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Different mechanisms trigger an increase in freezing tolerance in *Festuca pratensis* exposed to flooding stress

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ABSTRACT

Increased precipitation and snow melt during warmer winters may lead to low temperature flooding and ice encasement formation. These conditions are stressful to plants and may affect their winter survival and spring regrowth. The aim of this study was to assess the effects of low temperature flooding on frost tolerance, photosynthetic performance, osmotic potential, water soluble carbohydrate content and expression of *CBF6*, *Cor14b* and *LOS2* genes in four genotypes of *Festuca pratensis* with distinct levels of frost tolerance. It was shown that plants cold acclimated under flooding increase their frost tolerance faster and/or to a greater extent than in non-flooded controls. Changes in the induction kinetics of transcription factors encoding genes are connected with transient growth of frost tolerance in two out of the four genotypes, irrespective of their frost tolerance. A significant and stable increase in frost tolerance observed in the genotype with the lowest tolerance under control conditions was related to higher carbohydrate concentration in the flooded plants. In more frost tolerant genotypes, low temperature flooding also improved their resistance to low-temperature induced photoinhibition of photosynthesis. In conclusion, low-temperature flooding of the plant roots and crowns may boost cold acclimation efficiency in *F. pratensis*, but this effect is genotype-dependent and varies according to the background.

1. Introduction

The environmental stresses faced by plants in the winter seem to be enhanced by global warming. While low temperatures and frost events have probably become less frequent over most areas (IPCC, 2007), other detrimental phenomena, like increased winter precipitation (Osborn and Hulme, 2002) and snow melt during warmer winters, may lead to low temperature flooding and the formation of ice encasement. These stresses may affect plant overwintering and yield. It was shown that low temperature flooding is not as damaging as high temperature flooding (Beard and Martin, 1970). Growth at higher temperature is accompanied by reduced oxygen solubility and higher enzyme activity, but if the exposure is protracted, accumulating anaerobic products may damage the overwintering plants (Beard, 1964; Pomeroy and Andrews, 1979; McKersie et al., 1982).

Exposure to low temperature increases the tolerance of many species to freezing stress and to ice stress, and this is termed an acclimation process. Plant cold acclimation (CA) is associated with various biological changes. A major change in gene expression pattern (Gilmour et al., 1998) triggers a set of metabolic and physiological reactions, including the accumulation of compatible solutes, such as soluble sugars (Hoffman et al., 2010), and changes in photosynthetic machinery (Rapacz et al., 2008). It was shown that flooding of the soil surface during low temperature growth disturbed CA process of winter wheat, but this effect was genotype-dependent (Pomeroy and Andrews, 1989).

The accumulation of water soluble carbohydrates (WSC) has been associated with improved freezing tolerance in many grass species (Harrison et al., 1997; Patton et al., 2007). Water soluble carbohydrates are accumulated in the leaf and crown tissues vacuoles (Pollock and Cairns, 1991), and act as osmolites lowering

Abbreviations: CA, cold acclimation; CBF/DREB, C-repeat binding factor/dehydration-responsive element-binding protein; F_0 and $F_{0'}$, fluorescence when all PSII reaction centres are open in dark- and light-acclimated leaves, respectively; F_d, fluorescence decrease; F_m and $F_{m'}$, fluorescence when all PSII reaction centres are closed in dark- and light-acclimated leaves, respectively; F_s, steady state fluorescence in light exposed leaves; F_v and $F_{v'}$, variable fluorescence in dark- and light-acclimated leaves, respectively; F_v/F_m, apparent quantum yield of PSII; PSII, photosystem II; $F_{v'}/F_{m'}$, photosystem II antenna trapping efficiency; Fp8 and Fp13, *Festuca pratensis* genotypes with lower frost tolerance, according to Kosmala et al. (2009); Fp1 and Fp37, *Festuca pratensis* genotypes with higher frost tolerance, according to Kosmala et al. (2009); *LOS2*, low expression of osmotically responsive genes 2 (bifunctional enolase with transcriptional repression activity); t_{EL50}, temperature at which 50% of the plants were killed by frost; NPQ, non-photochemical quenching of chlorophyll fluorescence; q_P, photochemical quenching of chlorophyll fluorescence; *STZ/ZAT10*, zinc finger protein; WSC, water soluble carbohydrates.

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the crystallization temperature inside cells. Main storage carbohydrates in temperate forage grasses are fructans (Pontis, 1989; Cairns et al., 2002), and their major role is maintaining high photosynthesis rate in low temperature conditions by decreasing sucrose accumulation in photosynthetically active cells (Thorsteinsson et al., 2002). High photosynthetic activity protects plants from cold-induced photoinhibition of photosynthesis (Huner et al., 1993). Freezing tolerance is related to the tolerance against the cold-induced photoinhibition of photosynthesis. This is a consequence of common mechanisms shared by acclimation to freezing stress and cold-induced photoinhibition of photosynthesis (Rapacz et al., 2004, 2011). Comparative proteomic research published by Kosmala et al. (2009) revealed that most of the differentially accumulated proteins in cold acclimated *Festuca pratensis* genotypes of diverse frost tolerance were directly involved in photosynthesis.

Festuca pratensis is the most frost-tolerant species within the *Lolium–Festuca* species complex, and may be a possible effective donor of frost tolerance alleles for closely related *Lolium multiflorum* (Italian ryegrass) (Kosmala et al., 2006). Studies involving *F. pratensis* (Kosmala et al., 2009; Jurczyk et al., 2012) have shown its improved frost tolerance conferred by the CA, and that is why this plant is considered an excellent model for studying CA mechanisms in grasses.

The aim of the present study was to verify the hypothesis that low temperature flooding modifies the CA process, leading to changes in frost tolerance of *F. pratensis*. We focused on different aspects of CA: acclimation of the photosynthetic apparatus to cold, changes in WSC concentration, changes in the osmotic potential and induction kinetics of some genes. Three genes of *F. pratensis* were selected for molecular tests; two of them encoding transcription factors, activating the cold regulated genes expression (*CBF6* and *LOS2*), and one effector gene, encoding a protective protein (*FpCor14b*) (Lee et al., 2002; Chinnusamy et al., 2007; Nakayama et al., 2007). It has been reported that the induction of *FpCor14b*, *CBF6* and *LOS2* genes in *F. pratensis* is controlled not only by temperature, but also by light and the time of day when the low-temperature shift occurs. Specific induction of *CBF6* expression was detected after first few hours of cold treatment (Jurczyk et al., 2012).

2. Material and methods

2.1. Plant materials and stress treatments

The experiments were performed on the clones of four *F. pratensis* (Huds.) cv. Skra genotypes, Fp1, Fp8, Fp13 and Fp37, selected as previously described (Kosmala et al., 2009). Fp1 and Fp37 were shown to be high frost tolerant and Fp8 and Fp13 to be low frost tolerant genotypes. The selection was based on: (*i*) plant's ability to regrow after freezing at -8, -11 and −14 °C, following CA (4/2 °C, 10/14 h photoperiod, 200 μmol m^{-2} s^{-1} PPFD), and estimated using Larsen's (Larsen, 1978) visual score, and (*ii*) plant's t_{EL50} (temperature causing a 50% electrolyte leakage) after different periods of CA (Flint et al., 1967; Kosmala et al., 2009). The three year old plants were grown in an open-air vegetation room and at the beginning of the experiment they were about 17 cm high. The plants were grown in 20 cm diameter pots, and the substrate was a mixture of loam soil: sand: peat (1:1:1; v:v:v). In the autumn they were transferred to an air-conditioned greenhouse and grown at 20 °C in daylight, which was (if necessary) increased to 12 h and supplemented automatically on cloudy days to a PAR of 200 μmol m^{-2} s^{-1} using Agro HPS lamps (Philips, Brussels, Belgium). Relative humidity in the greenhouse was maintained at about 60%. Water was supplied as required and plants were fertilized once a week with a half-strength Hoagland's solution.

The experiments were performed in two independent series. Plants were transferred from the greenhouse to controlled environment chambers (+15 °C, 12/12 h photoperiod, 200 μmol m^{-2} s^{-1} PAR provided by Agro HPS lamps, Philips). Ten clones of each genotype were divided into two groups (flooded and control). In the case of the flooded plants the pots were partially filled with tap water to obtain the effect of partial submergence (2 cm above soil level), a state that was maintained until the end of the experiment. After two weeks the plants flooded at 15 °C and control plants were subjected to CA (21 days at 4/2 °C, 10/14 h photoperiod, 200 μmol m^{-2} s^{-1} PAR provided by Agro HPS lamps, Philips). The experiment was concluded after three weeks. The temperature and light intensity given above were measured on the upper surface of the leaves using a PAR/temperature microsensor, an integral part of the FMS2 chlorophyll fluorescence measuring system (Hansatech, Kings Lynn, UK).

2.2. Frost tolerance

Samples (about 3-cm fragments from the middle part of the youngest, but fully developed leaf) were collected after 7, 14 and 21 days of CA. Frost tolerance was determined as described earlier (Kosmala et al., 2009). Leaf fragments were placed into 20 cm^3 plastic vials on ice (5 cm^3 of frozen deionized water) to ensure ice nucleation in conductivity vessels. The total volume of plastic vials was 20 cm^3. The vessels were stored in darkness for 1.5 h at −3, −6, −9, −12, −15 °C in a programmed freezer. The temperature was lowered at a rate of 2 °C h^{-1}. Freezing temperatures were maintained for 1.5 h and then the temperature was increased up to 0 °C at a rate of 3 °C h^{-1}. After thawing, damage to the leaf tissue was estimated based on electrical conductance measurements (CC501, Elmetron, Zabrze, Poland). To achieve 100% electrolyte leakage, leaves of the control plants were frozen in liquid nitrogen. The percentage of electrolyte leakage (%EL) was determined according to Flint et al. (1967) and t_{EL50} was calculated using linear regression fitted to the central (linear) part of the relationship between the freezing temperature (at least three points) and %EL. The results are the means of two experimental series (20 replicates for each freezing temperature/genotype/treatment).

2.3. Photosynthetic acclimation to cold

Measurements of chlorophyll fluorescence parameters were performed before CA and after 7, 14 and 21 days of CA in flooded and control plants, using a pulse amplitude modulation chlorophyll fluorescence imaging system FluorCAM (PSI, Brno, Czech Republic). Four leaves from each genotype (flooded and control) were detached, attached to a piece of black paper with paper adhesive tape, and placed for measurements inside the FluorCAM chamber. Chlorophyll fluorescence induction kinetics and quenching parameters were evaluated at 20 °C following an experimental protocol comprising 20 min of dark adaptation and the measurements of: F_0 (minimal fluorescence intensity measured in the dark-adapted leaves with all PSII reaction centres opened), F_m (maximum fluorescence intensity of the dark-adapted leaves with all PSII reaction centres closed) after a light saturating pulse of about 2000 μmol m^{-2} s^{-1}, F_s (steady state fluorescence in the actinic radiation-exposed leaves) after 400 s of actinic light exposure (150 μmol m^{-2} s^{-1}) combined with saturating light pulses every 25 s, F_d (fluorescence decrease), $F_{m'}$ (maximum fluorescence intensity with all PSII reaction centres closed in the light-adapted leaves) during the last saturating pulse and $F_{0'}$ (minimal fluorescence intensity with all PSII reaction centres opened in the light-adapted leaves) measured with actinic light source switched off after a far-red light pulse. Variable fluorescence (F_v) was calculated as $F_m - F_0$. Photochemical quenching coefficient (q_P) was calculated according to Schreiber et al. (1994) as