Quantification of free and conjugated abscisic acid in five genotypes of barley (Hordeum vulgare L.) under water stress conditions

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Abstract

The goal of the present study was to obtain new insights into the mechanisms underlying drought stress adaptations in barley plants. For this purpose we evaluated changes in endogenous abscisic acid (ABA) and its glucose ester conjugate (ABAGE), as well as changes in proline content, water relations and growth parameters of five barley genotypes with different drought resistance characteristics. Different responses among the five genotypes studied (Ardahoui, Pakistan, Rihane, Manel ad Roho) led to changes in their pattern of growth and development under drought conditions. Water stress induced a reduction in relative water content, as well as an increase in proline content and endogenous ABA concentrations in all tested genotypes. The lack of water led to a 2-fold increase in proline content for var. Rihane and to a 5-fold increase in endogenous ABA for cv. Ardhaoui. Also, increases in endogenous ABAGE in all genotypes except for cv. Ardhaoui were observed. Our results show that changes in ABA and ABAGE correlated with variations in proline content and growth parameters of these genotypes which present different mechanisms to cope with water stress. We also suggest that new regulatory mechanisms implying ABA mobilization can be of great importance in adaptation of barley to drought.

Keywords: ABA; ABA-GE; d₆-ABA; d₅-ABA-GE; Hordeum vulgare; LC-MS/MS; Proline; Water deficit

1. Introduction

Barley is one of the most important cereal crops in many countries. In most of these countries, barley is often the most important rainfall crop that farmers can grow, and is often subjected to extreme water deficit during the dry season (Ceccarelli, 1994). Therefore, drought stress is a serious problem for barley production in these areas, because it affects simultaneously many traits through morphological, physiological, and metabolic changes occurring in all plant organs. It is known that under water deficit conditions plants decrease shoot growth in order to limit transpiration (Sansberro et al., 2004; Thompson et al., 2007). In addition, proline accumulation has been reported in different plant species (Choudhary et al., 2005; Fabro et al., 2004; Haudecoeur et al., 2009; Saradhi et al., 1995; Schat et al., 1997; Shao et al., 2006; Thiery et al., 2004; Yang et al., 2009; Yoshiha et al., 1995; Zhang et al., 2007), and a protective role for this amino acid in plant stress adaptation has been strongly suggested (Verbruggen and Hermans, 2008). Nevertheless, a correlation between proline accumulation and abiotic stress is not always so apparent and is not correlated with salt tolerance in barley (Chen et al., 2007, Widodo et al., 2009). However, increasing amounts of data suggest that proline has certain regulatory functions, controls plant development and acts as a signaling molecule (Szabados and Savouré, 2010).

The phytohormone abscisic acid (ABA) is well known for its regulatory role in integrating environmental constraints with the developmental programs of plants (Chow and McCourt, 2004; Christmann et al., 2006; Yamaguchi-Shinozaki and Shinozaki, 2004; Shinozaki and Shimamoto, 1997) and in triggering adaptive responses to water stress (Davies, 2005; Thorne et al., 2003).

Abbreviations: ABA, abscisic acid; d₆-ABA, deuterium-labeled abscisic acid; ABAGE, abscisic acid glucose ester; d₅-ABAGE, deuterium-labeled abscisic acid glucose ester; LC-MS/MS, liquid chromatography coupled to mass spectrometry in tandem mode; RWC, relative water content; SLA, specific leaf area; GLN, green leaf number; TN, tiller number; LA, leaf area; H, height.

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ABA affects a broad range of physiological processes at different developmental stages such as embryo development, germination, vegetative development, flowering, and organogenesis (Barrero et al., 2005; De Smet et al., 2006; Finkelstein et al., 2002; Liang et al., 2007; Razem et al., 2006; Xu et al., 1998). Continuous synthesis, transport and degradation dynamically maintain ABA levels in plant cells. Thus, plants control their responses to environmental stresses such as drought, cold and high temperatures, or salt stresses by modulating endogenous ABA levels (Schwartz et al., 2003; Shinozaki and Yamaguchi-Shinozaki, 1999) and it is also involved in the regulation of stress-induced gene-expression, allowing adaptation to environmental stress (Bray, 2002). On the other hand, catabolism can occur by several routes involving oxidation, reduction and conjugation (Cutler and Krochko, 1999) and a rapid and sudden increase in ABA content in drought-stressed leaves derived from carotenoid precursors has been shown (Quin and Zeevaart, 1999). So, ABA metabolic profiles can help to define the relative importance of these competing pathways, the conversion efficiencies within pathways, and the relationship between ABA metabolism, environmental conditions and development (Zhou et al., 2003). ABA can be conjugated through a multitude of pathways, the nature of which varies depending upon the species, developmental stage or tissue (Feurtado et al., 2007). ABA glucosyl ester (ABA-GE) is considered one of the major inactive forms of ABA and is widespread in the plant kingdom (Hartung et al., 2002); furthermore, its oxidized products can also be conjugated as glucosides. Even the inter-relationship of these pathways is not completely clear, ABA-GE appears to be a transported form of ABA.

In order to have a better understanding of barley adaptations to dry environments, five genotypes of hordeum differing in drought tolerance were chosen for this study. To achieve this purpose, changes in free and conjugated ABA concentrations as well as in proline contents have been analyzed, taking into account both water status and growth parameters of plants grown under drought stress conditions.

2. Materials and methods

2.1. Plant material and growth conditions

The experiments were conducted in a controlled growth chamber at the Faculty of Biology of the University of Barcelona, Spain. The five genotypes of barley selected are reported to show the following characteristics: (1) cv Ardhaoui: the unique local barley cultivar of the south of Tunisia. Six-rowed barley, resistant to drought (Deghais et al., 2007); (2) cv Pakistan: was introduced from Pakistan. Six-rowed barley, high yield in water and salt stress conditions; (3) cv Roho: was introduced from Denmark. Very well adapted to low rainfall regions (from 200 to 300 mm) (Deghais et al., 2007); (4) var Rihane: is adapted to semi-arid climate (Deghais et al., 2007) and (5) var. Manel: recommended for cultivation in humid and sub-humid climates (Deghais et al., 2007). Seeds were sown in pots containing a mixture of peat/perlite/vermiculite (1:1:1, v/v/v) in a growth chamber at controlled growth conditions (16/8 h photoperiod, 100 μmol m\(^{-2}\) s\(^{-1}\) light intensity, 25±2 °C temperature and 65–85% of relative humidity). Plants with 5 fully expanded leaves were divided into 2 groups: control plants (well irrigated) and water stressed plants (by withholding irrigation). Hoagland's nutrient solution (Hoagland and Arnon, 1950) was used to nourish the growing seedlings. The first sampling was made before inducing stress (7 days after sowing), and then on day 12, five days after onset of stress treatment. In both groups of plants, the plant height, leaf number/plant, tiller number/plant and specific leaf area were quantified as growth parameters. In order to determine plant water status relative leaf water content was recorded. Finally, free proline content and endogenous ABA and ABAGE were analysed. Plant height, leaf number and tiller number were determined at day 13, six days after treatment, both for control plants and drought-stressed plants of each genotype.

2.2. Determination of relative leaf water content (RWC)

Plant water status was determined by measuring the relative water content (RWC) of leaves as: 100×(FW−DW)/(TW−DW) where FW is fresh mass, DW is dry mass and TW is turgid mass after re-hydrating the leaves. The leaves were kept in distilled water in a closed glass flask at 5 °C in darkness (to minimize respiration losses) until they reached a constant weight. The FW of the leaves was determined immediately after collecting samples. Leaves were then dried for 24 h at 85 °C to determine DW. Three replicates per cultivar and treatment were obtained.

2.3. Specific leaf area (SLA)

Specific leaf area (SLA) was calculated in three leaves per cultivar and treatment as the ratio of leaf area to leaf dry mass. Each replicate was obtained from different individuals. First, the leaf area was determined according to Houala (1999) using the next equation of regression: LA=(0.7624∗L∗1.8841), where L was the length; l was the wide and LA was the leaf area. Then, the dry mass of these leaves was determined after oven drying for 24 h at 85 °C.

2.4. Proline determination

The proline content was determined spectrophotometrically in leaf samples (14 mg DW each sample) according to Bates et al. (1973) by measuring the quantity of the colored product of proline reaction with ninhydrin acid. The absorbance was read at 520 nm using a spectrophotometer. The proline concentration was determined from a standard curve and calculated on a fresh weight basis (μmol praline/g DW).

2.5. ABA and ABAGE analysis

Samples were carefully weighed and extracted by triplicate, as previously described by López-Carbonell et al. (2009). Deuterium-labeled internal standards (20 μl of a solution of
2000 ng/ml containing both \( \text{d}_{5}\text{-ABA} \) and \( \text{d}_{5}\text{-ABA-GE} \) were added to each of the samples and replicates at the beginning of the extraction procedure; this was repeated at the beginning of the extraction procedure. The extraction solvent used was acetone/water/ acetic acid (80:19:1, v/v) at \(-20^\circ\text{C}\). The extracts obtained were vortexed and centrifuged at 15,000 rpm, 4 °C, 10 min; the supernatants were collected and the pellets were re-extracted twice more with 700 \( \mu\text{l} \) of the extraction solvent and centrifuged again. Then, supernatants were pooled together, dried under a nitrogen stream and reconstituted in 200 \( \mu\text{l} \) of water/acetonitrile/acetic acid (90:10:0.05, v/v), stirred, vortexed, centrifuged (10,000 rpm, 10 min), filtered through a 0.45 \( \mu\text{m} \) PTFE filter (Waters, Milford, MA, USA). Finally, 5 \( \mu\text{l} \) of each sample were injected into the LC-MS/MS system.

### 2.6. Statistics analysis

The pots were placed in a completely randomized block design to eliminate any interference on plant growth among the pots. All data were analyzed for significance (\( P \leq 0.05 \)) by ANOVA with mean separation by Duncan’s test using statistical software SPSS (16.0).

### 3. Results

#### 3.1. Crop development and leaf growth parameters

Changes in height (H), green leaf number (GLN), tiller number (TN), leaf area (LA) and specific leaf area (SLA) parameters are shown in Table 1. Genotypic response in GLN to water stress was almost the same for cv. Ardhaoui, cv. Pakistan and var. Rihane (reduced by 20%). On the other hand, var. Roho displayed the lowest reduction (9%), but var. Manel was more susceptible and showed a reduction of 44%. GLN and SLA were positively correlated (\( r=0.15 \)) under drought conditions and values of both parameters were high in var Roho; the rest of genotypes showed intermediate values. Genotypes Ardhaoui, Pakistan, Rihane and Manel exhibited the most important reduction of SLA under stress conditions. In the same way, TN was reduced by 72% at the beginning of the tillering stage. Among drought-stressed plants, TN was high for cv. Ardhaoui and var. Rihane, but was low for var. Manel and intermediate for cv. Pakistan and var. Roho. The highest leaf area (LA) of irrigated plants was recorded for cv. Ardhaoui and var. Manel; nevertheless, it was not in agreement with genotypic ranking for the number of green leaves for var. Manel, due to some differences in leaf size. Under water stress conditions, LA was sharply reduced due to a combination of leaf growth reduction and to abscission. Ardhaoui cv, which presented the highest decline in LA under stress, also experienced a high decline in SLA (46%). Both the loss of leaves and the reduced leaf expansion caused a decrease in LA in water stressed plants; moreover, a negative correlation between LA and SLA was seen (\( r=-0.036 \)).

#### 3.2. Leaf relative water content

In the present study, mean RWC for all cultivars in control and stressed plants is recorded in Fig. 1. Under drought conditions, the RWC differed significantly (\( p<0.001 \)) among the genotypes studied. As can be seen by the 12th day, five days after treatment, and under well watered conditions, the RWC of the studied genotypes were around 98%. The reduction of water availability at the end of the treatment, caused a decline of 5% in the RWC of genotypes corresponding to varieties Rihane, Roho and cv Ardhaoui (showing a decline of 5%), while cv Pakistan and variety Manel declined to 6%. The RWC at day 12 was almost the same for cv. Ardhaoui, cv. Pakistan and var. Rihane; the rest of genotypes showed intermediate values. Genotypes Ardhaoui, Pakistan, Rihane and Manel exhibited the most important reduction of SLA under stress conditions. In the same way, TN was reduced by 72% at the beginning of the tillering stage. Among drought-stressed plants, TN was high for cv. Ardhaoui and var. Rihane, but was low for var. Manel and intermediate for cv. Pakistan and var. Roho. The highest leaf area (LA) of irrigated plants was recorded for cv. Ardhaoui and var. Manel; nevertheless, it was not in agreement with genotypic ranking for the number of green leaves for var. Manel, due to some differences in leaf size. Under water stress conditions, LA was sharply reduced due to a combination of leaf growth reduction and to abscission. Ardhaoui cv, which presented the highest decline in LA under stress, also experienced a high decline in SLA (46%). Both the loss of leaves and the reduced leaf expansion caused a decrease in LA in water stressed plants; moreover, a negative correlation between LA and SLA was seen (\( r=-0.036 \)).
positively correlated with LA (r=0.53, p<0.01) and GLN (r=0.50, p<0.01) under drought conditions. The analyses of variance indicate (Table 2) that the effect of the treatment and the genotype was highly significant on the RWC at the end of the treatment, and the interaction GxT was also statistically significant (p<0.01).

3.3. Proline content

Water stress treatment clearly increased proline concentration in leaves for almost all genotypes examined, compared with their respective controls (irrigated plants). As can be seen in Fig. 2, under favorable water conditions var. Manel showed the highest content of proline (9 μmol/g DW) and var. Rihane exhibited the lowest proline values (4 μmol/g DW) of the five genotypes. Under water stress conditions, var Roho exhibited the maximum content of proline (13 μmol/g DW); in contrast, var Rihane showed the lowest (8 μmol/g DW) and Pakistan cv showed intermediate values of about 456, 451, 294 ng/g (fw) for cv. Ardhaoui, cv. Pakistan, and var. Manel. When water stress conditions were imposed to plants, all five genotypes increased their proline content by 34%, 42%, 51% and 36% for cv. Ardhaoui, cv. Pakistan, var. Rihane and var. Roho respectively and only 26% for var. Manel. According to Duncan’s test (Table 3) most of the tested genotypes were classified into A group under drought conditions, which indicated that most barley genotypes had the anti-drought performance. In a similar way, Roho has the potential to be selected for planting and breeding in arid and semi-arid areas. The effect of the treatment and the genotype were very highly significant (p<0.001).

3.4. ABA and ABAGE content

The variations in total endogenous ABA and ABAGE concentrations in the five barley genotypes studied are shown in Fig. 3. As can be observed in Fig. 3a, ABA levels of well watered plants (control plants) showed clear differences between genotypes, ranking from 129 ng/g (fw) for Ardhaoui cv. to 762 ng/g (fw) for var. Roho and intermediate values of about 456, 451, 294 ng/g (fw) were obtained respectively for Rihane, Pakistan and Manel. When water stress conditions were imposed to plants, all five genotypes increased their

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Table 2

| Source of variation | H     | GLN   | TN    | LA    | SLA   |
|---------------------|-------|-------|-------|-------|-------|
| Genotype            | ns    | **    | ns    | ns    | ns    |
| Treatment           | ns    | *     | *     | *     | *     |
| G x T               | ns    | *     | ns    | ns    | ns    |

Within each column, different letters indicate significant differences at P<0.05 (Duncan’s test). n.s., *, ** and *** indicate non-significant or significant differences at P<0.05 or 0.01 respectively.

Table 3

| Source of variation | Proline content | RWC   |
|---------------------|-----------------|-------|
| Genotype            | d1              | d12   |
| Treatment           | ***             | ns    |
| G x T               | ***             | ns    |

Within each column, different letters indicate significant differences at P<0.05 (Duncan’s test). n.s., *, ** and *** indicate non-significant or significant differences at P<0.05, 0.01 or 0.001, respectively.
endogenous ABA concentrations which lead to 668 ng/g (fw) for cv Ardhaoui, 821 ng/g (fw) for cv Pakistan, 1111 ng/g (fw) for var Rihane, 504 ng/g (fw) for var Manel and 1046 ng/g (fw) for var Roho.

Changes in total endogenous ABAGE are shown in Fig. 3b. Under well watered conditions, the five genotypes studied had ABAGE levels which varied from 218 ng/g (fw) for Pakistan cv. to 530 ng/g (fw) for var. Roho. The other genotypes showed similar ABAGE contents between them (303 ng/g (fw) for cv. Ardhaoui, 307 ng/g (fw) for var. Rihane and 327 ng/g (fw) for var. Manel). Except for cv. Ardhaoui (which ABAGE decreased to 276 ng/g (fw)) the lack of water produced small increases in endogenous conjugated ABA. So, for Pakistan cv. ABAGE increased to 388 ng/g (fw), for Rihane var. values reached 362 ng/g (fw), for Manel var. 508 ng/g (fw) and for Roho 707 ng/g (fw).

4. Discussion

Drought treatment affected leaf production since appearance of green leaves (GLN) was reduced in all cultivars at the end of the drought period and was more marked in var. Manel; this genotype exhibited similar values in leaf production to cv. Pakistan and var. Rihane in non-stressed conditions, but var. Rihane and cv. Ardhaoui recorded less reduction (20%) in green leaf number. According to Poorter (1989), the advantage of cultivars with a slower growth in harsh environments is related to low demands of water and therefore will not exhaust the limited soil water reserve. So, the decrease in leaf number can be of great interest in reducing water losses under conditions of lack of water. Although water stress limits plant growth and the productivity of many crops (Lopes et al., 2004), barley is among the main temperate cereals that best adapts to water shortage (Sanchez-Diaz et al., 2002). In the present work, the height (H) was almost unaffected by the treatment; this is probably due to the fact that our investigation covered a limited period of time in comparison to the life span of the plant and it occurred during a steady state phase which was coincident with tillering stage. It has been reported that plant height and tiller number (TN) decrease significantly under drought in barley (Ivanic et al., 2000); nevertheless, our treatment had a significant effect on TN but no significant differences between genotypes were found. It is known that large tillers may improve survival and we found. It is known that large tillers may improve survival to leaf water status were different depending on the cultivar, and it was in accordance with the highest total amount of proline observed in var Roho, similar to those of cv. Ardhaoui. On the other hand, under stress conditions var Rihane seems to adjust its water status with the lowest proline content and a reduction of 5% in RWC. Interestingly, the extent of osmotic adjustment was high in cv. Pakistan and var. Manel (the most susceptible genotypes) which exhibit the lowest values of RWC compared to the other three genotypes, and a high proline content of stressed plants. These findings suggest that genotypes cv. Ardhaoui, var. Rihane and var. Roho, could present different water resistance strategies and have favorable water content in stress conditions. Moreover, it suggests that osmotic adjustment could be a part of the drought resistance mechanisms developed by Hordeum vulgare and could be exploited in breeding programs for improved water stress tolerance. Although the total concentration of proline in our genotypes was low, these genotypes exhibited clear changes in proline content when subjected to water stress. As has been suggested (Szabados and Savouré, 2010) the stimulation of synthesis can be an important factor in stress adaptation and for tolerance to certain adverse environment conditions (Hong et al., 2000; Mattioli et al., 2008; Miller et al., 2009; Székely et al., 2008). Likewise, the negative correlation we have found between proline content and turgor maintenance during stress in var. Rihane indicates that its role in osmotic adjustment (OA) is very important even at low concentration and that it contributes to maintaining leaf turgor in these plants. Moreover, differences between genotypes in water status support mechanisms such as the abscisic acid action (Passioura, 2002) or osmolyte accumulation (Serraj and Sinclair, 2002), so more studies on proline and ABA metabolism are needed to improve tolerance of these plants to water stress conditions.

Water shortage induces accumulation of ABA in stressed barley plants (Popova, 1998) and a reduction in cell size is related to an increase ABA synthesis (Jia et al., 2001). Our results are in this line and show that when water stress conditions were imposed to plants, all five genotypes increased their endogenous ABA concentrations. Moreover, the highest increase in ABA was recorded by the drought resistant cultivar cv. Ardhaoui (5-fold) which, in turns, also suffered the highest decline in leaf area (32%) compared to the susceptible genotypes Pakistan, Rihane, Roho and Manel. A classical model has proposed that drought-stressed roots produce ABA that is transported to leaves via the xylem as a part of the “root-to-leaf” drought signal. However, Christmann et al. (2005) provides evidences that this “root-to-leaf” drought signal can cause ABA production in leaves too. Our results are in the same line than those obtained in recent studies on two different barley cultivars with contrasting drought resistance characteristics (Veselov et al., 2008) and show that leaves of drought tolerant cultivars have more ABA than susceptible ones, thus helping those plants to adapt to dry environmental conditions.

Nevertheless, under water deficit conditions changes in ABAGE content of the 5 genotypes studied were not so homogenous like those of ABA. Our results revealed that
endogenous ABAGE decreased in cv. Ardhaoui, in contrast to the increases observed in cv. Pakistan (1.7-fold), var. Rihane (1.2-fold), var. Manel (1.5-fold) and var. Roho (1.3-fold); this may indicate a different behavior for cv. Ardhaoui in comparison to the other four genotypes. According to the concept of reversible conjugation, it may be assumed that ABAGE is the source for the accumulation of ABA in the xylem sap of drought-stressed barley roots; thus it would be expected that, under drought conditions, the concentration of ABAGE in leaves decreases whereas the concentration of free ABA increases. This could explain why cv. Ardhaoui showed a decrease in endogenous ABAGE being responsible for ABA increase and the source of the enhanced levels of free ABA in these plants. This fact is supported by Lee et al. (2006) who demonstrate that ABAGE can be hydrolyzed in response to stress by AtBG1, thus leading to an increase in the active ABA concentration. These authors have demonstrated that β-glucosidase synthesis can be induced by stress factors, thus being a new route of ABA production from ABAGE cleavage under osmotic and drought stress. In a different way, the other four genotypes (cv. Pakistan, var. Rihane, var. Manel and var. Roho) showed an increased in the ABAGE concentrations of leaves at the same time that endogenous ABA increases. It has been reported (Sembdner et al., 1994) that the accumulation of ABAGE could be the result of an enhanced ABA metabolism. Dietz et al. (2000) showed that ABAGE was located in intracellular storage organelles, xylem sap and probably cytosol and cell wall. Also, Lee et al. (2006) demonstrate that AtBG1 beta-glucosidase was located in the endoplasmic reticulum and remains there during stress responses. Interestingly, these findings could have implications for the local production of ABA in leaves during stress via hydrolysis of a pre-existing pool of inactive ABAGE and to evaluate whether or not the novo biosynthesis is also involved in the rapid increase in ABA levels. Changes observed in ABA and ABAGE concentrations suggested that they can play a key role in barley adaptations to drought conditions and open a door to future investigations on new regulatory mechanisms on ABA metabolism. Thus, they will contribute to a better understanding of barley responses modulation to drought tolerance in Mediterranean environments.

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