at 25±1°C, and then the calli were transferred to IEFH medium at 25±1°C and a 16/8 h photoperiod (photosynthetic photon flux density of 400 μmol m^{-2} s^{-1}, relative humidity of 45%). Differentiated calli and plantlets were scored after culturing for two weeks. Regeneration efficiency (%) was calculated according to the number of regenerated plantlets divided by the total number of immature embryos incubated in the experiment.

Measurement of relative soil water content: During the sampling time for the immature embryos, we measured the relative soil water content (RSWC) at the 20 cm, 40 cm, 60 cm, 80 cm, and 100 cm soil depth in the field, for both the drought treatment and the control. The relative soil water content measurements were made using an AP-

204 Moisture Meter AquaPro-Sensors. The sensors of the instrument system for measuring the relative soil water content were buried in soil in auto-anti-canopy at ICS-CAAS. We obtained the corresponding data from the control system when collecting wheat immature embryos.

Measurement of ABA, IAA, and H_2O_2 content: Immature wheat seeds 13 days post anthesis (DPA)were collected, quickly frozen using liquid nitrogen, and ground prior to measurement of ABA, IAA, and H_2O_2 content. ABA and IAA content were measured at the Institute of Genetics and Developmental Biology of Chinese Academy of Sciences according to the protocol described by Fu *et al.* (2012). H_2O_2 content was analyzed according to the protocol described by Zhang *et al.* (2010).

Statistical analysis: The data on regeneration efficiency and the endogenous content of ABA, IAA, and H_2O_2 were analyzed using ANOVA implemented in the R language. The differences between different treatments were evaluated with Turkey's pairwise comparison method.

Results

Effects of drought stress of wheat donor plants on the regeneration of immature embryos: The immature embryos isolated from the wheat plants grown under different water conditions were cultured on MSS medium for three weeks for callus induction. The embryonic calli were then transferred to IEFH medium and cultured for two weeks for the production of green shoots. The regeneration efficiencies for the different genotypes and the different treatments were calculated based on the number of immature embryos inoculated, the number of embryonic calli produced, and the number of green shoots regenerated (Table 1). The regeneration efficiency was 401.11% for CB037 under the drought condition (Fig. 1D), which was about two folds higher than that for the CB037 in the normal irrigation condition (Fig. 1A). The regeneration efficiency was 122.30% for YM158 under drought stress; this was higher than the value (103.88%) for YM158 under the normal irrigation condition (Table 1, Fig. 1B and 1E). For KN199, however, the regeneration efficiency under drought stress (118.99%) was lower than that under the normal irrigation condition (180.51%) (Table 1, Fig. 1C and 1F). Based on inferential statistical analysis, the differences in the regeneration efficiencies between the drought and control conditions were significant at $p<0.05$ level for each of the three genotypes (Table 1).

Effects of relative soil water content on the regeneration capacity of immature wheat embryos: Wheat roots are typically distributed within 100 cm (depth) of soil (Barraclough & Leigh, 1984). To understand the strength of the drought stress condition in our experiment in soil at the time of collecting the immature wheat seeds for tissue culture and the endogenous hormone assay, we measured the relative soil water content at different soil depths within the root zone for both the drought treatment and the normal irrigation treatment soils (Fig. 2). The RSWC for each soil depth for the drought treatment was significantly lower than that of the corresponding layer for the normal irrigation treatment (Fig. 2). There was a particularly pronounced difference in RSWC at the soil depth of 40-60 cm. For this range of soil depth, the RSWC was 33 to 60% for drought stress and 46 to 70% for the control. Irrigating or not irrigating the experimental fields caused different drought stress of soil and further led the physiological change of wheat donor plants. Lower RSWC was beneficial to plant regeneration of wheat immature embryos.

Analysis of ABA, IAA, andH_2O_2 content in immature wheat embryos grown under drought and normal irrigation treatments: To further explore the physiological basis through which drought or low RSWC enhanced the regeneration capacity of the immature wheatembryos, the concentrations of endogenous ABA, IAA, and H_2O_2 were measured in the immature grains of the tested wheat genotypes (Fig. 3).

The ABA content in the immature seeds of each of the three wheat cultivars grown in the drought condition were higher than those grown under the normal irrigation condition. The differences in ABA were statistically significant for CB037 and YM158 between the drought and normal irrigation treatment. For example, ABA contents were 47.14 pg mg^{-1} under drought stress and 31.09 pg mg^{-1} under control condition for CB037, and its regeneration efficiencies were 401.11% under drought stress and 230.02% under control condition, both differences being significant (Table 1, Fig. 3). A similar trend was observed for KN199, but the differences were not statistically significant between the two treatments. These results suggested that drought stress led to a increased ABA content in donor wheat plants. We observed that increased ABA levels had a positive effect on the regeneration capacity of immature wheat embryos (Table 1).

Table 1. Regeneration of wheat immature embryos under drought stress treatment.

Genotypes	Treatments	Immature embryos	Embryonic calli	Differentiated calli	No. of plantlets	Regeneration efficiency (%)	
						0.05 level	0.01 level
CB037	Control	493	468	429	1134	230.02b	B
	Drought	360	343	320	1444	401.11a	A
YM158	Control	541	485	258	562	103.88b	A
	Drought	592	534	324	724	122.30a	A
KN199	Control	467	420	326	843	180.51a	A
	Drought	437	349	274	520	118.99b	B

Note: Small letters and capital letters stand for statistically significant differences at $p<0.05$ and $p<0.01$ probability levels, respectively. Within a column, means denoted by the same letter are not significantly different according to the test at $p< 0.05$ or $p< 0.01$

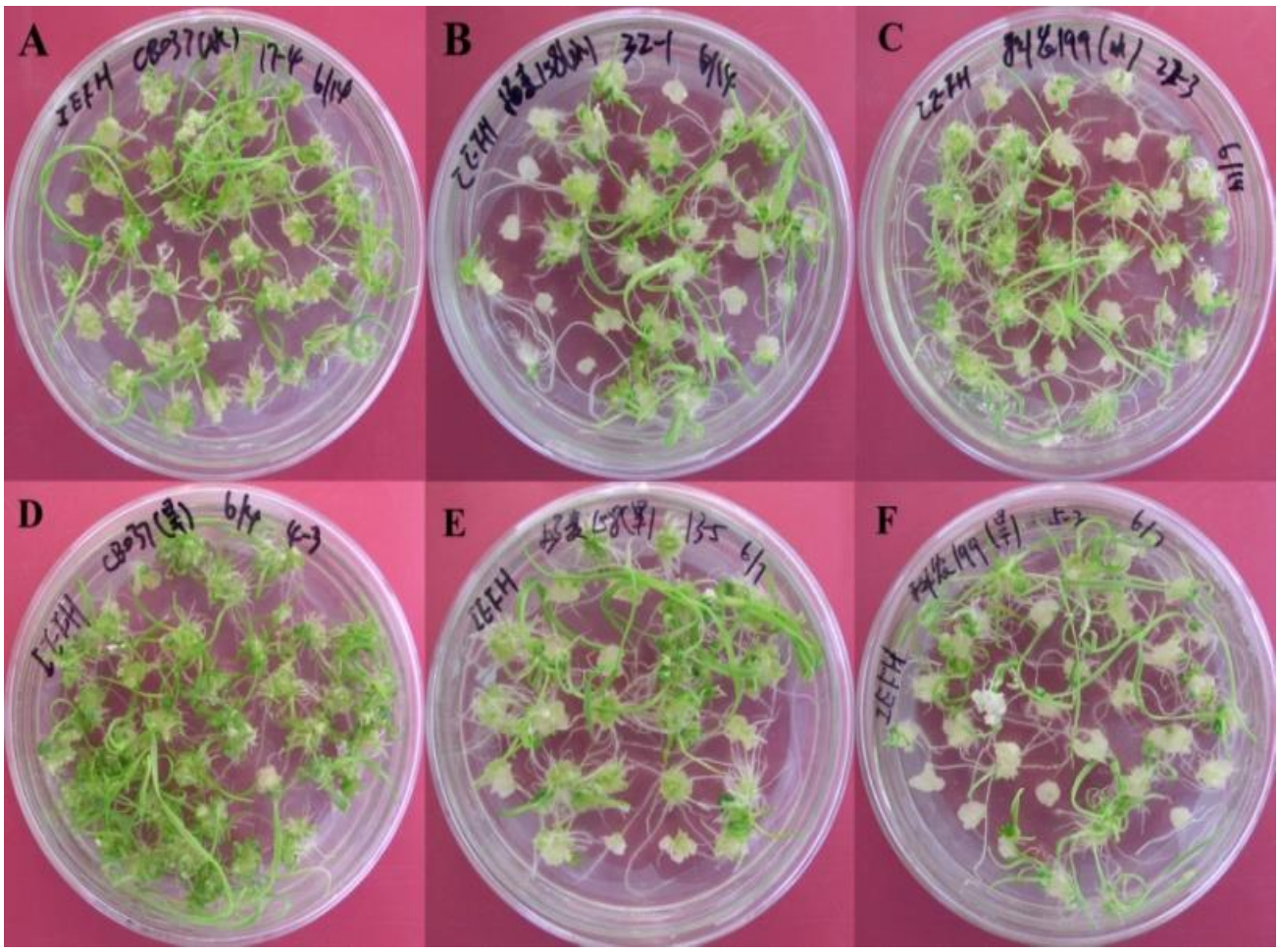

Fig. 1. Regeneration status of the immature embryos from different wheat cultivars grown under the drought stress and the normal irrigation conditions. A, B, C: shoot regeneration under the control treatment for CB037, YM158, and KN199, respectively; D, E, F: shoot regeneration under the drought stress treatment for CB037, YM158, and KN199, respectively.

Compared to the normal irrigation samples, drought stress led to a decrease in the endogenous IAA content in CB037 and YM158 (Fig. 4), which were 75.63 pg mg^{-1} under control condition and 45.00 pg mg^{-1} under drought stress for CB037, and 57.57 pg mg^{-1} and 34.02 pg mg^{-1} for YM158, respectively. While, the IAA content in KN199 was 24.40 pg mg^{-1} under the control condition and 33.51 pg mg^{-1} under drought stress. These results indicated that drought stress reduced IAA levels in the immature seeds of the majority of the wheat genotypes we tested; we speculate that lower IAA levels might be propitious for the regeneration of wheat immature embryos (Table 1, Fig. 4). Though the IAA content in KN199 under the drought condition was increased compared to the normal irrigation condition (Fig. 4), however, its regeneration efficiency under the drought condition was decreased compared to with the normal irrigationcondition (Table 1).

H_2O_2 content in the immature grains displayed similar trends with ABA content. In the three wheat genotypes, the H_2O_2 content in the immature seeds under the drought condition was all higher than that under the control condition (Fig. 5). The H_2O_2 content in the immature grains of CB037 grown under drought stress was 7-foldhigher that grown under the normal irrigation condition.For the KN199 wheat variety, the H_2O_2 content in its immature grains under drought stress was more than 3-fold greater than that of grown under normal irrigation condition.For the YM158 wheat variety, the H_2O_2 content in its immature grains under both conditions was highly similar (not significantly different). The differences in H_2O_2 levels in the immature kernels between the two conditions was highly significant for both the CB037 and the KN199 genotypes, but was not significant for YM158. We infer that drought stress resulted in higher H_2O_2 levels in the target tissues, and that the increased concentration of this signal molecule led to the increased regeneration of the target tissues (Table 1).

In summary, the ABA and H_2O_2 content in immature wheat seeds grown under drought stress were increased, and the IAA content was decreased, as compared with the samples from the normal irrigation condition. We conclude that drought stress led to increase in ABA and H_2O_2 and decreases in IAA in the immature embryos of donor wheat plants, and suppose that these changes resulted in the observed elevated regeneration capacity of the immature embryo (Table 1).

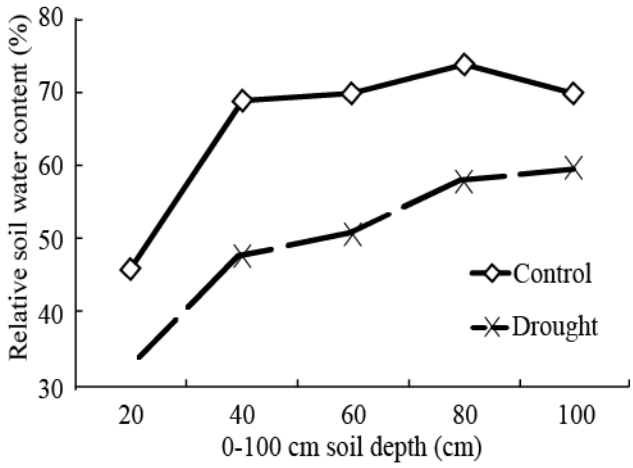

Fig. 2. Trend of the relative soil water content (RSWC) in various soil layers for the drought stress and normal irrigation treatments. The RSWC in each soil layer under drought stress was significantly lower than that in the corresponding layer for the normal irrigation condition. The largest difference between the two treatments was observed at the 40 to 60 cm soil depth.

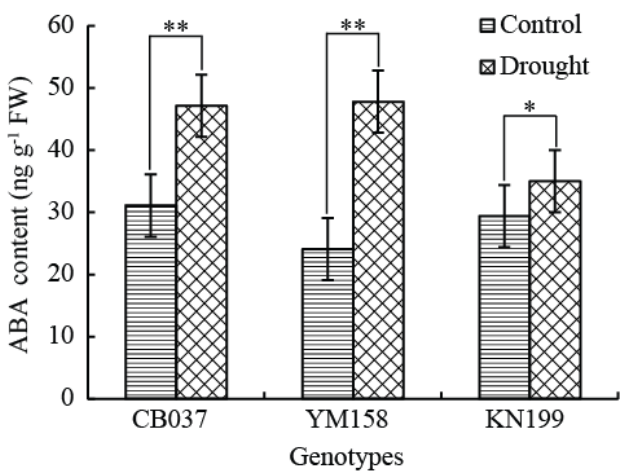

Fig. 3. ABA content in immature wheat grains grown under drought and control conditions. One or two asterisks over the histogram represent statistically significant differences at the $p<0.05$ or $p<0.01$ probability levels, respectively.

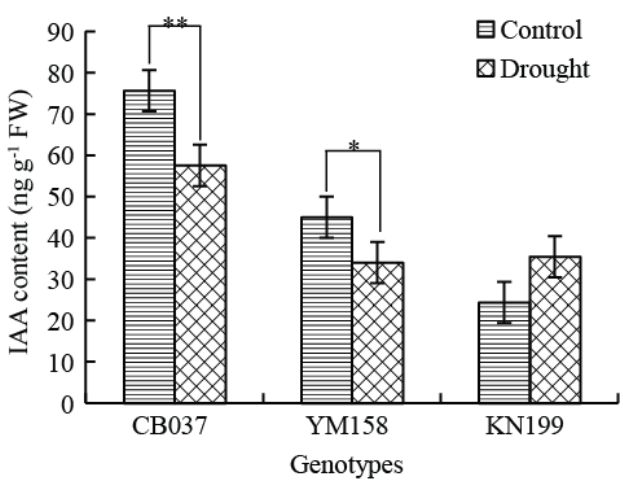

Fig. 4. IAA content in immature wheat grains grown under drought and control conditions. One or two asterisks over the histogram represent statistically significant differences at the $p<0.05$ or $p<0.01$ probability levels, respectively.

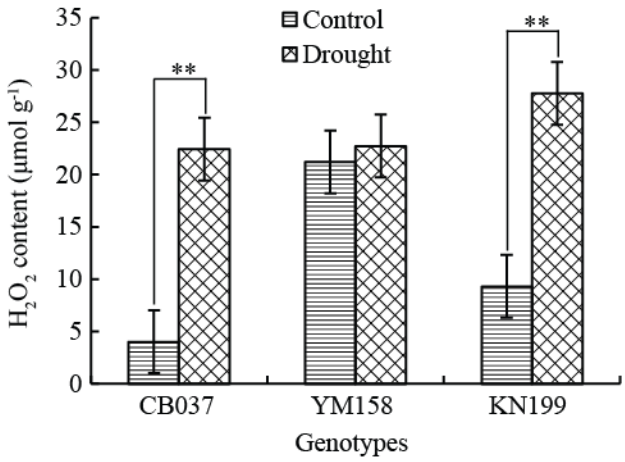

Fig. 5. H_2O_2 content in the immature grains of wheat grown under drought and control conditions. One or two asterisks over the histogram represent statistically significant differences at the $p<0.05$ or $p<0.01$ probability levels, respectively.

Discussion

Compared with some model plants such as tobacco, rice, and *Brachypodium distachyon*, wheat exhibits low regeneration rates and strong genotype dependence in tissue culture applications. This situation has resulted in lagged progress for wheat in genetic modification research. Many factors, including genotypes, phytohormone levels, medium, and physiological status are known to crucially influence the regeneration efficiency of immature wheat embryos *In vitro*. Optimization of these factors has greatly contributed to the improvement of wheat regeneration and transformation efficiencies, and immature embryos are now typically used as explants.Drought stress treatment of *In vitro* soybean (*Glycine max* L.) callus cultures was found to increase plant regeneration potential (Hammatt & Davey, 1987). This was also observed with *japonica* rice (Tsukahara & Hirosawa, 1992) and *indica* rice (Rancé *et al.*, 1994). Moreover, it is known that desiccation of immature embryos increases the stable transformation efficiency in wheat (Cheng *et al.*, 2003). In this study, we found that drought stress to donor plants during the entire growth period improved the regeneration capacity of immature embryos in wheat. To explore the possible reasons of this observed improvement, the concentrations of ABA, IAA, and H_2O_2 in the immature seeds from the drought-treated donor plants were measured. The result suggested that drought stress caused the changes in the levels of endogenous ABA, IAA, and H_2O_2levels, and the endogenous PGRs then influenced the regeneration efficiency of wheat immature embryos.

ABA biosynthesis ability in the wheat callus originated from the immature embryos cultured on osmolarity and ABA containing medium was significantly strengthened and promoted embryogenesis and organogenesis (Brown *et al.*, 1989). A previous study found that the application of exogenous ABA was advantageous for increasing the regeneration frequency of rice callus via both organogenesis and somatic embryogenesis (Jiang *et al.*, 2006). Application of 0.3 mg L^{-1} ABA in callus induction medium can significantly improve quality and regeneration efficiency of wheat calli (Bie *et al.*, 2011). In this study, we found that the ABA level in the immature CB037, YM158,

and KN199 seeds grown under drought stress condition was higher than that in the corresponding immature seeds grown under normal irrigation condition. Combined with increased regeneration performance of CB037 and YM158 under drought stress condition, we summarize that the regeneration efficiency of wheat immature embryos can be improved by regulating endogenous ABA levels and adding exogenous ABA.

It is interesting that drought stress led to a decrease in the endogenous IAA content in CB037 and YM158. Combining the regeneration results of the immature embryos, we found that endogenous IAA content was closely and positively associated with increased regeneration efficiency. It is known that the IAA concentration, within a suitable range, is advantageous for the regeneration of immature wheat embryos and that excessive endogenous IAA will inhibit regeneration capacity. Our results are in agreement with the previously reported findings on this subject (Charriére*et al.*, 1999; Suzuki *et al.*, 2004; Zhang *et al.*, 2008).

In this study, we also found that H_2O_2 content of immature wheat embryos had a close relationship with regeneration capacity. With larger differences in H_2O_2 content, CB037 and KN199 showed high regeneration efficiency in this study. The H_2O_2 content in the immature grains of YM158 under both conditions was almost the same, and its regeneration efficiencyunder both conditions was similar. Most recently, Zhang *et al.* (2015) found that adding 0.005 to 0.01‰ H_2O_2 in callus induction medium enhanced embryonic calli production and plant regeneration from the immature embryos of wheat. Therefore, suitable higher H_2O_2 levels in explants or culture medium can be helpful for increasing regeneration capacity in immature wheat embryos.

The present study revealed that drought stress was beneficial in improving the regeneration capacity of immature wheat embryos. Drought stress caused changes in the accumulationlevels of endogenous IAA, ABA, and H_2O_2 in immature wheat grains. In fact, our recent publications have reported that adding ABA and H_2O_2 in culture medium resulted improved regeneration efficiency of wheat immature embryos (Bie *et al*., 2011; Zhang *et al*., 2015). Comparing with media modification, drought stress might better balance the endogenous hormones in wheat immature embryos which benefit to embryogenesis. Therefore, drought stress treatment of the mother plants showed ideal improvement of plant regeneration from wheat immature wheat embryos. Combination of drought stress and plant growth regulators adding in culture media might enhance plant regeneration efficiency of immature wheat embryos dramatically. Our results have the potential to improve wheat genetic transformation efficiency.

Acknowledgments

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