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Effects of exogenous abscisic acid and gibberellic acid on filling process and nitrogen metabolism characteristics in wheat grains

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Abstract

Hormones play important roles in regulating the grain filling process and protein accumulation. In this study, two wheat cultivars differing in grain protein concentration were used to investigate the changes in endogenous hormone contents and the mechanism of hormonal regulation of grain filling and protein accumulation under exogenous application of abscisic acid (ABA) or gibberellic acid (GA_3). Results showed that the mean grain weight of the ABA treatment was 11.8% and 5% higher than that of control in Shannong1391 (SN1391) and Gaocheng8901 (GC8901), respectively. The equivalent values for SN1391 and GC8901 were 16.9% and 6.9% after GA_3 was applied. The protein concentration was significantly increased by 17.6% and 15.3% in response to application of ABA in SN1391 and GC8901, respectively, and by 21.2% and 12.0% responding to GA_3. GA_3 showed much higher effects than ABA on glutamine synthetase (GS) activity and free amino acid contents. The elevated contents of endogenous ABA and gibberellins (GA_s) were closely associated with the exogenous ABA and GA_3, respectively. The indole-3-acetic acid (IAA) contents reduced at the early grain filling period under the application of ABA, but it increased responding to exogenous ABA and GA_3 at the mid and later grain filling period. Correlation analysis demonstrated that grain filling rate positively and significantly correlated with the contents of GA_s and IAA and ABA, free amino acid contents and GS activity significantly correlated both with the contents of ABA and GA_s, and soluble protein contents significantly correlated with the contents of ABA and IAA. It can be concluded that the effects of exogenous hormones were predominantly mediated by changes in endogenous hormone contents, which affected grain filling process, nitrogen metabolism characteristics, and, therefore, changed protein accumulation amounts.

Keywords: Protein accumulation; Grain filling; Hormone contents; Nitrogen metabolism characteristics.
Abbreviations: ABA-Abscisic acid; DGF-Duration of grain filling; DPA-Days post-anthesis; GA_3-Gibberellic acid; GA_s-gibberellins; GC8901-Gaocheng8901; GS-Glutamine synthetase; HPLC- High performance liquid chromatography; IAA-Indole-3-acetic acid;SN1391-Shannong1391; IGFP-Initial grain filling potential.

Introduction

Protein accumulation is the fundamental process determining the quality of wheat grains (Jiang et al., 2009; Li et al., 2011; Payne et al., 1981; Weegels et al., 1996). Studies indicated that protein accumulation is related to grains filling process and protein concentration, which were regulated by environmental factors, such as water conditions, nitrogen application, heat stress etc. Plant growth regulators play important roles in the grain filling process and determining the level of proteins, which respond differently to the environmental stimuli (Bano et al., 1993; Jackson et al., 1988). Drought condition is closely associated with the reduced levels of endogenous IAA, ZR and GA_s and elevated level of ABA in organs, especially in grains (Davies et al., 1986; Xie et al., 2003). Numerous studies proved that changes in endogenous hormone contents affected grain filling process and protein concentration under different cultivation practices (Majid et al., 2011; Xie et al., 2003; Zahir et al., 2001), but the effects were unclear under exogenous application of ABA or GA_3.
Grain filling process was closely associated with the changes in hormone contents (Yang et al., 2001; Yang et al., 2003). The increase in ABA contents at the end of grain filling and its rapid fall during maturation have leads to an assumption that ABA plays an important role in dry matter accumulation (Beruter et al., 1983; Eeuwens et al., 1975; Schussler et al., 1984; Travaglia et al., 2007; Wang et al., 1999). Gibberellins (GA_s) have been proved to be an important phyto-hormone that regulates the duration of grain filling in crops (Zhang et al., 2009). Lur and Setter (1993) observed that IAA contents abruptly increased in the endosperm of maize kernels at about 10 d after pollination, which was in coincidence with the increase in deoxyribonucleic acid content per nucleus. Previous studies have shown that endogenous hormones are essential regulators for grain filling in cereal crops, and regulate the grain weight via influencing grain filling process and activities of key enzymes involved in sucrose-to-starch conversion in cereal organs (Ahmadi et al., 1999; Brenner et al., 1995; Lee et al., 1989; Yang et al., 2004). Currently, there is a lack of understanding of how exogenous hormones influence grain filling process, and the proposal that exogenous hormones are involved in the regulation of endogenous hormones levels remains disputable. Nitrogen metabolism characteristics, such as GS activity, soluble

protein contents and free amino acid contents were known to be involved in the changes of protein concentration (Zhao et al., 2005). Integrating the previous reports in wheat and other crops, Xie et al. (2003) concluded that enhanced ABA contents generally increases protein concentration, the relationship between the IAA contents and protein accumulation in grains was negative at the grain enlargement stage, but positive at the grain filling stage and the effects of enhanced ABA and IAA contents on protein accumulation were mainly mediated via enhancing mRNA production and protein gene expression. Only few studies have been reported that elucidate relationships between protein concentration and the activity changes in key regulatory enzymes of the free amino acid to protein pathway under exogenous application of ABA or GA_3. Furthermore, it is of interest to understand the relationships between the variations in hormone contents and nitrogen metabolism in grains under exogenous application of ABA or GA_3. The results mentioned above indicated that the grain filling process and protein concentration were influenced by changes of endogenous hormone levels. However, little was known about the implication of post-anthesis exogenous application of ABA or GA_3 on grain filling process and protein concentration. Therefore, the objective of this study was to elucidate the relationship between endogenous hormones variations and grain filling process and nitrogen metabolism characteristics under the application of ABA or GA_3, and to provide a theoretical basis for guiding regulation of wheat productivity gains and grain quality formation in wheat by exogenous application of growth substances.

Results

Exogenous ABA or GA_3 effects on the grain weight and grain filling characteristics

The mean grain weight was significantly increased after exogenous application of ABA or GA_3, but this increasing varied with cultivars and treatments. The mean grain weight of the ABA treatment was 11.8% and 5% higher than that of control in SN1391 and GC8901, respectively. The equivalent values for SN1391 and GC8901 were 16.9% and 6.9% when GA_3 was applied (Fig.1), indicating that the effects of exogenous GA_3 was much higher than exogenous ABA on increasing the grain weight. The grain filling process in both cultivars was well described by the logistic growth equation. The grain filling characteristics varied with cultivars and treatments. The coefficients K, C_0, T_{max} and T were significantly increased after ABA or GA_3 was applied. For SN1391, compared with the ABA treatment, application of GA_3 significantly increased the coefficients K, C_0 and T, while R_{max} and T_{max} showed a little variation (Table 2). Application of GA_3 significantly increased the R_{max} and T_{max} and decreased the C_0 in comparison with the ABA treatment in GC8901, whereas had no significant effect on T and K. Exogenous ABA or GA_3 induced changes in endogenous contents, which were associated with the varied grain filling process, have been shown in our previous studies (Yang et al., 2011).

Exogenous ABA or GA_3 effects on protein concentration, protein accumulation and protein components

Protein concentration transiently decreased in both cultivars (Table 3). They reached a minimum at 21DPA, and increased gradually thereafter. As shown in Table 3, application of ABA or GA_3 significantly increased the protein concentration in both cultivars, but the difference varied with cultivars and treatments. The protein concentration was significantly increased by 17.6% and 15.3% in response to exogenous ABA in SN1391 and GC8901, respectively, and by 21.2% and 12.0% responding to GA_3. In order to understand the impacts of exogenous ABA or GA_3 on protein accumulation, we calculated the changes of protein accumulation amounts. As shown in Table 4, protein accumulation amounts increased gradually with grain development in both cultivars. Application of ABA or GA_3 significantly increased the final protein accumulation amounts. Compared with the control, the final protein accumulation amounts increased 26.9% and 19.5% responding to exogenous ABA in SN1391 and GC8901, respectively, and increased 24.7% and 37.2% responding to GA_3, respectively. Compared with application of ABA, application of GA_3 significantly increased the amounts of protein accumulation, whereas no significant effect was observed in SN1391. Exogenous application of ABA or GA_3, compared to the control, significantly increased concentration of glutenin and gliadin in the two cultivars and globulin in SN1391 (Table 5), whereas the effects on albumin concentration in the two cultivars and globulin concentration in GC8901 were not significant, indicating that application of ABA or GA_3 was responsible for the increase in concentration of glutenin and gliadin for both cultivars.

Exogenous ABA or GA_3 increases the GS activity in grains

The activity of glutamine synthetase (EC 6, 3.1.2) was closely associated with the strength of grain ammonia assimilation. Our results suggested that GS activity in grains decreased gradually until 35 DPA in SN1391 and GC8901, but a transient increase was observed after application of GA_3 in SN1391 at the early grain filling stage (Fig. 2). Compared to the control, exogenous application of ABA or GA_3 significantly increased the GS activity from 7 to 21 DPA in grains, and the effects of GA_3 were much higher than ABA at the initial stage of grain filling in SN1391. A transient increase of GS activity from 7 to 14 DPA under application of ABA was uncovered in both cultivars.

Exogenous ABA or GA_3 increases the free amino acid contents in grains

Free amino acids play key roles in *in-vivo* transport of nitrogen assimilation and provide the substrate for protein synthesis. The levels of these amino acids determine the capacity of grain nitrogen assimilation and protein synthesis. Our results suggested that free amino acid contents in grains gradually increased in both cultivars, they reached a maximum at 14 DPA, and sharply decreased thereafter (Fig. 3). As compared to the control, application of exogenous ABA or GA_3 had a marked influence on free amino acid contents at the early and mid grain filling stage. Exogenous GA_3 treatment alone increased the free amino contents more than exogenous ABA treatment from 7 to 21 DPA, suggesting that free amino acid contents varied with the different hormones.

Exogenous ABA or GA_3 effects on soluble protein content in grains

Soluble protein includes kinds of enzymes related to the protein metabolism and its contents were an important indicator of protein metabolic intensity. Our results showed

Table 1. Weather conditions in 2009 and 2010, Tai'an, Shandong Province, China.

Year	Weather condition		
	Max.temp (℃)	Min.temp (℃)	Rainfall (mm)
2009	31.4	13.7	50.2
2010	31.9	10.1	43.3

Data were collected from 1 May to 5 June. temp. = temperature.

Table 2. Exogenous ABA or GA_3 effects on grain filling characteristic

Varieties	Treatments	Parameters characteristic							
		K	A	B	C_0	T_{max}	R_{max}	T	R^2
SN1391	CK	49.65c	4.418	-0.2	0.592c	22.09b	2.48a	33.07c	0.9878
	ABA	57.36b	4	-0.17	1.03b	23.53a	2.43a	36.45b	0.9876
	GA_3	62.58a	3.621	-0.152	1.63a	23.82a	2.178a	38.22a	0.9942
GC8901	CK	34.75b	4.48	-0.25	0.27c	17.92b	2.172b	26.71bc	0.9862
	ABA	37.75a	4.056	-0.227	0.64a	17.86b	2.14b	27.54ab	0.9946
	GA_3	36.32a	4.82	-0.25	0.49b	19.28a	2.67a	28.07a	0.9772

K, final grain weight (mg grain^{-1}); C_o, initial grain filling potential; R_{max}, maximum grain filling rate (mg g^{-1} grain^{-1} d^{-1}); T_{max}, appearance time of maximum grain filling Rate (d); T, duration of grain filling period (d); A and B are coefficients determined by regression; R^2, related coefficients.
CK, The control groups; ABA, exogenous abscisic acid was sprayed to the spikes; GA_3, exogenous gibberellin was sprayed to the spikes.

that soluble protein contents were increased constantly before 21 DPA, reaching a maximum at 21 DPA and falling quickly afterward (Fig. 4). Compared to the control, exogenous application of ABA or GA_3 significantly increased the soluble protein contents in grains. Again, exogenous application of GA_3 had a higher effect than the exogenous ABA for both cultivars from 7 to 21 DPA, whereas it was the opposite for the hormonal treatments from 21 to 35 DPA.

Effects of exogenous ABA or GA_3 on endogenous hormone contents in grains

The changes of endogenous hormone contents in response to exogenous ABA or GA_3 during grain filling were shown in Fig. 5. ABA and GA_s contents showed a similar pattern of changes during grain filling in the two cultivars. They both had a transient increase from 7 to 14 DPA, reaching a maximum at 14 DPA, and then gradually decreased. Contrast to ABA and GA_s contents, IAA contents were low at the initial grain filling stage. They reached a maximum at 21 DPA and declined slowly afterward. Compared to the control, application of ABA significantly increased the ABA contents and decreased the GA_s contents at the early and mid grain filling stage. The endogenous GA_s contents were significantly increased and ABA contents decreased after the GA_3 was applied. However, IAA contents were reduced at the early grain filling period with application of ABA, and it enhanced responding to application of ABA or GA_3 at the mid and later grain filling period.

Relationships of hormones contents to the grain filling rate and GS Activity and free amino acid contents and soluble protein contents

Correlation analysis demonstrated that contents of ABA and GA_s were positively and significantly correlated with grain filling rate (r=0.45* and 0.46*, P<0.05), GS activity (r=0.31*, r= 0.42*, P<0.05) and free amino acid contents (r= 0.74**, 0.72**, P<0.01). The correlation between the ABA contents and soluble protein contents was positive and significant (r=0.30*, P<0.05). IAA contents were neither significantly correlated with the GS activity nor significantly correlated with free amino acid contents, whereas positively and significantly correlated with soluble protein contents (r=0.32*, P<0.05).

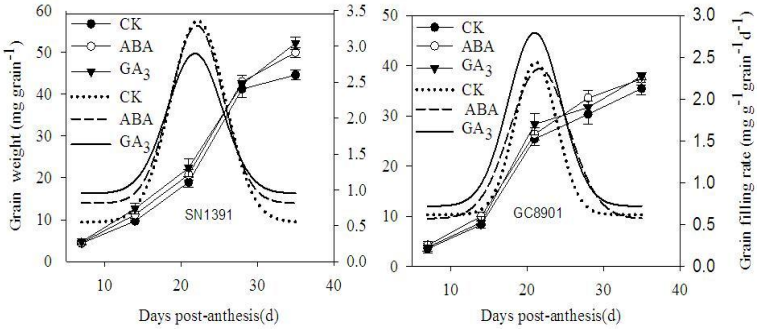

Fig 1. Exogenous ABA or GA_3 effects on single grain weight and grain filling rate of SN1391 and GC8901. The broken line and the smoothed curve represent the variation tendency of single grain weight and grain filling rate, respectively.

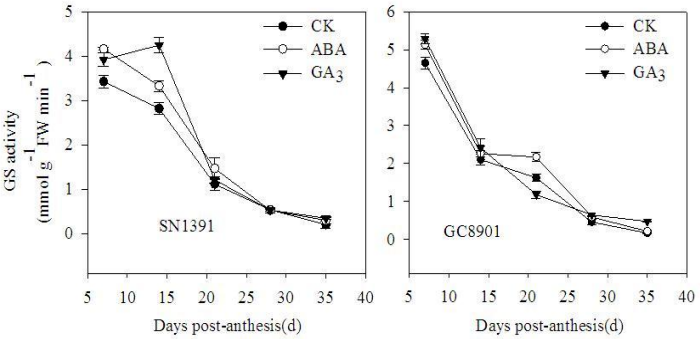

Fig 2. Exogenous ABA or GA_3 effects on GS activity in grains

Discussion

Protein concentration and grain weight were the fundamental process determining the protein accumulation, to a great extent, determine the quality of wheat grains. Phyto-hormones, such as ABA, GA_s and IAA, are known to be involved in the regulation of grain weight and protein concentration (Ahmadi et al., 1999; Xie et al., 2003).

Table 3. Exogenous ABA or GA_3 effects on protein concentration (g kg^{-1}, dry basis) in grains.

Treatments	Days post-anthesis (d)									
	7		14		21		28		35	
	GC8901	SN1391	GC8901	SN1391	GC8901	SN1391	GC8901	SN1391	GC8901	SN1391
CK	180.127a	154.72b	141.79b	144.81b	127.68b	120.25c	135.19b	131.33c	140.69b	127.73b
AB A	182.88a	152.68b	177.32a	138.94b	132.18b	143.27a	154.58a	169.45a	162.15a	150.33a
GA_3	171.24b	164.27a	182.26a	174.46a	140.15a	133.38b	152.54a	147.71b	157.65a	154.82a

CK, The control groups; ABA, exogenous abscisic acid was sprayed to the spikes; GA_3, exogenous gibberellin was sprayed to the spikes.

Table 4 Exogenous ABA or GA_3 effects on protein accumulation amounts（g 1000-$kernels^{-1}$) in grains.

Treatments	Days post-anthesis (d)									
	7		14		21		28		35	
	GC8901‡	SN1391	GC8901	SN1391	GC8901	SN1391	GC8901	SN1391	GC8901	SN1391
CK	0.550a	0.700ab	1.8438a	1.479b	2.415b	4.609a	3.825b	5.619a	3.916c	6.584b
ABA	0.693a	0.581b	1.394b	2.399a	3.583a	3.774b	4.112a	5.504a	4.860b	8.359a
GA_3	0.611a	0.812a	1.476b	1.718b	3.756a	3.756b	4.347a	5.024b	5.388a	8.212a

CK, The control groups; ABA, exogenous abscisic acid was sprayed to the spikes; GA_3, exogenous gibberellin was sprayed to the spikes.

Our data clearly demonstrated that application of ABA significantly increased grain weight, which was attributed to the changes in grain filling process (extended DGF and increased IGFP) and endosperm cell division (Yang et al., 2011). However, the mechanism by which the increased ABA facilitates grain filling process and endosperm cell division after exogenous application of ABA was not well understood. It has been observed that ABA regulates epidermal cell-type-specific gene expression in the meristematic zone of Arabidopsis (Barber et al., 2004), and it enhances the movement of photosynthetic assimilates toward to developing seeds (Brenner et al., 1995). It was also proposed to play an important role in relation to sugar-signaling pathways, enhance the ability of plant tissues to response to subsequent sugar signals and promote grain filling through regulating activities of key enzymes involved in sucrose-to-starch conversion in cereal organs (Kato et al., 1993; Yang et al., 2004). In the present study, the increased starch accumulation (unpublished data), extended duration of grain filling and improved initial grain filling potential, at least partly, lead to the increased grain weight. However, it has been reported that application of ABA significantly decreased duration of grain filling and these differences are most likely due to different concentration of exogenous hormones, and our data proved that the application of ABA (24mg L^{-1}) significantly reduced duration of grain filling (unpublished data). In addition, our results confirmed that the elevated ABA contents in grains regulated by exogenous ABA were closely associated with the increased protein concentration (Xie et al., 2003). However, there were focal questions about how the applied ABA leads to an increase in protein contents. Previous reports indicated that hormones acting as responding signals to abiotic stress play essential roles in regulating the characteristics of nitrogen metabolism (Bano et al., 1993; Davies et al., 1986; Jackson et al., 1988). Results were also proved in rape and alfalfa that elevated ABA contents favored grain growth in some cases, especially in storage protein accumulation and storage protein gene expression (Wilen et al., 1990; Xu 1995). In our study, ABA contents positively and significantly correlated with free amino acid contents and GS activity and soluble protein contents in grains, suggesting that the increased protein concentration was mainly due to the enhanced enzyme activity of ammonia assimilation and substrate concentration of protein synthesis under exogenous application of ABA. Yang (2001) observed that high contents of gibberellins (specifically GA_1 and GA_4) during rice grain filling stages

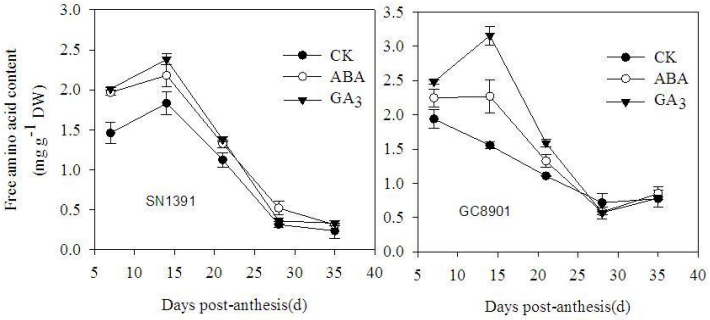

Fig 3. Exogenous ABA or GA_3 effects on free amino acid contents in grains.

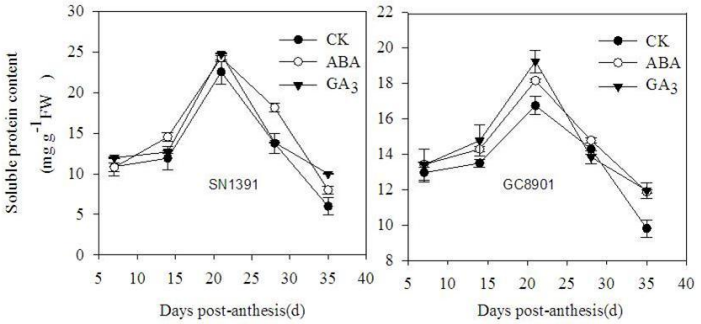

Fig 4. Exogenous ABA or GA_3 effects on soluble protein contents in grains.

were associated with rapid enlargement of the embryo, whereas it had no significant effect on grain filling. However, our result reveals positive correlation between grain filling rates and GA_s levels, which requires further investigation to uncover the phenomena. In agree with the early reports (Xie et al., 2003; Yang et al., 2011), our results clearly demonstrated that GA_s contents in grains were positively related to the increased grain weight, which was mainly ascribed to the extended duration of grain filling (Table 2) and enhanced endosperm cell division(Yang et al., 2011). The fact that GA_s contents in grains negatively associated with the protein concentration has been well reported by Xie (2003), which was closely associated with the increased activity of enzymes involved in protein degradation (Wang et al., 2002), whereas positively and significantly correlation has been observed between the GA_s contents and GS activity and free amino acid contents in grains, indicating that the increased protein concentration under application of GA_3 was

Table 5. Exogenous ABA or GA_3 effects on protein components in grains at 35 DPA.

Treatments	Protein components (g kg^{-1},dry basis)							
	Albumin		Globulin		Gliadin		Glutenin	
	GC8901	SN1391	GC8901	SN1391	GC8901	SN1391	GC8901	SN1391
CK	14.199a	11.93a	16.43a	17.93c	23.682c	15.278b	32.9b	31.55c
ABA	13.952a	12.751a	15.36a	25.63a	32.015a	22.363a	38.38a	42.85a
GA_3	13.383a	12.978a	16.36a	21.01b	26.968b	23.303a	39.2a	34.59b

CK, The control groups; ABA, exogenous abscisic acid was sprayed to the spikes; GA_3, exogenous gibberellin was sprayed to the spikes.

closely related to the enhanced GS activity and free amino acid contents. However, possible genetic and physiological involved with the changes in nitrogen metabolism under application of ABA or GA_3 needs to further investigated. It has been reported that IAA contents in wheat grains reached maximum at grain filling stage, and played an important role in regulation of grain filling (Brenner and Cheikh., 1995). The positive effects of IAA on grains capacity and photo-assimilate translocation within development wheat grains have been already reported in wheat (Majid et al., 2011). In the present study, IAA contents positively and significantly correlated with grain filling rate and soluble protein contents, which are consonant with the previous studies, indicating that IAA may be involved in regulation of protein accumulation. Obviously, further investigation is needed to understanding the mechanism by which IAA facilitates the changes in nitrogen metabolism characteristics.

Materials and methods

Plant materials and growth conditions

The field experiments were carried out in growing seasons from October, 2008 to June, 2009 and from October, 2009 to June, 2010 at the Experimental Station of Shandong Agricultural University, Tai'an, China (36°18' N, 117°13' E). Two high-yield winter wheat cultivars currently used in local wheat production, Shannong1391 and Gaocheng8901 were used. The soil was a sandy loam, and the 0-20 cm soil layer contained 13.70 g total organic matter · kg^{-1}, 0.87 g total N/kg, 76.55 mg available N · kg^{-1}, 50.53 mg available P_2O_5 · kg^{-1} and 86.3 mg available K_2O · kg^{-1}. Maize was the previous crop. The wheat cultivars were planted in plots (3 m × 3 m) with 180 plants m^{-2}. Nitrogen (120 kg · ha^{-1} as urea), P (75 kg · ha^{-1} as single superphosphate) and K (120 kg · ha^{-1} as KCl) were applied as basal fertilizer before planting, with nitrogen being top-dressed at the jointing stage. No noticeable crop damage from weeds, insects, or diseases, and no special weather events during the grain filling period (1 May to 5 June) of 2009 and 2010 were observed (Table1).

Chemical applications

ABA (±-S-cis, trans abscisic acid, Beijing, Kelinon Agrochemical Co., China) and GA_3 (Shanghai Chem & Biol Co., China) were dissolved in ethanol or 0.1M NaOH (the final pH was adjiusted to 7.1). After anthesis completed, 45μM ABA or 60μM GA_3 was applied to spikes by using an atomizer. The chemicals were applied daily for 4 d from 1 to 4 post-anthesis (DPA) at rate of 500mL m^{-2} at each application, with 0.05% (v/v) Tween 20 as surfactant. All solutions used contained ethanol and Tween 20 at final concentrations of 0.1% (v/v) and 0.05% (v/v), respectively. The same volume of deionized water containing same concentrations of ethanol and Tween 20 were applied to the control plants. The experiment was a completely randomized design with three replications.

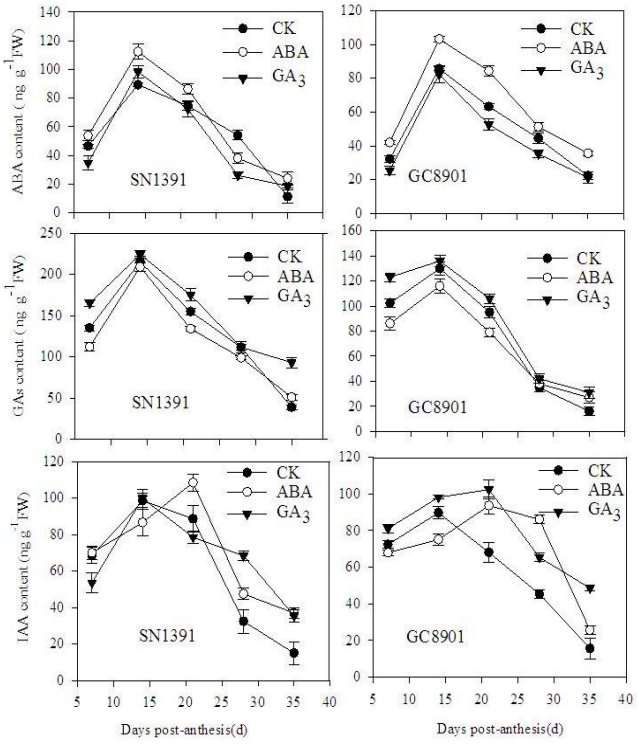

Fig 5. Exogenous ABA or GA_3 effects on endogenous hormone contents in grains

Sampling

Two hundred spikes flowering on the same day were chosen and tagged for each experimental plot. From 7 days post anthesis, 20 labeled spikes of each plot were sampled at 6-day interval till 35DPA. These spikes were killed by heating to 105℃ for 30 min and then dried at 70℃ for the determination of grain filling process, protein concentration, protein components and free amino acid content. On the same sampling date, another 20 spikes were sampled and immediately frozen in liquid nitrogen for at least 30 min and kept at -40 ℃ till enzyme and hormone assay. Grains of the basal 4 to 8 spikelets on the spikes of SN1391 and GC8901, having little variation in flowering time and the amount and size of vascular bundles among florets in a wheat spike, were used to investigate the filling process and nitrogen metabolism characteristics.

Grain filling process analysis

The grain filling process was fitted by logistic growth equations as described by Darroch and Baker (1990): $Y=K/(1+e^{A+Bt})$, where Y is the single grain weight (mg · $grain^{-1}$), K is the final single grain weight (mg · $grain^{-1}$), A and B are the coefficients determined by regression and t is the time

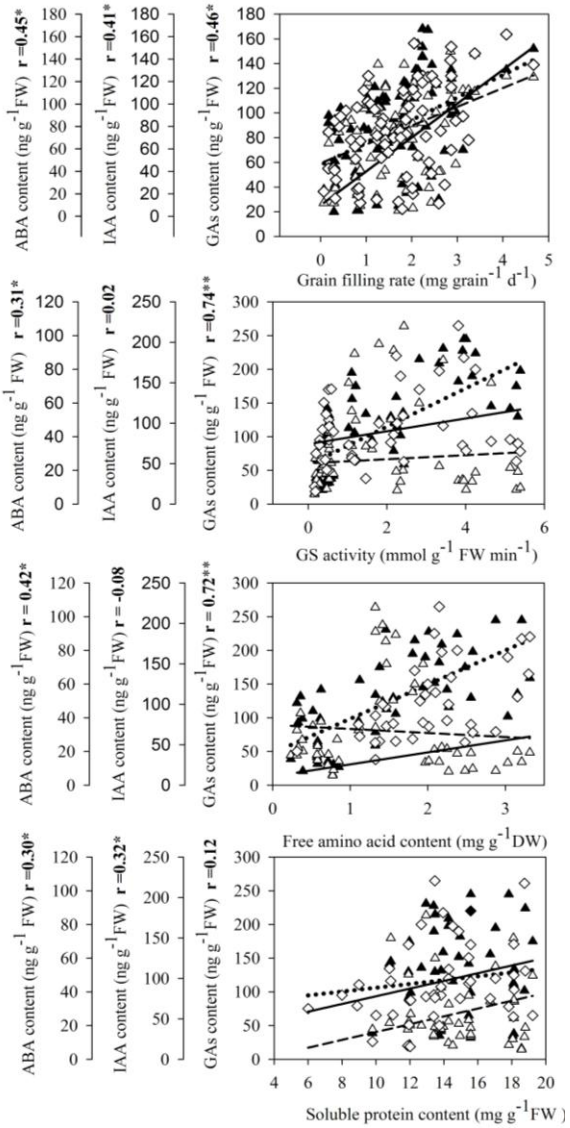

Fig 6. Relationships of hormone contentsto the grain filling rate and GS activity and free amino acid contents and soluble protein contents. Correlation coefficients (r) are calculated and asterisks (**) represent significance at the 0.01 probability level and asterisks (*) represent significance at the 0.05 probability level.

after anthesis. The grain filling rate was defined as the derivative of $V_t = y' = -KBe^{A+Bt}/(1+e^{A+Bt})^2$. The active duration of grain filling period was defined as that when Y was from from 5% (t_1) to 95% (t_2) of K. The active duration of grain filling period (T) was estimated and initial grain filling potential(C_0) was calculated as the derivative of T= 〔In(1/9)-A〕/ B and $C_0 = K/(1+e^A)$. The maximum grain filling rate and appearance time of maximum grain filling were calculated as R_{max}= - KB/ 4 and T_{max}= -A/ B, respectively.

Determination of protein concentration, protein accumulation amounts and protein components

Total N in grains was measured with the Semi-Micro Kieldahl method (AOAC, 1984), and multiplying total N by 5.7 gave the protein concentration. The protein accumulation amounts was calculated by multiplying 1,000-grain weight by protein concentration, which were expressed as g·1000-kernels^{-1}. Protein components were determined according to procedures described by He (1985), with the unit of g · kg^{-1} dry matter.

Determination of GS activity and soluble protein content

To extract GS, about 1 g fresh grain samples were homogenized using a chilled mortar and pestle in 5 ml of 0.1 M Tris–HCl buffer (pH 7.5), containing 0.5 mM EDTA (disodium salt) and 1 mM $MgCl_2$. Enzyme was assayed by the procedure described by Boyer (1959). The standard curve was performed with graded concentration of γ-glutamylhydroxamate. Absorbance value was read at 540 nm using a spectrophotometer (Shimadzu UV-2450, Tokyo, Japan). Activity of enzyme was discribed with μmol · g^{-1} · FW · min^{-1}. Soluble protein contents were determined according to Bradford (1976), taking bovine serum albumin as the standard. The absorbance value was detected at 595 nm using a spectrophotometer and the soluble protein contents were expressed as mg · g^{-1} · FW.

Determination of free amino acid contents

The free amino acid contents were quantified according to the Ninhydrin-dyeing method described by Zou (2000). About 1 g grains were ground into a fine powder with pestle in the mortar. Five milliliter acetic acid solutions (10%) was added into mortars and then filtered to the flask. The filterate was stained with 3 ml of ninhydrin liquid for 15 minutes in water bath at 100 ℃. The absorbance was measured at wavelength of 570 nm using a spectrophotometer and the free amino acid contents were expressed as mg · g^{-1}· DW.

Hormone extraction, purification and determination

Hormone contents were measured by High Performance Liquid Chromatography (HPLC) as described by Yang (2007). Fresh grains (1g) was ground into powder with liquid nitrogen and 5 ml cold acetonitrile (100%) containing 30 ug · ml^{-1} diethyl dithiocarbamate sodium (anti-oxidant) was added to extract for 12 h in refrigerator at 4 ℃. Extracts were then centrifuged at 5,000 rpm for 15 min and the supernatant liquid were evaporated in vacuum at 37-40℃. The solid residue was resuspended in 4 ml of a phosphate buffer/chloroform solution (50: 50, V: V). The PH of the aqueous phase, in which 150 mg PVP was added, was adjusted to 3 with pure formic acid and extracted with 4 ml ethyl acetate. After discarding the aqueous phase, the organic fraction was evaporated in vacuum at 37-40℃ and the solid residue was resuspended with 1 ml of an acetonitrile/methanol/0.6% acetic acid solution (5: 50: 45, V: V: V), which was filtered through an organic membrane with pore size of 0.22 μm. A 20 μl aliquot of this solution were injected into a fixed 20μL loop for loading onto a 4.6 mm by 150 mm, 5μm particle size reverse-phase (C18) column (Waters, USA). Samples were eluted from the column by a Waters series 515 pump at 25℃ with a flow rate of 0.4 ml · min^{-1}. Hormone peaks were detected by a Photodiode Array Detector (Waters 2998 Sparations Module, USA) absorbance at 254 nm, and hormone contents was expressed as ng · g^{-1} · FW. The recovery of each hormone was calculated to be >80% based on assays that added known amounts of standard hormone solutions to a split extract.

Statistical analysis

Since grain weight of the two years behaved the same, the grain weight presented (Fig. 1) is an average of the two years. The single grain weight was calculated by the average weight of 1,000-grains. The data for analysis of grain filling process, nitrogen metabolism characteristics and hormone contents were mainly from the growing season of 2009 to 2010. Analysis of variance was performed with PASW software version 18.0. Data from each sampling date were analyzed separately. Duncan's new multiple range test (DMRT) was employed to assess differences between the treatment means at the 0.05 probability level.

Conclusion

The changes, at least partly, in endogenous ABA, GA_s and IAA contents under application of exogenous ABA or GA_3 may indirectly affect protein accumulation via influencing the grain filling process and activities of enzymes related to nitrogen metabolism. While the possible regulation mechanisms of exogenous hormone in grain filling process and nitrogen metabolism, and its relations to endogenous hormones require further investigation.

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