Brief communication

Variation in ion leakage parameters of two wheat genotypes with different *Rht-B1* alleles in response to drought

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The reaction to soil drying was evaluated in two *Triticum aestivum* near-isogenic lines carrying different alleles of the height-reducing gene *Rht-B1* based on an improved method for assessment of electrolyte leakage. The two lines were previously shown to differ in their physiological responses to induced water deficit stress. Drought was imposed for 6 days on 10-day-old seedlings. Ion efflux from leaves was measured conductometrically in multiple time points during the 24 h incubation period, and the obtained biphasic kinetics was interpreted according to a previously developed theoretical model proposing different leakage rates through the apoplast and the symplast. Most of the model parameters were able to properly differentiate the two closely related genotypes. The mutant *Rht-B1c* displayed lower and slower electrolyte leakage in comparison with the wild-type *Rht-B1a*. It was speculated that the *Rht* genes expressing defective DELLA proteins might be involved in water stress response through modulation of cell wall stiffness, which influences its capacity for ions retention, and also by their contribution to ROS detoxification, thus indirectly stabilizing cellular membranes. The presented analytical approach relating processes of ion and water flow in and out of the cell could be used for characterization of membrane and cell wall properties of different genotypes under normal and stress conditions.

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1. Introduction

Development of water deficit stress in plants is the common consequence of the unavailability of water to cells in conditions such as drought, high salinity and extreme temperatures (Hare *et al.* 1998; Sperdouli and Moustakas 2012). Cell membranes are the main targets of damage, which is generally associated with metabolic disturbances, leading to generation of reactive oxygen species (ROS) (Hare *at al.* 1998; Thapa *et al.* 2011; Kocheva *et al.* 2014b).

The most widely employed technique for assessment of membrane integrity is by estimating the Injury Index based on ion leakage from damaged plant tissues (Blum *et al.* 1997; Prášil and Zámečník 1998; Farooq and Azam 2006; Chipilski *et al.* 2012). Electrolyte leakage was used as a selection marker for differentiation of drought-tolerant genotypes, which tended to leak at lower rate compared to sensitive ones (Whitlow *et al.* 1992; Pelah *et al.* 1997; Bajii *et al.* 2002; Roy *et al.* 2009). However, inability to distinguish ion fluxes through different cellular compartments is one disadvantage of the method. This problem was recently solved by establishing a kinetic approach based on measurement of ion leakage from plant tissues at multiple time points (Kocheva *et al.* 2005). Diffusion processes between the apoplast and the symplast have been elucidated lately by introducing the rates of ion fluxes through cell walls and membranes, respectively, as promising parameters for evaluation of leaf water status (Kocheva et *al.* 2014a). The proposed improved model suggested that not only membrane permeability but also diffusion through the cell wall (apoplast) were influenced by dehydration.

Keywords. Cell wall; drought; electrolyte leakage; membranes; semi-dwarfing genes; wheat

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Stress response at the whole plant level involves processes of precise phytohormonal balance and signal transduction. Gibberellic acid (GA) is a specific regulator of cell growth and differentiation under normal and stress conditions and promotes tissues expansion through degradation of certain growth inhibitors known as DELLA proteins, or DELLAs (Harberd *et al.* 2009; Claeys and Inze 2013). In wheat, DELLAs are products of the reduced-height genes (*Rht*), orthologous to the Arabidopsis *GAI* gene (Peng *et al.* 1999). The mutations at the *Rht* loci encode defective DELLAs that are irresponsive to GA, decrease cell number and cell dimensions, and lead to reductions at tissue, organ and whole plant levels (Hoogendoorn *et al.* 1990). An additional pleiotropic effect of the *Rht* genes on stress tolerance was recently suggested (Kocheva *et al.* 2014b). The wheat mutant line carrying the severe dwarfing allele *Rht-B1c* was more tolerant to water deficiency than the tall isogenic line (*Rht-B1a*), as evidenced by its overcoming of oxidative damages, increased osmolyte accumulation (Kocheva *et al.* 2014b), retained photosynthetic activity and less affected leaf anatomy (Nenova *et al.* 2014).

In this study we applied the improved kinetic approach describing the water–ion balance between the apoplast and the symplast to wheat *Rht* near-isogenic lines in order to (1) test the suitability of the method to discriminate genotypes differing in a single gene; and (2) assess changes in ion fluxes through cell walls and membranes based on the diffusion model parameters under optimal and drought conditions in lines with *Rht-B1* alleles of contrasting size reducing effects and differential physiological responses to induced drought. This would help understand the possible role of the *Rht* genes expressing defective DELLAs in plant response to water stress.

2. Materials and methods

2.1 *Plant material and stress imposition*

Near-isogenic lines (NILs) of wheat (*Triticum aestivum* L.) cultivar April Bearded carrying *Rht-B1a* (wild type) and *Rht-B1c* (severe dwarfing) alleles were used. Seeds were sown in 1 kg pots with alluvial meadow soil (pH 6.2). Plants were grown in a climatic chamber with 22/17°C day/night temperature, 14 h photoperiod, irradiance of 250 μmol m^{-2} s^{-1} and 70% relative humidity. Tap water was supplied daily sustaining 60% of full soil moisture capacity. For drought stress, 10-day-old plants were left without watering for 6 days, while the controls were watered daily.

Leaf water content (LWC) in leaves was calculated as:

$$\mathrm{LWC} = (\mathrm{FW}–\mathrm{DW})/\mathrm{DW},$$

where FW is the fresh weight and DW is the dry weight of leaves, measured after drying at 104°C until constant weight was achieved.

2.2 *Measurement of ion leakage kinetics*

For assessment of ion leakage kinetics, 15 leaf pieces (1.5 cm in length) were cut from stressed or control plants of each line and were immersed in 20 mL distilled water at RT. Conductivity of the solutions was measured at multiple time points during 24 h incubation period and again after boiling the samples for 30 min. Results were expressed as the ratio $\kappa/\kappa_{\max}$ versus time, where κ is conductivity of samples at a particular moment and $\kappa_{\max}$ is total electrolyte content determined after boiling. Hence, a multiple-point kinetics curve was obtained. Fitting of experimental data was done using the Exponential Associate function of Origin 5.0 software:

$$\kappa/\kappa_{\max} = C_o(t) \approx A_1\left(1-e^{-t/t1}\right) + A_2\left(1-e^{-t/t2}\right) + C_o{}^0$$

Thus, the relation $\kappa/\kappa_{\max}$ is used as a measure of electrolyte leakage, and $C_o{}^0 \approx 0$ is the initial ion concentration in the outer solution where leaves were incubated. From this obviously biphasic kinetics, four main parameters were derived according to the previously developed diffusion model (Kocheva *et al.* 2005) describing ion efflux from plant tissues: Amplitude A_1 and time constant t_1 of the first phase, and amplitude A_2 and time constant t_2 of the second phase. A_1 and A_2 are dimensionless coefficients related to the volumes of apoplast (V_e) and symplast (V_i) and to the concentration of ions contained in these compartments, respectively. If $\alpha = V_e/V_o$ and $\beta = V_i/V_o$ are the ratios of the corresponding volumes to the volume of the external solution V_o, in which the measurement is carried out, then A_1 and A_2 can be represented as:

$$A_1 = C_e{}^0\alpha/\left(C_e{}^0\alpha + C_i{}^0\beta\right)(1+\alpha)$$

$$A_2 = C_i{}^0\beta/\left(C_e{}^0\alpha + C_i{}^0\beta\right)(1+\beta)$$

Here $C_e{}^0$ and $C_i{}^0$ are the initial ion concentrations in the apoplast and symplast, respectively. Thus, parameters A_1 and A_2 reflect the maximal capacity of each compartment for donating ions to the overall efflux. Time constants t_1 and t_2, representing the rate of efflux from the two compartments, are given by:

$$1/t_1 = (1+\alpha)(P_w A_w/V_e)$$

$$1/t_2 = (1+\beta)(P_m A_m/V_i)$$

Here P_w and P_m are the permeabilities of apoplast (wall) and symplast (membrane), while A_w and A_m are their surfaces.

Period of the prompt phase, T, was introduced as the moment of phase equilibrium and represented a combination of the four main parameters defined as:

$$T = \frac{t_1 t_2 \mathrm{In}\left[\left(A_1 - C_o^0\right)t_2 / \left(A_2 - C_o^0\right)t_1\right]}{t_2 - t_1}$$

Three additional parameters could be derived: ion concentration at the moment T, $C(T)$, in relative units; initial leakage rate calculated as $dC(0)/dt$ and expressed in relative units per min; and rate of leakage at the moment T defined as $dC(T)/dt$, in relative units per min. The physical meanings of these parameters were described by Kocheva *et al.* (2014a).

2.3 *Statistical analysis*

Two separate experiments were carried out. All parameters were measured in at least three replications for each of the experiments. The data were analysed by non-parametric statistical methods using the software package STATISTICA 7 (StatSoft 2005). The consistency of the results between the two experiments was tested with an experiment-to-experiment Spearman correlation analysis. In the figures, the average results are presented. Sample variability is given by the standard error (SE). The significance of differences in the pairwise comparisons between non-stressed and stressed plants and between the two *Rht* NILs was evaluated by the Mann-Whitney U test at overall significance level of 0.05.

3. Results

Under optimal watering conditions, plants of the two lines had similar leaf water content (table 1). Water deprivation caused considerable decrease in water content with no significant difference between the two isogenic lines.

It was shown earlier that the kinetics of electrolyte leakage from leaves of untreated (control) and stressed plants could be described by an exponential curve with two phases representing the movement of ion fluxes from the apoplast and the symplast (Kocheva *et al.* 2005). According to the proposed diffusion model, the first prompt phase was attributed to leakage through cell walls (apoplast), while the slower second phase was ascribed to efflux through cellular membranes (symplast) owing to different ion permeability of these two cellular structures. Under optimal conditions the two wheat genotypes carrying contrasting *Rht* alleles had similar degree of leaf ion leakage with *Rht-B1a* reaching slightly higher values of the $\kappa/\kappa_{\max}$ relation (figure 1A). Stressed tissues leaked more ions into the outer solution than untreated samples. *Rht-B1c* demonstrated lower efflux throughout the measurement compared to the wild-type allele.

Model parameters were derived after fitting experimental data with the Exponential Associate function. Under control conditions, amplitude of the prompt phase A_1 (indicating the potential efflux from the apoplast) was lower in the mutant line than in the wild type but demonstrated similar growth in the two genotypes under dehydration (figure 2A). The corresponding time constant t_1 was lower in *Rht-B1c* plants but drought caused more significant increase of this parameter in the dwarfs than in wild-type plants (figure 2B), implying slower leakage from the apoplast in *Rht-B1c* under stress. Under optimal conditions dwarfs had smaller amplitude A_2 and time constant t_2 of the slower phase in comparison with the wild-type plants (figure 2C and D). Drought increased A_2 in both genotypes, while t_2 decreased in the wild type but increased in the dwarfs.

The estimated period T of the prompt phase was lower in control dwarf plants than in the tall ones, but increased significantly after drought in both genotypes with higher values in the dwarf *Rht-B1c* line (figure 3A). Under normal watering ion concentration at the moment of phase equilibrium, $C(T)$, was lower in *Rht-B1c* and stress caused greater increase in the wild-type plants than in the dwarfs (figure 3B). The parameter $dC(0)/dt$ describing the magnitude of ion flux rate in the beginning of the measurement was slightly higher in *Rht-B1c* controls and increased after drought, more markedly in *Rht-B1a* (figure 3C). The rate of leakage at the moment T, $dC(T)/dt$, was initially similar in dwarfs and in tall plants but drastically decreased in *Rht-B1c*, while a slight increase was observed in the wild type (figure 3D).

Table 1. Effect of soil drought on leaf water content in two contrasting *Rht* genotypes. Data are means ± SE (n=3)

Treatments	Leaf water content [g H_2O g^{-1} DW]
Rht-B1a - Control	6.07 ± 0.22
Rht-B1c - Control	6.18 ± 0.28
Rht-B1a - Stress	1.85 ± 0.56
Rht-B1c - Stress	2.88 ± 0.57

4. Discussion

The applied diffusion approach was able to clearly distinguish the two near-isogenic lines differing by a single gene. Under optimal conditions most of the model parameters had the potential to segregate the reaction of *Rht-B1c* dwarfs, which had less leakage from the apoplast and the symplast in comparison with *Rht-B1a*, as evidenced by the lower values of phase amplitudes A_1 and A_2 and the smaller ion concentration $C(T)$ at the moment T of phase equilibrium. Under drought, the two genotypes were even better distinguished by their leakage kinetics and the model parameters

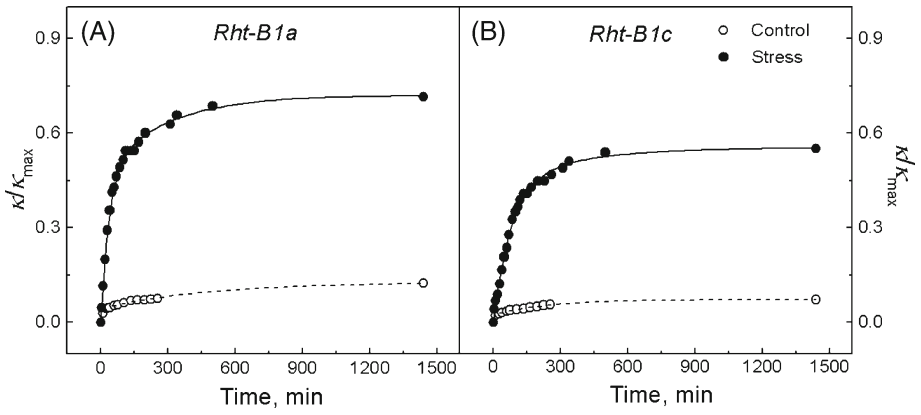

Figure 1. Kinetics of electrolyte leakage from leaves of two wheat genotypes carrying different *Rht* alleles: (**A**) wild-type *Rht-B1a*, and (**B**) severe dwarf *Rht-B1c* under optimal (Control) and drought (Stress) conditions.

describing ion fluxes through the two cellular compartments. Stressed *Rht-B1c* dwarfs had lower ion efflux (lower κ/κ_{max} and $C(T)$), slower initial leakage rate (lower values of $dC(0)/dt$), slower rate at the moment T, extended duration of the apoplastic leakage (higher values of period T and t_1), and lower membrane leakage (permeability) demonstrated by the smaller amplitudes A_2 of the slow phase than *Rht-B1a*. Based on the obtained results, we can hypothesize that *Rht*/DELLAs might be involved in the regulation of the amount and rate of ion fluxes from plant tissues under both non-stress and drought stress conditions.

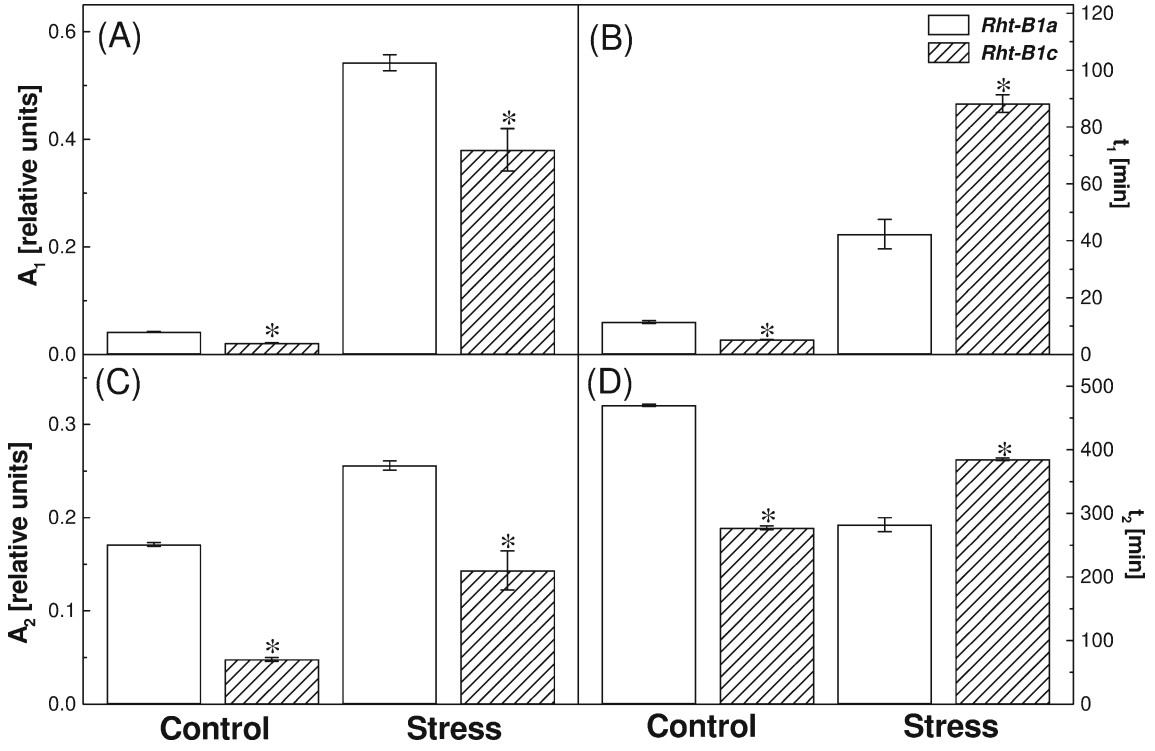

Figure 2. Changes in the main model parameters in wheat plants carrying contrasting *Rht* alleles under optimal and soil drought conditions: (**A**) amplitude of the first phase, A_1; (**B**) time constant of the first phase, t_1; (**C**) amplitude of the second phase, A_2; (**D**) time constant of the second phase, t_2. Values are means ± SE (n=4).

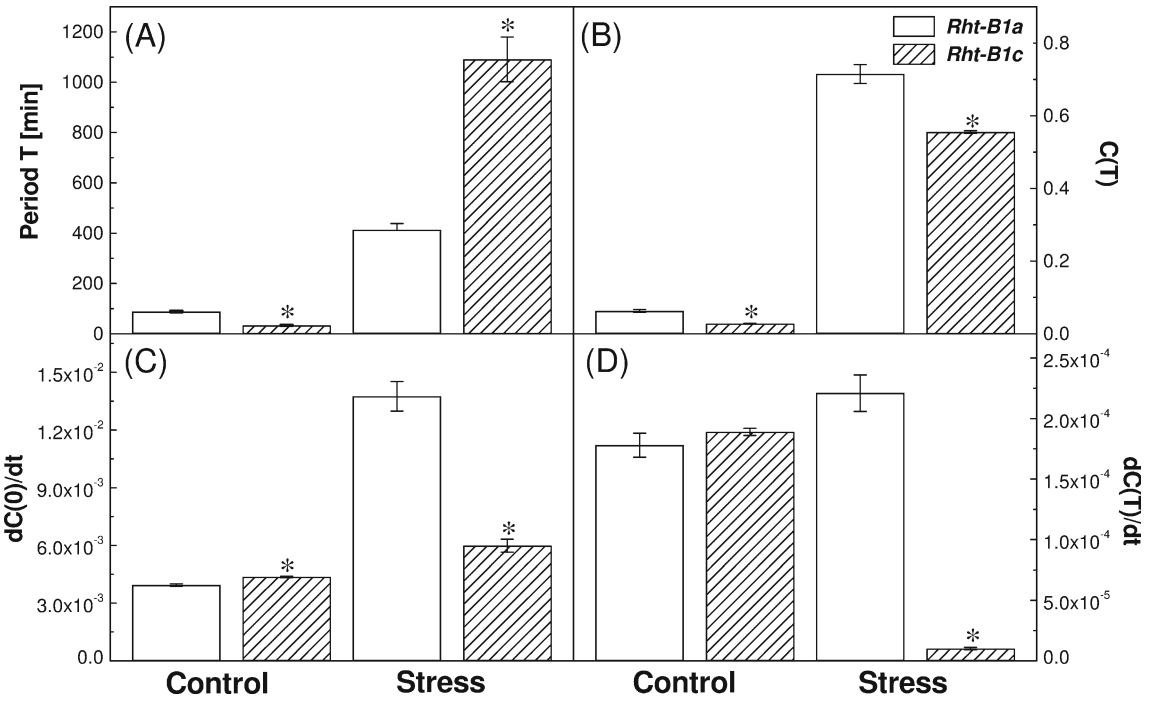

Figure 3. Effect of soil drought on the derivative parameters of the diffusion model in two wheat genotypes carrying different *Rht* alleles: (**A**) period *T*; (**B**) ion leakage at the moment *T*, *C*(*T*); (**C**) initial leakage rate, d*C*(0)/d*t*; (**D**) leakage rate at *T*, d*C*(*T*)/d*t*. Values are means ± SE (*n*=4).

Physiologists have come to appreciate that the regulation of plant growth under water-limiting conditions is a matter of achieving a balance between growth and survival (Claeys and Inzé 2013). DELLA proteins are among the master regulators that help maintaining this balance. In *Arabidopsis*, DELLAs have been shown to both arrest plant growth and promote survival under stress by enhancing the expression of ROS detoxifying genes, thus limiting stress-provoked damages (Achard *et al.* 2008). The orthologous della *Rht* genes in wheat were also supposed to affect plant responses to water deficit by promoting ROS detoxification and hence alleviating the consequences of stress (Kocheva *et al.* 2014b). The higher ability of *Rht-B1c* plants to modulate the antioxidant defence system in comparison with *Rht-B1a* contributed to their better sustained membrane integrity (Kocheva *et al.* 2014b). It is tempting to speculate that defective DELLAs in *Rht-B1c* plants could improve drought tolerance by modulation of cell membrane permeability, thus regulating ion efflux through the symplast.

On the other side, cell growth depends to a great extent on mechanical properties of cell walls. Under desiccation, tightening, or hardening, of cell walls occurs, which increases the capacity of expanding cells to maintain turgor pressure and restrains growth (Neumann 1995). Certain changes in cell wall structure could actually limit ion efflux and hence retain turgor. In addition, it was shown that ROS induce scissions in the cell wall structural polysaccharides (Taleisnik *et al.* 2009), and accordingly their lowered production could prevent cell wall loosening and expansion and thus contribute to reduced leaf elongation. Thus, the results presented here could imply that the DELLA-encoding *Rht* genes might have effect on water and ions fluxes through cell walls and membranes, thus contributing to the maintenance of cell turgor. Increased number of stiff cell walls could be one possible reason for the observed lower leakage from the apoplast in drought *Rht-B1c* mutant, as evidenced by the model parameters.

Besides ROS scavenging, an additional biophysical response to water deficit termed '*cell wall adjustment*' was suggested by some authors (Neumann 1995; Wu and Cosgrove 2000; Moore *et al.* 2008). It involves changes in cell wall extensibility as a result of restructuring of expanding cell walls. A recent work in *Arabidopsis* found that DELLA proteins regulate the expression of genes whose products are involved in cell wall structure and modification. Thus, a possible role of DELLAs in cell expansion was suggested (Hou *et al.* 2008). This is also in agreement with earlier work by Keyes *et al.* (1990) demonstrating that the

mechanical cell wall properties are strongly associated with the *Rht*/GA-mediated leaf cells expansion in wheat. In consonance with the latter findings, our results show quantitatively smaller and slower electrolyte leakage from the cell wall (apoplast) in the severe dwarf (expressing mutant DELLAs) both under well-watered conditions and under drought, implying tightening of cell walls.

In stressed plants, lower electrolyte leakage is evidenced with higher accumulation of osmolytes, which decreases the osmotic potential and enhances water retention capacity (Hare *et al.* 1998; Zivčák *et al.* 2009; Sperdouli and Moustakas 2012). In our previous study, we found higher drought-induced accumulation of the compatible solute proline in the dwarf plants than the wild-type plants (Kocheva *et al.* 2014b). Apart from its role in osmotic adjustment, proline is known to improve membranes stability by protecting their structural components (Verbruggen and Hermans 2008).

A number of different processes appear to constitute the complex stress response of the plant and it seems likely that the apoplast (cell wall) in tight cooperation with the plasmalemma is involved in perception, transduction and reaction to environmental signals (Humphrey *et al.* 2007). The observed relatively lower and slower electrolyte leakage from *Rht-B1c* leaves in comparison with *Rht-B1a* could be connected with processes that preserve membrane stability and contribute to the osmotic and cell wall adjustment. Moreover, it could be concluded that the proposed analytical approach has the potential of a valuable means for characterization of membrane and cell wall status under stress by relating it to their involvement in the ion and water flows in and out of the cell.

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