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Research Article

WHEAT SEMI-DWARFING GENES AFFECT PLANT RESPONSE TO DROUGHT-INDUCED OXIDATIVE STRESS IN A GENOTYPE DEPENDENT MANNER

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Summary: The genotype-specific impact of three gibberellin-insensitive height reducing genes (*Rht* genes) on wheat plant response to oxidative stress provoked by water deficit was investigated. Seedlings (6-day-old) of six near-isogenic lines (NILs) (*Rht-B1a+-D1a (rht), Rht-B1b, Rht-B1c, Rht-D1b, Rht-B1b+ -D1b* and *Rht-B1c+-D1b*) in four cultivar backgrounds were exposed to 15% polyethylene glycole-induced osmotic stress for 8 days. Main growth parameters and leaf content of free proline, hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) were measured to assess plant stress tolerance and the corresponding level of oxidative stress. All factors (treatment, *Rht* allele and cultivar) and their interactions had significant effects on the growth parameters and stress indicators. Tolerance index of root length on average over the four cultivar backgrounds decreased in the order: *Rht-B1c+-D1b* (63%) > *Rht-B1b+-D1b* ≈ *Rht-B1c* ≈ *Rht-D1b* ≈ *Rht-B1b* > *rht* (53%), while tolerance index of shoot length insignificantly depended on the *Rht* allele. However, the percentage reduction in root and shoot length in *Rht* NILs varied appreciably among the cultivars. On average over the genetic backgrounds, the stress markers assay of dehydrated plants showed the lowest H_2O_2 content in lines carrying the allele *Rht-D1b* in the background of the cultivars 'April Bearded', 'Bersée' and 'Maris Huntsman'. This allele in the 'Maris Widgeon' background had the opposite effect on the H_2O_2 contents under simulated drought. No difference in the leaf MDA content was observed between the *Rht* NILs both in control and stressed plants on average over cultivars. The highest accumulation of free proline in controls was measured in plants carrying the combinations *Rht-B1b+-D1b* and *Rht-B1c+-D1b*; however, under stress, the *Rht-B1b* plants accumulated proline to the highest degree. The observed general effect of individual *Rht* alleles varied depending on the genotypic background. This information accentuates the need for an accurate choice of an *Rht* allele when introducing them into a specific genetic background to develop a drought tolerant cultivar.

Keywords: Hydrogen peroxide; malondialdehyde; osmotic stress; proline; reduced height genes; wheat.

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INTRODUCTION

Drought is one of the most common environmental stresses compromising crop productivity. Particularly in the juvenile stages, water deficit can severely affect plant development and thereby reduce the potential yield. Most harmful consequences of drought in plants are associated with the evolved osmotic and oxidative stress. Markers of developing oxidative stress include generation of reactive oxygen species (ROS; for example, hydrogen peroxide, H_2O_2) and accumulation of malondialdehyde (MDA) as a by-product of polyunsaturated fatty acid oxidation in cell membranes (Kocheva et al., 2004). ROS are highly reactive and, in the absence of effective protective mechanisms, can cause lipid peroxidation, protein and DNA breakage, and, finally, cell death (Beligni and Lamattina, 1999; Quan et al., 2008). The accumulation of MDA results in impaired integrity and functioning of cell membranes (Kocheva et al., 2004). In response to stress, plants produce the alpha-imino acid proline. A possible role of free proline accumulation for alleviating cell membrane injuries in stressed leaves and supporting the stability of enzymes and other proteins has been proposed (Kocheva et al., 2004; Sperdouli and Moustakas, 2012).

Our recent research suggested that two mutant height reducing alleles in wheat mediate plant physiological response to drought (Kocheva at al., 2014; Nenova et al., 2014). It was shown that under soil drought seedlings carrying the dwarfing allele *Rht-B1c* and, to a lesser extent, those carrying the semi-dwarfing allele *Rht-B1b* suffered from less osmotic and oxidative damages in comparison with the tall control, carrying the wild-type allele *Rht-B1a* (*rht*). This was manifested by neutralization of the harmful consequences of high H_2O_2 concentrations by activation of some ROS detoxifying enzymes, lower content of MDA and, thereby, better sustained integrity and functionality of cell membranes, along with cell osmotic adjustment through osmolytes accumulation thus aiding the regulation of leaf hydration (Kocheva et al., 2014).

In wheat, more than 20 height reducing genes are known (McIntosh et al., 2008). Alleles at the *Rht-B1* and *Rht-D1* loci reduce stem extension by causing partial insensitivity to gibberellins (GA; Börner et al., 1993). This altered GA-response is caused by mutations in the homologous DELLA genes at the corresponding loci (Pearce *et al.,* 2011). The products of various mutations at *Rht* loci are differentially modified DELLA proteins, which probably cause different degrees of α-amylase gene depression and, accordingly, result in diverse growth inhibition (Börner et al., 1993; Hedden, 2003). A study on the co-ordination between gibberellins, plant height, and stress tolerance suggested that reduced sensitivity to GA, with a concomitant reduction in height are important for induction of stress tolerance in barley (Sarkar et al., 2004). The *Rht-B1b* allele (previously *Rht1* gene) together with another mutation, *Rht-D1b* (previously *Rht2* gene) have been extensively used since 1950s because of the considerable yield benefits associated with increased lodging resistance and higher fertilizer responsiveness (Hedden, 2003). The distribution of both alleles is restricted

mostly to regions of cooler climate (Knopf et al., 2008). However, a trend towards an increase of their proportion amongst the newly released cultivars in South-Eastern Europe has been observed (Chebotar et al., 2001; Ganeva et al., 2005; Tošović-Marić et al., 2008; Landjeva et al., 2012). The increased introduction of *Rht-B1b* and *Rht-D1b* in South-Eastern European wheat varieties over the last decades and the anticipated hydro-thermal change in this region (Kazandjiev et al., 2014) raise questions about the effect of these genes on the growth of young wheat plants at low water availability.

Drought tolerance is a complex quantitative trait controlled by numerous mechanisms and involving a large number of physiological responses (Wehner et al., 2015). That is why the manifestation of the impact of semi-dwarfing genes on plant physiological responses to drought is expected to vary depending on the genetic background. The present study was performed with the aim to determine the effect of *Rht* mutations in various genetic backgrounds on wheat seedlings response to dehydration-induced osmotic and oxidative stress.

MATERIALS AND METHODS

Plant material

The *Rht* genic effects were studied in a set of 24 near-isogenic wheat (*Triticum aestivum* L.) lines of wide range of plant height, governed by both the genotypic background (cultivars 'April Bearded', 'Bersée', 'Maris Huntsman' and 'Maris Widgeon'), and the *Rht* allele (*Rht-B1a*+-*D1a* (or *rht*), *Rht-B1b*, *Rht-B1c* and *Rht-D1b*, or their combinations *Rht-B1b*+-*D1b* and *Rht-B1c*+-*D1b*) (Flintham et al. 1997). Seeds were sterilized with 5% sodium hypochlorite for 10 min and washed extensively, left in water for 30 min and germinated on moistened filter paper at 21°C in the dark. Three-day-old seedlings were transferred to a ½ Hoagland solution and grown in a growth chamber with 16/8 h (day/night) photoperiod at 23/18°C for 3 days. Osmotic stress was imposed on 6-day-old seedlings by transferring them to 15% polyethylene glycol (PEG 6000, Merck) dissolved in full strength Hoagland solution for 8 days. Seedlings grown for 8 days on nutrient solution without PEG served as control.

Growth parameters

Growth parameters were determined in 14-day-old seedlings after thorough washing of roots under running tap water to remove PEG remains. Shoot and root lengths were measured from the seed to the tip of the longest leaf or root, respectively, in at least 15 well-developed plants from each line and treatment.

Tolerance index (TI)

Tolerance indices determine stress tolerance potential of genotypes and were calculated using the formulae:

$$\text{TI root (\%)} = (\text{Root length of stress plants}/\text{Root length of control plants}) \times 100$$

$$\text{TI shoot (\%)} = (\text{Shoot length of stress plants}/\text{Shoot length of control plants}) \times 100$$

Biochemical analyses

Accumulation of H_2O_2, MDA and free proline were determined in leaves.

Hydrogen peroxide content was measured according to Alexieva et al. (2001). Leaf samples (0.5 g) were

homogenized in 0.1 % trichloracetic acid followed by centrifugation at 12,000 x g for 30 min. 0.5 ml supernatant was added to 0.5 ml potassium phosphate buffer (pH 7) and 1 ml 1M potassium iodide. The reaction was developed for 25 min in darkness and absorbance was measured at 390 nm. The amount of H_2O_2 was calculated from a standard curve.

Lipid peroxidation was determined by measuring MDA content in 0.5 g leaf fresh weight using the thiobarbituric acid method of Cakmak and Horst (1991). Leaves were homogenized in 0.1 % trichloracetic (TCA) acid and centrifuged (5,000 x g for 20 min at 4°C). A 0.5 ml aliquot of supernatant reacted with 1.5 ml 0.5% (w/v) thiobarbituric acid in 20% (w/v) TCA. The mixture was kept in a boiling water bath for 30 min and then cooled on ice bath. Absorbance was measured spectrophotometrically at 532 nm and corrected for non-specific absorption at 600 nm. MDA content was calculated using a molar extinction.

Determination of free proline content was done according to the acid ninhydrin method of Bates et al. (1973). Leaf samples (0.3 g) were homogenized in 3 % sulphosalycylic acid, homogenate filtered through filter paper. After addition of ninhydrin and glacial acetic acid, test tubes were incubated in a boiling water bath for 1 h and then reaction was stopped by using ice bath for 5 min. The mixture was extracted with toluene and absorbance was measured spectrophotometrically at 518 nm. Proline concentration was estimated from a standard curve.

Statistical analysis

Two independent experiments were conducted. In each experiment, all biochemical parameters were measured in four replications for each *Rht* allele / cultivar / treatment combination (two biological samples each in two technical repetitions). The observed trend was consistent over the two experiments. The data were analyzed by factorial ANOVA using the statistical package STATISTICA 7 (StatSoft 2005) and are presented as means ± standard error, n = 4. Different letters indicate significant differences ($p \leq 0.05$).

RESULTS

All factors (*Rht* allele, cultivar and treatment) and their interactions had a significant effect on both growth and biochemical parameters ($p<0.001$).

Growth parameters

Treatment of 6-day-old seedlings with 15% PEG over a period of 8 days had a strong reducing effect on both root and shoot lengths. The tolerance index (TI) calculated based on the root/shoot length of stressed plants divided by root/shoot length of control plants, respectively, showed large genotypic variation determined by both cultivar and *Rht* allele effects (Figs.1, 2). The tolerance index of root length on average over the four cultivar backgrounds decreased in the order: *Rht-B1c+-D1b* (63%) > *Rht-B1b+-D1b* ≈ *Rht-B1c* ≈ *Rht-D1b* ≈ *Rht-B1b* > *rht* (53%). However, the percentage reduction in root length in *Rht* alleles varied appreciably among the cultivars. The highest TI of root length was observed at allele combination *Rht-B1c+-D1b* in cultivars 'April Bearded' and 'Maris Huntsman' (Fig. 1A, C), while the lowest TI was observed at *rht* line in cultivar 'April Bearded', and at *Rht-B1b*

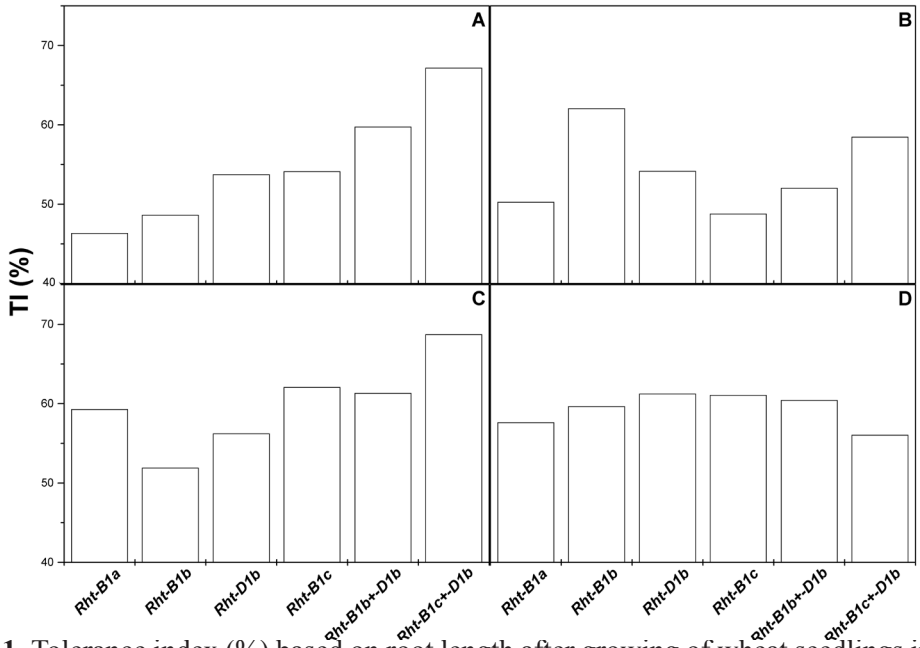

Figure 1. Tolerance index (%) based on root length after growing of wheat seedlings in 15% PEG in Hoagland solution for 8 days (stress) divided by root length in Hoagland solution (control) - effect of height reducing genes (*Rht* genes) and genetic background of four cultivars: A (April Bearded); B (Bersée); C (Maris Huntsman); D (Maris Widgeon).

in cultivar 'Maris Huntsman'. In cultivar 'Bersée', the highest TI of root length was recorded in *Rht-B1b* plants, and the lowest one – in *Rht-B1c* seedlings (Fig. 1B). In cultivar 'Maris Widgeon', the lowest TI of root length was noted in the presence of *Rht-B1c+-D1b,* while there was no considerable difference among the rest of near-isogenic lines regarding this trait (Fig. 1D).

The tolerance index of shoot length slightly depended on the *Rht* allele. The lowest decreasing effect was observed in lines carrying the wild-type allele *rht* (87%), followed by the lines carrying the genes of economic importance *Rht-D1b* and *Rht-B1b* (84 and 83%, respectively), and the two combinations *Rht-B1b+-D1b* and *Rht-B1c+-D1b* (81 and 80%, respectively). The greatest reducing effect was observed in the presence of *Rht-B1c* allele (78%). The genotypic background effects on shoot length reductions were also evident. Thus, in cultivar 'April Bearded', the allele combination *Rht-B1c+-D1b* showed the lowest reduction under stress (Fig. 2A), whereas in cultivars 'Maris Huntsman' and 'Maris Widgeon' the smallest reductions were observed in the line carrying the wild-type allele *rht* (Fig. 2C, D). At the same time, the allele *Rht-B1c* and/or the allele combination *Rht-B1c+-D1b* caused the highest reductions in shoot length in cultivars 'Bersée', 'Maris Huntsman' and 'Maris Widgeon' (Fig. 2B, C, D).

Stress markers

In all near-isogenic lines, the leaf content of the three stress markers (H_2O_2, MDA and free proline) increased after 8-day exposure of seedlings to 15%

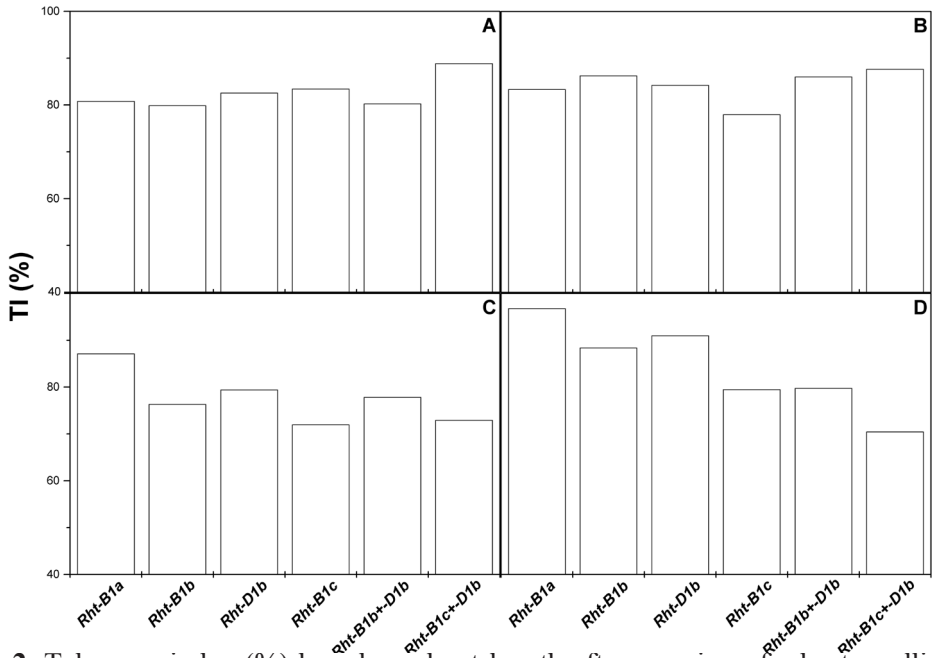

Figure 2. Tolerance index (%) based on shoot length after growing of wheat seedlings in 15% PEG in Hoagland solution for 8 days (stress) divided by shoot length in Hoagland solution (control) - effect of height reducing genes (*Rht* genes) and genetic background of four cultivars: A (April Bearded); B (Bersée); C (Maris Huntsman); D (Maris Widgeon).

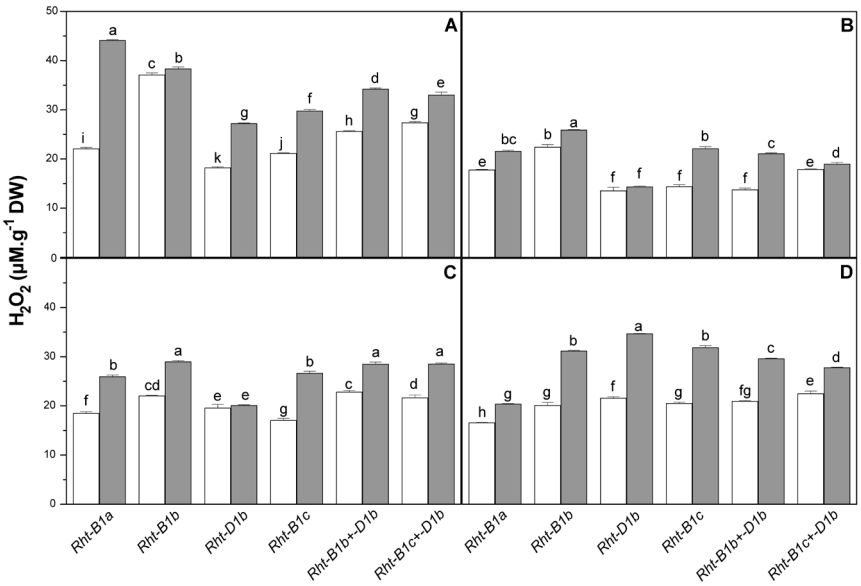

Figure 3. Changes in the leaf content of H_2O_2 after growing of wheat seedlings in Hoagland solution (control, white bars) and in 15% PEG dissolved in Hoagland solution for 8 days (stress, grey bars) - effect of height reducing genes (*Rht* genes) and genetic background of four cultivars: A (April Bearded); B (Bersée); C (Maris Huntsman); D (Maris Widgeon). Data represent the average of two experiments with two replicates (n=4). Vertical bars denote standard deviation. Different letters indicate significant differences ($p<0.05$)

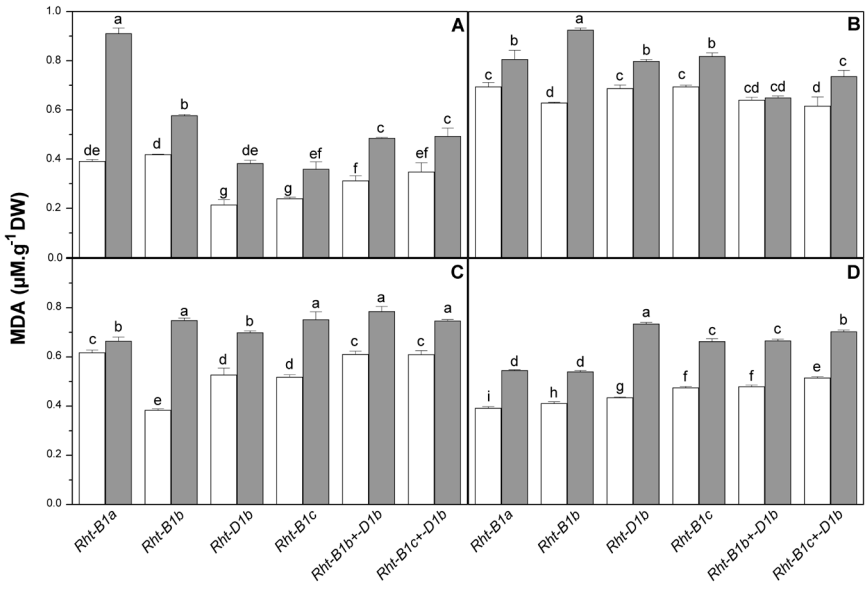

Figure 4. Changes in the leaf content of MDA after growing of wheat seedlings in Hoagland solution (control, white bars) and in 15% PEG dissolved in Hoagland solution for 8 days (stress, grey bars) - effect of height reducing genes (*Rht* genes) and genetic background of four cultivars: A (April Bearded); B (Bersée); C (Maris Huntsman); D (Maris Widgeon). Data represent the average of two experiments with two replicates (n=4). Vertical bars denote standard deviation. Different letters indicate significant differences ($p<0.05$)

PEG (Figs. 3-6). Treatment, genotype (cultivar), *Rht* allele and interactions effects were all highly significant ($p<0.05$).

The induced osmotic stress caused a 34% increase in the leaf H_2O_2 content on average over all genotypes. Under both control and stress conditions, the highest average values of H_2O_2 content were recorded in the set of lines in the background of cultivar 'April Bearded', and the lowest – in 'Bersée' (Fig. 3A, B). On average over the genetic backgrounds, the lowest values of H_2O_2 content were measured in the presence of alleles *Rht-B1c, Rht-D1b* and the wild-type allele *rht* (in controls) as well as in the presence of allele *Rht-D1b* (after stress) (Fig. 6A). The relative increase in the leaf H_2O_2 content after plant exposure to osmotic stress varied considerably depending on both *Rht* allele and cultivar background. Thus, in different genetic backgrounds, the highest and the lowest increase in H_2O_2 content was recorded in different near-isogenic lines (Fig. 3).

Following PEG treatment, the increase in the leaf MDA content averaged over all genotypes was 38%. On average over the *Rht* lines, the highest values of MDA in both control and stressed plants were detected in 'Bersée', and the lowest – in 'April Bearded' (Fig. 4A, B). The effect of *Rht* alleles depended considerably on the genotypic background. The highest and lowest increase in the MDA content after stress was recorded in different near-isogenic lines depending on the cultivar background (Fig. 4A-D). However, on average over the genetic backgrounds, no difference was observed between the *Rht* phenotypes both in control and stressed

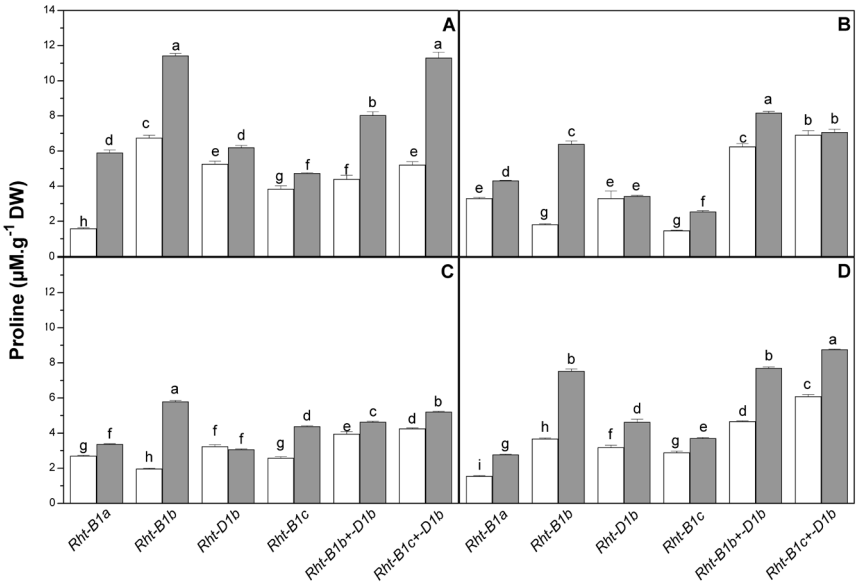

Figure 5. Changes in the leaf content of free proline after growing of wheat seedlings in Hoagland solution (control, white bars) and in 15% PEG dissolved in Hoagland solution for 8 days (stress, grey bars) - effect of height reducing genes (*Rht* genes) and genetic background of four cultivars: A (April Bearded); B (Bersée); C (Maris Huntsman); D (Maris Widgeon). Data represent the average of two experiments with two replicates (n=4). Vertical bars denote standard deviation. Different letters indicate significant differences ($p<0.05$)

plants (Fig. 6B)

Water deficiency induced significant accumulation of free proline (58%) on average over all near-isogenic lines. In both control and stressed plants, the near-isogenic lines in 'April Bearded' had the highest proline content, and those in the background of 'Maris Huntsman' – the lowest proline content (Fig. 5A, C). In controls, lines carrying the combination *Rht-B1c+-D1b* had the highest proline content in leaves (Fig. 6C). The same line together with lines *Rht-B1b* and *Rht-B1b+-D1b* demonstrated the highest proline content under stress. Under stress, the highest proline increase was detected in dehydrated *Rht-B1b* plants, which represented a nearly 180% increase in comparison with untreated controls. The lowest relative increase (16%) was detected in *Rht-D1b* line (Fig. 6C). This trend was consistent over all genetic backgrounds.

DISCUSSION

The pleiotropic effects of wheat height reducing genes have been mostly associated with greater productivity (Flintham et al., 1997), increased photosynthetic capacity (Morgan et al., 1990) and improved nutrient use efficiency (Gooding et al., 2012). Few studies have examined the influence of wheat *Rht* genes on the response of plants to water deficiency. The reactions of *Rht* genes to induced drought during booting and anthesis were studied in terms of effects on grain set (Alghabari et al., 2014). At a juvenile stage, analogous studies were carried out on growth, biomass accumulation, leaf surface, water

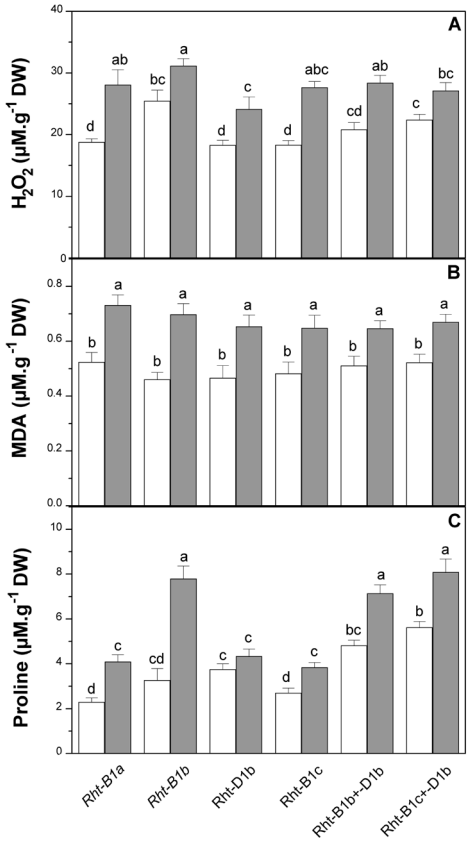

Figure 6. Influence of wheat height reducing genes (*Rht* genes) and their combinations on leaf content of H_2O_2 (A), MDA (B) and free proline (C) after growing of seedlings in Hoagland solution (control, white bars) and in 15% PEG dissolved in Hoagland solution (stress, grey bars) for 8 days. Data represent average over four genetic backgrounds. Vertical bars denote standard deviation. Different letters indicate significant differences ($p<0.05$)

balance and osmotic regulation (Blum et al., 1997; Landjeva et al., 2008a). By using a series of near-isogenic *Rht* lines in different cultivar backgrounds, growth inhibition at water deficiency was found to be inversely proportional to plant size. Thus, plants with higher potential to accumulate biomass (e.g., tall *rht* controls, *Rht-B1b* and *Rht-D1b*) retain growth under stress conditions, while those with lower growth potential (e.g., *Rht-B1c*) have a strongly inhibited stress growth (Landjeva et al., 2008a). At the same time, plants with lower biomass show better tolerance (Blum et al., 1997). Based on these studies, it may be assumed that the better tolerance of shorter-stemmed plants is due to their relatively smaller size and slower growth. In compliance with earlier works, the present study conducted at a different developmental stage, showed that in conditions of water deficiency the shoot growth was less inhibited in plants with higher potential to accumulate biomass (the wild-type *rht* allele and the two alleles with economic importance, *Rht-B1b* and *Rht-D1b*), and to a higher extent in lines with lower growth potential (*Rht-B1c* and double dwarfs). At the same time,

root growth was generally more strongly suppressed in lines with higher potential for biomass accumulation. However, these allele effects were different in the different genotypic backgrounds (Figs. 1, 2). The opposite effects of osmotic stress on root and shoot growth are probably related to the redistribution of assimilates from the roots to the forming leaves. The distribution of assimilates between roots and shoots is one of the adaptive mechanisms in plants. According to the obtained results, the re-distribution of assimilates after stress is associated with a less inhibition of the growth of shoots compared to the growth of roots. At earlier ontogenetic stages, the root growth is less inhibited, especially in the wild-type allele, *Rht-B1b* and *Rht-D1b* (Landjeva et al., 2008a). These differences suggest activation of various protective mechanisms at different vegetative phases. Revealing the effect of GA-insensitive *Rht* genes on the shoot length in conditions of low water availability is important in relation to the trend for early season drought in many regions in South-Eastern and Central Europe (Alexandrov et al., 2004; Kazandjiev et al., 2014). The distribution of *Rht-B1b* and *Rht-D1b* genes in Central European (Miazga et al. 1998; Šíp et al. 2010) and South-Eastern European countries (Chebotar et al. 2001, Ganeva et al. 2005, Tošović-Marić et al. 2008) increases the importance of this study. The low soil humidity due to scarce precipitation in the period of time around sowing (September-October) hampers seed germination and seedling emergence, which in turn affects subsequent plant development, winter survival and can lead to substantial yield loss. Accordingly, the choice of less susceptible to water deficit stem-reducing genes is of importance. The present work demonstrates that among the studied alleles, *Rht-B1b* and *Rht-D1b* were associated with higher tolerance. However, a recent study suggests that the combination of either gene with the GA-responsive *Rht8* represents a more appropriate solution for drought prone regions (Landjeva et al., 2012).

The results obtained in the present study are consistent with other studies on the effects of dehydration induced osmotic and oxidative stress on plant growth (Kerepesi and Galiba, 2000; Kocheva et al., 2004). In the current study, the presence of *Rht-D1b* allele and its combination with *Rht-B1c* was associated with the lowest degree of oxidative stress, manifested by the lowest increase in the content of H_2O_2 on average over the cultivar backgrounds (Fig. 6A). On average over the four cultivars, no significant difference in the content of accumulated MDA was observed between the different *Rht* phenotypes both in controls and after stress. However, the four sets of lines differed considerably with respect to MDA accumulation in terms of response of individual *Rht* alleles. This suggests activation of different defense mechanisms in the different genetic backgrounds. The considerable increase in the free proline content in stressed leaves of *Rht-B1b* lines suggests the role of this allele in the activation of cell mechanisms of osmoregulation. Proline accumulation is considered one of the cell adaptation mechanisms in response to osmotic and oxidative stress developing during water shortage (Alexieva et al., 2001; Sperdouli and Moustakas, 2012). It is

obvious, however, that the lines carrying *Rht-B1b* allele still maintained high levels of H_2O_2 despite the high proline accumulation (Fig. 6A, C). Clarifying the reasons for this requires further studies on antioxidant protection. In the *Rht-D1b* allele, the increase in proline accumulation was insignificant (Fig. 6C). It could be suggested that maintenance of relatively lower levels of oxidative stress in *Rht-D1b* lines was possibly associated with other mechanisms, including activation of enzymatic and non-enzymatic antioxidant components.

Our study shows that, in addition to the *Rht* genes, the genotypic background exerts a significant effect on the growth responses to induced osmotic and oxidative stress. This is most likely due to the involvement of a large number of genes, which are associated with plant tolerance as well as genes still uncharacterized. It is known that numerous genes are up- and/or down-regulated under dehydration (Bartels and Souer, 2003), which further complicates the identification of genes or chromosomal regions that confer drought stress tolerance. Using a set of chromosome substitution lines, Farshadfar et al. (1995) showed that tolerance related genes were localized to at least eight of the 21 pairs of wheat chromosomes. Landjeva et al. (2008b) reported quantitative trait loci (QTLs) affecting seedling growth of young wheat plants during induced dehydration on 10 chromosomes.

Achieving stable crop production in environments with frequent drought periods depends largely on the ability of plants to maintain their functions under low water status. In relation to the climate change scenarios with expected increase of drought risk, research in drought stress tolerance has become more important than ever. The results of the present study point to the differential response of GA-insensitive *Rht* genes to drought induced oxidative stress depending on the genetic background. They might be of importance for breeders when introducing height reducing genes into wheat cultivars designed to be grown in drought liable regions.

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