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Secondary metabolism and antioxidants are involved in the tolerance to drought and salinity, separately and combined, in Tibetan wild barley

Imrul Mosaddek Ahmed [1], Umme Aktari Nadira [1], Noreen Bibi, Fangbin Cao, Xiaoyan He, Guoping Zhang, Feibo Wu *

Institute of Crop Science, Department of Agronomy, College of Agriculture and Biotechnology, Zijingang Campus, Zhejiang University, Hangzhou 310058, China

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ABSTRACT

Drought and salinity are the two most common and frequently co-occurring abiotic stresses constraining crop productivity worldwide. Secondary metabolites play a major role in the adaptation of plants to the environment and in overcoming stress conditions. To reveal the physiological and molecular differences between Tibetan wild (XZ5, drought-tolerant; XZ16, salinity/aluminum-tolerant) and cultivated barley (salinity-tolerant *cv* CM72), secondary metabolites and their related genes were studied in response to the individual and combined stresses (D + S) of drought (4% soil moisture) (D) and salinity (S) and the subsequent recovery. Callose content and activities of chitinase, sucrose synthase (SuSy), sucrose phosphate synthase (SPS) and acid invertase (AI) increased more in XZ5 and XZ16 under drought and salinity, both alone and combined, compared with control than in CM72. Elevated phenol and flavonoid content were also observed under single and combined stresses in the two wild genotypes relative to control. The induced expression of genes related to secondary metabolism (*GST1, PPO, SKDH, PAL, CAD* and *chi2*) was demonstrated under all stress conditions in wild barley and accompanied an increase in activities of the respective enzymes, with the greatest increase observed in XZ5. During rehydration and recovery, activities of all enzymes increased except for phenylalanine ammonialyase (PAL) and cinnamyl alcohol dehydrogenase (CAD), which only increased in XZ5. Moreover, microscopic imaging of leaves revealed DNA damage with increasing tail moment under all stress treatments, but XZ5 and XZ16 were less affected than CM72. Our findings suggest that high tolerance to D + S stress in Tibetan wild barley is closely related to enhanced callose, chitinase and carbohydrate metabolism as well as ROS level control through modulation of antioxidant enzymes, their secondary metabolism and their translation level.

1. Introduction

Drought and salinity are the two most common and frequently co-occurring abiotic stresses constraining crop productivity (Ali and Abbas, 2003). According to one estimate, increased soil salinization will affect more than 50% of all arable lands by the year 2050 (Wang et al., 2003), and approximately 1/3 of the world's arable land currently faces yield reduction due to cyclical or unpredictable drought, a great threat to agricultural production (Chaves and Oliveira, 2004). To meet the needs of the growing world population, it is essential to effectively utilize dehydrated and salted soil by developing crop varieties that are well adapted to drought and salt stress. However, the progress toward developing drought- and salt-tolerant crops is significantly hampered by the physiological and genetic complexity of these traits. It is therefore important to understand the mechanisms of drought/salinity tolerance in plants and to identify the genetic resources that could result in high levels of tolerance for multiple stresses.

In plants, the physiological impacts of salt stress at both the tissue and cellular levels resembles those of other hyperosmotic stresses such as drought, cold and freezing (Xiong et al., 2002). In addition to causing tissue and cellular dehydration, salt stress also imposes ionic stress on plants, which creates a 'chemical drought' (Zhu, 2001). Plants are normally subjected to a combination of various types of abiotic stresses in nature, such as the combined effects of drought and salinity. Studies have revealed that the physiological and biochemical responses of plants to the

Abbreviations: AI, acid invertase; ANOVA, analysis of variance; CAD, cinnamyl alcohol dehydrogenase; Ci, intercellular CO_2 concentration; DPPH, 1,1-diphenyl-2-picryl-hydrazyl; gs, stomatal conductance; GST, glutathione-S-transferase; $O_2^{\bullet-}$, super oxide; PAL, phenylalanine ammonialyase; Pn, net photosynthetic rate; PPO, polyphenol peroxidase; SKDH, shikimate dehydrogenase; SMC, soil moisture content; SPS, sucrose phosphate synthase; SuSy, sucrose synthase; TP, total phenol; Tr, transpiration rate.

* Corresponding author. Tel.: +86 57188982827; fax: +86 571 88982827.
E-mail address: wufeibo@zju.edu.cn (F.B. Wu).

[1] These authors contributed equally to the work.

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interaction of drought and salinity are unique and cannot be directly extrapolated from the responses to the two stresses individually (Mittler, 2006). However, little information is available regarding the combined stresses of drought and salinity. Therefore, studies on these stresses in combination are of considerable ecological significance and are practical for improving abiotic stress tolerance in plants.

Plant secondary metabolism covers all physiological and biochemical facets of "secondary products" including the functional and evolutionary aspects (Iriti and Faoro, 2009). Different stresses regulate the production of secondary metabolites, which are most often used for plant defense and stress tolerance (Zhao et al., 2005). Secondary metabolites, such as phenol, flavonoids and callose, are involved in the defense against biotic and abiotic stresses and contribute significantly to the antioxidant activity of plant tissues (Bharti et al., 2013; Ferrat et al., 2003; Pourcel et al., 2007). Secondary metabolites are natural compounds produced by plants and perform a variety of physiological roles (Chen et al., 2009). In plants subject to drought and salinization, we hypothesized that high solute concentrations of secondary metabolites could facilitate osmotic adjustment during stress and then recovery following re-irrigation. In addition, some of the biological processes and metabolic pathways that are affected by salt stress or involved in stress response and tolerance have been identified in gene expression studies. This is in line with the knowledge that drought and salt stress, like all abiotic stresses, can alter gene expression, which ultimately brings change at the protein level (Seki et al., 2003).

Barley (*Hordeum vulgare* L.) is the fourth most important cereal in the world, and it is considered an ideal model for studies of heredity and physiology (Forster et al., 2000). However, due to the rapid loss of genetic variation from replacement, modern barley cultivars have become more sensitive to abiotic and biotic stresses, and their monotonous genetic background has been an obstacle to breeding improved cultivars. Wild barley germplasm is a treasure trove of useful genes and offers rich sources of genetic variation for crop improvement. Our previous studies demonstrated that Tibetan wild barley XZ5 (high drought tolerant)/XZ16 (salinity/Al tolerant) is more tolerant to the combined stresses of drought and salinity than *cv* CM72, and there are distinctive genotypic differences in plant growth, antioxidant enzyme activities and metal concentration between Tibetan wild and cultivated barley genotypes (Ahmed et al., 2013a,b,b). However, the enzymes and gene expression involved in secondary metabolism in Tibetan wild type barley have never been investigated and compared with elite cultivars under combined stress. Thus, the question arises whether the mechanism for stress tolerance in wild barley is associated with secondary metabolism and its related gene expression. This knowledge is important for understanding the mechanisms underlying tolerance to the combined stresses of drought and salinity in wild barley. In the present study, we examined the secondary metabolism and related gene expression involved in tolerance to the combination of drought and salinity in wild barley by comparing the two Tibetan wild barley genotypes, XZ16/XZ5 and salt-tolerant *cv* CM72. We also analyzed the capacity of plants to recover following rehydration. These results will be useful for understanding the mechanisms of drought and salinity tolerance in barley and will provide an effective pathway for the exploration of tolerant genes that might improve drought and salinity tolerance in plants.

2. Materials and methods

2.1. Plant materials and experimental design

The pot experiment was carried out in a greenhouse under natural light condition during December to February, 2012–2013 at Zijingang Campus, Zhejiang University, Hangzhou, China. The plant genotypes used in the experiment and treatment methods were the same as those introduced in our previous study (Ahmed et al., 2013a) except subsequent recovery. After acquiring 4% soil moisture by withholding irrigation for 35 days during the vegetative stage, the pots was subsequently re-watered and allowed to recover for 5 days. During this period, especially soils in the pots of the drought and D + S plants were remained humid at a 60–80% water holding capacity. Soil moisture was measured daily using an HH2 Moisture Meter (Delta-T Devices, Cambridge, UK). For physiological, biochemical and molecular studies, second uppermost fully expanded leaf samples were collected at 4% soil moisture level (stress) and 5 days of recovery period (recovery). The experiment was arranged in a split-plot design with treatments as the main plot and genotype as the sub-plot with nine replicates. The measurement was done with five replicates on physiological parameters, and four replicates on all enzymes (secondary metabolism and antioxidants) activities, and three replications for secondary metabolism-related gene expression and DNA damage. All reagents were analytical grade and all stock solutions were prepared with deionized water.

2.2. Measurement of photosynthetic parameters

A LI-6400 portable photosynthesis system (LI-COR, Lincoln, NE) was used to measure net photosynthetic rate (Pn), stomatal conductance (*gs*), transpiration rate (Tr) and intercellular CO_2 concentration (Ci) of the second fully expanded leaf.

2.3. Determination of callose content

For the quantitative measurement of callose content, plant samples were extracted with 1 N NaOH at 80 °C. Supernatants were mixed with aniline blue solution (40 vol of 0.1% aniline blue in water, 21 vol of 1 N HCl, and 59 vol of 1 M glycine/NaOH buffer, pH 9.5), and fluorescence was measured using a fluorospectrometer (Jobin-Yvon/SPEX) as previously described (Köhle et al., 1985).

2.4. Determination of the activity of enzymes involved in carbohydrate metabolism

The activity of SuSy, SPS and AI were determined according to the method of Ruan et al. (2003) with minor modifications. Leaf tissue (0.5 g) was ground to a fine slurry in 5 mL of grinding buffer (100 mM Tris–HCl, 2 mM EDTA-Na_2, 5 mM $MgCl_2$, 2% ethylene glycol, 0.2% BSA, 2% PVP and 5 mM DTT prepared in sodium acetate, pH 5.2) using a pre-chilled mortar and pestle. Samples were then centrifuged at 10,000 rpm, and the supernatant was collected. The supernatant (3 mL) was further subjected to dialysis in dialysis bags dipped in dialysis buffer (25 mM Tris–HCl pH 7, 2.5 mM $MgCl_2$, 1 mM EDTA-Na_2, 1% ethylene glycol and 1 mM DTT) overnight. During this time, the dialysis buffer was changed thrice and the final volume was maintained at up to 5 mL with Milli-Q water. The amount of water added was recorded as a dilution factor, and the supernatants were used for the analysis of acid invertase (AI), sucrose synthase (SuSy) and sucrose phosphate synthase (SPS).

The activity of SuSy was measured by incubating the enzyme at 30 °C for 10 min. The reaction mixture contained 50 μL of enzyme, 0.4 mL of reaction buffer (100 mM Tris–MES, pH 7, 10 mM fructose, 5 mM magnesium acetate, 5 mM DTT), 0.1 mL UDPG (10 mM) and 1 mL of distilled water in 10 mL tubes. Tubes were kept in boiling water for 3 min, followed by the addition of 100 μL of 2 M NaOH, and were then kept in boiling water again for 10 min. The reaction mixture was then cooled to room temperature, and 3.5 mL of HCl (30%) and 1 mL of 1,3-dihydroxybenzene (0.1%) were added. The tubes were mixed well and again