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Research article

The effect of H$_2$O$_2$ and abscisic acid (ABA) interaction on β-amylase activity under osmotic stress during grain development in barley

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Abstract

The effects of exogenous abscisic acid (ABA) and polyethylene glycol (PEG 6000) treatments on grain H$_2$O$_2$, ABA and β-amylase activity were studied during grain development in the spike culture experiments with variety Triumph and its ABA-insensitive mutant TL43 as the plant materials. The results showed that during grain development the two genotypes were similar in the pattern of ABA concentration change, but differed greatly in the pattern of H$_2$O$_2$ concentration and β-amylase activity changes. The β-amylase activity was positively correlated with H$_2$O$_2$ concentration, negatively correlated with ABA concentration, and it is mainly closely associated with continued high levels of ABA with respect to H$_2$O$_2$. Water stress (PEG treatment) induced β-amylase was associated with H$_2$O$_2$ concentration but not with ABA concentration. Exogenous application of H$_2$O$_2$ and Ascorbic acid (AsA) increased β-amylase activity in Triumph but reduced that of TL43. However, the endogenous H$_2$O$_2$ concentration in grains was always consistent with β-amylase activity. A novel model was hypothesized from the current results to illustrate the relationship between H$_2$O$_2$, ABA and β-amylase synthesis for the barley exposed to abiotic stresses.

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1. Introduction

β-Amylase (1,4-α-D-glucan malto-hydrolase, EC 3.2.1.2), is an exoamylase that catalyses hydrolysis of 1,4-α-glycosidic linkages of polyglucan chains at the non-reducing ends to produce maltose and it is one of the most important starch hydrolysis enzymes in plants [1]. β-Amylase may be synthesized in different parts of plants, including leaves, stems and grains. The genes controlling synthesis of β-amylase may vary with plant tissues; however, their functional domain should be similar as they are of the same gene family. In malting barley, β-amylase activity is a critical quality parameter, being closely associated with malting value and quality [2]. The physiological role of β-amylase in starch degradation was once considered to be catalytically inactive on starch granules without sufficiently prior digestion by other amylolytic enzymes like α-amylase on the basis of in vitro studies [3]. However, the importance in leaves of β-amylase in transitory starch breakdown and its induction under a number of abiotic stresses have been demonstrated recently [4–9].

In fact, the β-amylase induction could be regarded as a type of tolerance metabolism to help plants cope with unfavorable growing conditions. For example, in Arabidopsis, temperature stress up-regulated two members of β-amylase gene family (BMY7 and BMY8), which resulted in maltose accumulation [9]. Evidence obtained from in vitro assays shows that maltose has ability to protect proteins, membranes, and photosynthetic electron transport chain from injury caused by temperature stress. Therefore, enhanced β-amylase activity and the resultant maltose accumulation may function as a compatible-solute stabilizing factor. Conversely, the induction of β-amylase activity was also observed in cucumber cotyledons under water stress and in filling wheat grains under heat stress [10,11]. In germinating barley grains, β-amylase activity is closely associated with total amylolytic activities. Differing from other hydrolytic enzymes synthesized de novo, β-amylase accumulates mainly during grain development [12]. However, during grain development, enhanced β-amylase activity would be expected to restrain the transformation of soluble sugars into starch, thus reduce the source-sink alternation. Therefore, the carbohydrate metabolism adaptation of plants against stresses will be conducted at cost of reduced productivity.

Abscisic acid (ABA) is considered as a key plant hormone in regulating stress adaptation and seed development [13–16]. It has
been proposed that ABA plays a major role in relation to sugar-signaling pathways and enhances the ability of plant tissues to respond to subsequent sugar signals [17]. It has also been suggested that ABA enhances the movement of photosynthetic assimilates towards to developing seeds [14]. However, the possible interaction between β-amylase and ABA in regulating stress adaptation remains unclear. Exogenous addition of ABA inhibited the accumulation of β-amylase activity and mRNA in germinating seeds [18]. Moreover, the enhancement of ABA synthesis by increasing soluble sugars suggested that ABA restricts starch to conversion of soluble sugars [19]. Meanwhile, exposure of plants to abiotic stresses increased both β-amylase activity and ABA concentration [11,20]. Therefore, the response of β-amylase activity to ABA seems a paradox, indicating there are other elements participating in balance with ABA and the regulation of β-amylase. Recently, hydrogen peroxide (H2O2) has been considered as an essential second messenger involved in both stress response and seed development [21,22]. Both H2O2 content and β-amylase activity were increased when plants were exposed to abiotic stresses, and there was the interaction between H2O2 and ABA in their effects on the β-amylase activity in barley aleurones [23]. Therefore, it may be assumed that H2O2 is also involved in interaction with ABA and induction of β-amylase.

In this investigation, an ABA-insensitive (abi) barley mutant TL43, which exhibits fast germination and reduced seed dormancy, and its parent variety (Triumph) were used to determine the change of H2O2, ABA concentrations and β-amylase activity in response to osmotic stress in filling grains. It was reported that TL43 had distinctly higher β-amylase activity and smaller kernels in comparison with its parent [24,25]. However, the underlying mode of β-amylase regulation in plants remains uncertain. The objective of the present study was to make clear of the mechanism with respect to H2O2 and ABA by which their concentration influences β-amylase activity accumulation in filling grains.

2. Materials and methods

2.1. Plant material and cultivation

The experiment was conducted in the experimental farm on the Huaijachi Campus (Zhejiang University, Hangzhou, China) from November 2007 to May 2008. Two barley genotypes (Hordeum vulgare cv. Triumph and its abi-like mutant TL43) were grown in a field. The similar spikes were chosen and tagged at anthesis. Eight spikes were sampled at 3, 6, 9 days post anthesis (DPA), and all samples were frozen in liquid N for 1 min before being stored at −80 °C. At 9 and 18 DPA, which were at the end of division and the start of dehydration of endosperm cells, respectively, the tagged spikes were cut at about 35 cm below a spike, containing the flag leaf, 3 top-most internodes and their sheaths, and were subsequently used for spike culture. The shoots were surface-sterilized by immersion in 1% (v/v) mercury bichloride for 1 min, then in 20% (v/v) of a commercial bleach solution for 5 min, rinsed twice in sterile distilled water, and then transferred to sterilized conical flask containing 150 ml nutrient solution, and sealed by foam and distilled water, and then transferred to sterilized conical flask containing 150 ml nutrient solution, and sealed by foam and aluminum foil. In each conical flask eight spikes were inserted, which were then placed in a growth incubator programmed for photoperiod of 16 h, relative humidity (RH) of 75% and day/night temperature regime of 22/16 °C.

The composition of the basic nutrient solution was as follows (μmol L−1): (NH4)2SO4, 264.8; MgSO4, 547.5; K2SO4, 91.3; KNO3, 183.0; Ca(NO3)2, 253.7; KH2PO4, 182.2; C6H5O7Fe·5H2O, 14.9; MnCl2·4H2O, 5.0; ZnSO4·7H2O, 0.38; CuSO4·5H2O, 0.16; H3BO3, 46.9; and H2MoO4, 0.06. The solution pH was adjusted to 6.5 with HCl or NaOH, as required.

2.2. Experimental design

There were 4 treatments for the culture of the spikes taken at 9 DPA, i.e. (1) basic nutrient solution, control; (2) 10−5 M ABA; (3) 0.75% polyethylene glycol 6000 (PEG) and (4) the combined treatment with 10−5 M ABA and 0.75% PEG 6000 (A + P treatment). PEG treatment was used to form the osmotic stress. The nutrient solution was renewed at 2-day intervals and 8 spikes were sampled at 3-day intervals.

There were 3 treatments for the culture of the spikes taken at 18 DPA, i.e. (1) control; (2) 0.01% Ascorbic acid (AsA); (3) 0.01% H2O2. The nutrient solution was renewed at 2-day intervals and the 16 spikes were collected at 5-day intervals, frozen in liquid N for 1 min before being stored at −80 °C.

2.3. Determination of hydrogen peroxide concentration

The H2O2 concentration of grains was determined according to the method described by O’Kane et al. [26]. Eight frozen grains from middle of the spike were used to determine fresh weight (FW) and then ground in a mortar with a pestle by being homogenized with 5 ml of 0.2 M perchloric acid. After 15 min of centrifugation at 13,000 × g at 4 °C, the resulting supernatant was neutralized to pH 7.5 with 4 M KOH and then centrifuged at 1000 × g for 3 min at the same temperature. The supernatant was immediately used for spectrophotometric determination of H2O2 at 590 nm using a peroxidase-based assay. The reaction mixture contained 12 mM 3-dimethylaminobenzoic acid in 0.375 M phosphate buffer (pH 6.5), 1.3 mM 3-methyl-2-benzothiazolidone hydrazide, 20 μl (0.25 U) horseradish peroxidase (Sigma, St Louis, Mo) and 50 μl of the collected supernatant to a total volume of 1.5 ml. The reaction was started by the addition of the peroxidase. The increase in absorbance was measured at 590 nm using a spectrophotometer (Hitachi U-2000, Tokyo, Japan) at 60 s intervals. The absorbance was expressed as μmol H2O2 g−1 DW and each measurement consisted of 3 replications.

2.4. ABA extraction and measurement

Twenty frozen grains from the middle of the spike were ground with an iced mortar in 10 ml 80% (v/v) methanol extraction containing 1 mmol l−1 butylated hydroxytoluene (BHT) as an antioxidant. The extracts were incubated at 4 °C overnight and centrifuged at 5000 rpm for 10 min at the same temperature. The supernatants were passed through Chromosep C18 columns (C18 Sep-Pak Cartridge, Waters Corp., Millford, MA, USA). The hormone fractions eluted from the columns were dried in a freeze dryer (Labconco, England), and dissolved in 2 ml phosphate buffer saline (PBS) containing 0.1% (w/v) Tween 20 and 0.1% (w/v) gelatin (pH 7.5). The composition of PBS was as follows (mmol L−1−1): NaCl 136.8, KH2PO4 1.5 and Na2HPO4 8.3, and its pH was 7.5.

The content of abscisic acid (ABA) was determined with an enzyme-linked immunosorbent assay (ELISA) according to that described by Yang et al. [15]. A 96-well micro-titration plate was coated with 100 μl coating buffer (1.5 g l−1 Na2CO3, 2.93 g l−1 NaHCO3, 0.02 g l−1 Na2N3, pH 9.6) containing synthetic ovalbumin conjugates for ABA and incubated for 4 h at 37 °C for ABA. After washing with PBS containing 0.1% (v/v) Tween 20 for 4 times, 50 μl of either samples or standard ABA (0–2000 ng ml−1 dilution range) and 50 μl antibodies were added in each well and incubated for 45 min at 37 °C. The antibody against ABA was prepared according to the method described by Weiler et al. [27]. After washing as above, 100 μl 1.25 g ml−1 horseradish peroxidase labeled goat anti-rabbit immunoglobulin was added to each well and incubated for 1 h at 37 °C. After washing for 5 times, 100 μl buffered enzyme substrate
containing 1.5 mg ml\(^{-1}\) ortho-phenylenediamine and 0.008\% (v/v) \(\text{H}_2\text{O}_2\) was added, and the enzyme reaction was carried out in the dark at 37 \(^{\circ}\)C for 15 min, and then stopped by adding 50 \(\mu\)l 3 M \(\text{H}_2\text{SO}_4\) per well. The absorbance was recorded at 490 nm. ABA content was calculated according to Weiler et al. [27]. Three replicates were performed for each measurement. The percentage recovery of ABA was calculated by adding known amounts of standard ABA to a split extract. In this study 79.6–85.8\% of total ABA was recovered.

2.5. Measurement of grain dry weight and \(\beta\)-amylase activity

The half remaining grains of the spikes were dried at 105 °C for 2 h and 80 °C for 72 h, and then grain dry weight (DW) was determined. The other half grains were dried at 65 °C for 2 days, and milled for use in further \(\beta\)-amylase activity measurement.

Both “free” and “bound” forms of \(\beta\)-amylase were extracted by extraction buffer with 100 mM cysteine. The activity was measured using the Betamyl assay kit (Megazyme International, Ireland Ltd) according to McCleary and Codd [28]. The results are expressed as U g\(^{-1}\) DW and each measurement had 3 replicates.

2.6. Statistical analysis

Analysis of variance was performed with Data Processing System (DPS) developed by Tang and Feng [29]. Differences among means were evaluated using the Duncan’s multiple range test [30].

3. Results

3.1. Grain filling during seed development

Fig. 1 illustrates the changes in grain fresh and dry weight during seed development. Both Triumph and TL43 followed a similar trend of grain filling (Fig. 1a and b, c and d). The grain FW reached its peak at 12 DPA, remained stable until 21 DPA and then declined sharply, irrespective of treatment, except for TL43 in the combined (A + P) treatment, which showed a dramatically decrease from 18 to 24 DPA (Fig. 1a and b). For grain DW, the highest increase in rate of accumulation occurred from 9 to 12 DPA and a slight rise was observed after that (Fig. 1c and d).

ABA was considered to have dual effects during grain filling, which would be favorable for carbohydrate transportation but also affects stomatal conductance and photosynthesis. Compared to the control, exogenous ABA application from 9 DPA resulted in the reduction of both grain FW and DW, irrespective of genotypes, which suggested an inhibitory role for exogenous ABA that was overwhelmed by a positive response during early grain filling stage (Fig. 1a, b, c, d). Moreover, in the A + P treatment, grain dry weight of both genotypes was further reduced as compared with PEG treatment, indicating that exogenous ABA can not alleviate the negative effect of PEG treatment at that stage (Fig. 1c and d).

3.2. Effects of exogenous ABA and PEG treatments on the concentrations of ABA and \(\text{H}_2\text{O}_2\), and \(\beta\)-amylase activity during grain development

The curves of response to different treatments during grain development for Triumph and TL43 are shown in Fig. 2. The endogenous ABA concentration was quite low initially and ascended rapidly during early grain development (Fig. 2a and b). The distinct difference in ABA concentration could be found among the treatments when spike culture commenced at 9 DPA. PEG addition increased ABA concentration during the whole of grain development, except for 18 DPA, when grains had the highest ABA concentration. The exogenous ABA application not only caused a significant increase in endogenous ABA concentration, but also postponed the decline in grain ABA concentration. In addition, there was no genotypic difference in grain ABA concentration for the control and PEG treatment. However, Triumph had much higher grain ABA concentration than TL43 when exogenous ABA was added in the culture solution.

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In contrast to ABA, the change of grain H$_2$O$_2$ concentration differed between TL43 and Triumph (Fig. 2c and d). In the control, Triumph showed a decrease at the early filling stage and then an increase from 18 DPA, while TL43 showed an increase from 12 DPA. The PEG treatment greatly enhanced grain H$_2$O$_2$ concentration while ABA treatment only induced a slight increase in H$_2$O$_2$ concentration for both genotypes. It is notable that, regardless the initial induction of H$_2$O$_2$ by the PEG and A + P treatments, the increment of H$_2$O$_2$ concentration occurred from 18 to 21 DPA for Triumph (Fig. 2c), and from 9 to 12 DPA for TL43 (Fig. 2d).

The accumulation of β-amylase activity during grain filling is shown in Fig. 2e and f. For Triumph, β-amylase activity had a rapid increase from 9 to 18 DPA and 24 to 30 DPA, respectively, and showed a relatively small increase between 18 and 24 DPA in the control (Fig. 2e). However, the duration with less increase was shorter for TL43 (from 18 to 21 DPA). The higher increase rate and shorter duration with less increase might be associated with the higher final β-amylase activity in grains of TL43 (Fig. 2e and f).

The PEG treatment increased β-amylase activity, whereas the ABA treatment reduced β-amylase activity, irrespective of genotype. It is notable that the time of slower increase in β-amylase activity (18–24 DPA) was coincident with the time of lowest H$_2$O$_2$ and highest ABA concentrations for Triumph, and moreover the rapid increase after that (24–27 DPA) was generally consistent with rise of H$_2$O$_2$ concentration and decline of ABA concentration (Fig. 2a, c, e).

In addition, TL43 showed constant increase of H$_2$O$_2$ concentration from 9 DPA and shorter duration of less increase in β-amylase activity (18–21 DPA) in comparison with Triumph (Fig. 2b, d, f).

### 3.3. ABA/H$_2$O$_2$ changes during grain development

As the balance between ABA and H$_2$O$_2$ appeared to be correlated with β-amylase accumulation during grain development, the ratio of ABA to H$_2$O$_2$ concentration (ABA:H$_2$O$_2$) was calculated and its relation to β-amylase accumulation was also examined (Fig. 3a and b). ABA:H$_2$O$_2$ ratio under the PEG treatment was relatively
lower and its peak was narrower, but this stage of development had higher β-amylase activity relative to any other treatment, irrespective of genotype. Meanwhile, the increase of β-amylase activity was slower when ABA:H2O2 value was higher, and faster when ABA:H2O2 value was lower, indicating that ABA:H2O2 value was negatively associated with β-amylase activity (Figs. 2e and f, 3a and b). Between the two genotypes, TL43 had lower ABA:H2O2 value, but the lowest β-amylase activity was observed in the A + P treatment for both genotypes, which may be attributed to longer lasting time of high ABA:H2O2 for the A + P treatment (until 27 DPA) relative to that of ABA treatment (until 24 DPA) (Fig. 3a and b).

3.4. Effects of exogenous AsA and H2O2 on β-amylase activity

Ascorbic acid (AsA), a chemical scavenger of hydrogen peroxide, was used in the current study to determine its effect on grain ABA, H2O2 concentrations and β-amylase activity (Table 1). In the control, TL43 had significantly higher H2O2 concentration and β-amylase activity, but basically similar ABA concentration, in comparison with Triumph, which again indicates disturbed H2O2 metabolism in TL43. In the AsA and H2O2 treatments, grain H2O2 concentration and β-amylase activity were increased in Triumph, but reduced in TL43, indicating that grain H2O2 concentration and β-amylase activity might be closely associated.

The significant difference in grain ABA concentration could be found between the two genotypes and among treatments. Both exogenous AsA and H2O2 addition significantly reduced ABA concentration in Triumph at 23 DPA, but significantly increased it at 28 DPA. However, for TL43, no significant difference in grain ABA concentration was observed between each treatment and the control, except a significant increase in H2O2 treatment at 28 DPA.

4. Discussion

TL43 is an abi (ABA-insensitive) barley mutant that can germinate at 10 times higher ABA concentration than its wild-type parent Triumph despite the mutant containing a similar level of endogenous ABA. Moreover, the ABA sensitivity of post harvest mature grains of Triumph is GA independent [32]. TL43 is an abi (ABA-insensitive) barley mutant that can germinate at 10 times higher ABA concentration than its wild-type parent Triumph despite the mutant containing a similar level of endogenous ABA. Moreover, the ABA sensitivity of post harvest mature grains of Triumph is GA independent [32]. The effects of AsA and H2O2 treatments on grain ABA and H2O2 concentration and β-amylase activity during grain development in two barley genotypes.

Table 1

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Treatments</th>
<th>AsA (µg g⁻¹ DW)</th>
<th>H2O2 (µmol g⁻¹ DW)</th>
<th>β-Amylase activity (U g⁻¹ DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>18 d</td>
<td>23 d</td>
<td>28 d</td>
</tr>
<tr>
<td>Triumph</td>
<td>Control</td>
<td>145</td>
<td>169 a</td>
<td>152 d</td>
</tr>
<tr>
<td></td>
<td>AsA</td>
<td>115 b</td>
<td>220 b</td>
<td>249 a</td>
</tr>
<tr>
<td></td>
<td>H2O2</td>
<td>77 c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TL43</td>
<td>Control</td>
<td>145</td>
<td>172 a</td>
<td>180 c</td>
</tr>
<tr>
<td></td>
<td>AsA</td>
<td>161 b</td>
<td>171 c</td>
<td>230 ab</td>
</tr>
<tr>
<td></td>
<td>H2O2</td>
<td>158 a</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Spike culture was started on 18 DPA. Different letters indicate statistical significance at P < 0.05 within a column; ** represents high significance of interaction between genotypes and treatments.
Fig 4. The proposed mechanism of β-amylase synthesis in barley grains under osmotic stress.

The exact pathway for ABA and H₂O₂ regulation of β-amylase synthesis still remains to be elucidated. A cDNA array study of flooding barley grains identified the down regulation of genes encoding ROS-scavenging enzymes in the later stages of grain development, which was consistent with the increased grain H₂O₂ concentration in this experiment and suggestive of programmed cell death (PCD) [22]. PCD is also recognized as an essential part of stress response and in fact, β-amylase induction is coincident with PCD in most cases, especially abiotic stresses and the desiccation stage of seeds [4–9,22,34]. β-Amylase synthesis in these cases might be involved in response to PCD for protection and mobilization of carbohydrate for reutilization. The roles of H₂O₂ and ABA on PCD have already been studied in barley aleurone layer, and showed that H₂O₂ triggered PCD and ABA postponed PCD [43,44]. Therefore, the effect of H₂O₂ and ABA on β-amylase accumulation might be a component of the PCD process.

In summary, the current investigation suggests a novel mechanism of β-amylase accumulation during barley grain filling. The mechanism proposed involves a positive effect of H₂O₂ and a negative effect of ABA on β-amylase synthesis. This regulation system of β-amylase may be a component of the process of PCD. Furthermore, it was found that there was a distinct difference in grain H₂O₂ concentration between the mutant TL43 and Triumph during grain development, and H₂O₂ concentration was highly correlated with β-amylase accumulation, which may account for ABA insensitivity and weaker dormancy in TL43.

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References


