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Effects of growth stage and hardening conditions on the association between frost resistance and the expression of the cold-induced protein COR14b in barley

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Abstract

Sowing date, being determinant for growth stage, may play a decisive role in optimising freezing resistance of winter annual plants. In cereal species, in spite of the abundant literature analysing the factors responsible for the acquisition of frost resistance through the cold hardening process, the involvement of the growth stage *per se*, has been seldom considered, especially at the earlier vegetative phases. In this work the contribution of growth stage in determining resistance to freezing temperature has been analysed in field and growth chamber experiments using winter and spring barley cultivars exposed to different hardening conditions. Field damage was assessed twice during winter on plants sown at three different dates. In the growth chamber experiments several acclimation treatments at 11/7 and/or 3/1 °C (day/night) were simulated. In both field and laboratory experiments the development of cold acclimation was monitored by means of a COR14b specific antibody, since in previous studies the expression of COR14b was found genetically linked to frost resistance. The lowest resistance, found in the youngest plants and in spring cultivars, however, was not always associated with the lowest level of COR14b accumulation. COR14b accumulation correlated with frost resistance at the earlier field sampling date and in plants grown at 11/7 °C. In a following phase of the hardening process (second sampling in field and 4 weeks at 3/1 °C in growth chamber) the accumulation of COR14b was independent of plant stage and genotype, showing no association with freezing resistance. Results suggest that growth stage is crucial for the achievement of maximal resistance in barley, but not for COR14b expression.

Keywords: Cold acclimation; Barley; COR14b; Phenological development

1. Introduction

To achieve high yield capacity and yield stability over-winter crops have to survive and grow during the cold season. A fundamental component of the winter survival capacity is represented by the freezing tolerance, which is based on an inducible process, known as hardening or cold acclimation, that occurs when plants are exposed to low non-freezing temperatures (Levitt, 1980). Interaction between genotype and sowing time was found when frost tolerance of legume plants was assessed at different developmental stage, suggesting that survival improved when planting was delayed and the plants had not reached flowering (Brandsæter et al., 2002). Similarly, legume seedling freezing tolerance was higher in young than in older plants (Meyer and Badaruddin, 2001).

In winter cereals, sowing time should rather be optimized to allow plants to reach the three to four-leaf-early tillering stage before frost. At this stage frost resistance reaches its peak, as reported for winter wheat by Lecomte et al. (2003), citing earlier works. In the more recent literature little information is available on the role of the growth stage *per se* in cereal species, especially if focused at the vegetative phases. Instead, several reports have investigated the effect of factors determining hardening duration and rate. A curvilinear relationship between freezing tolerance and days of acclimation was described by Fowler et al. (1996a) for rye and wheat cultivars exposed at 4 °C. A rapid initial rate in cold acclimation occurred in all cultivars, followed by a gradual slowing and, later, a decline. The transition from the vegetative to generative phase was assumed to be responsible for the duration of freezing tolerance (Mahfoozi et al., 2001a; Prášil et al.,

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2004). The critical role of two major mechanisms, vernalization requirement and photoperiod, with a distinct effect in the frost tolerance of winter and spring wheat and barley, was highlighted (Mahfoozi et al., 2001b). Vernalization and photoperiod requirements were identified as the physiological switches sustaining a high expression level of low temperature up-regulated genes. This idea was supported by a number of studies on plants grown and hardened under controlled environmental conditions (Fowler et al., 1996b, 2001; Danyluk et al., 2003).

The cold regulated genes have been suggested to play a role in frost resistance since their expression, under controlled conditions, correlate with the development of freezing resistance in wheat (Fowler et al., 1996b) and barley (Pearce et al., 1996). In both species winter resistant cultivars accumulated cold induced mRNA (Ohno et al., 2001; Takumi et al., 2003) or the corresponding proteins (Danyluk et al., 1998; Giorni et al., 1999) more rapidly and for a longer period than the spring susceptible ones.

Among the cold regulated genes described so far in cereals, COR14b is one of the best characterized (Crosatti et al., 2003). Although the COR14b gene is located on the homeologous group 2, the expression of COR14b mRNA is controlled by a chromosome region (i.e QTL) that coincides with a major QTL (gene) for cold resistance located on the homoelogous group 5 of barley (Francia et al., 2004) and wheat (Vagujfalvi et al., 2000 and Vagujfalvi et al., 2003).

In this work we have investigated, in field and growth chamber experiments, the effect of the growth stage and of cold acclimation conditions on frost tolerance as well as on the expression of COR14b in winter and spring cultivars of barley. Since the expression of this gene is associated with the acquisition of frost tolerance, variations in frost tolerance depending on growth stage as well as on genotype should be reflected by different amounts of COR14b.

2. Materials and methods

2.1. Plant materials

The experiments were carried out using five barley (*Hordeum vulgare* L.) cultivars: three with winter habit ("Aliseo", "Nure" and "Onice") and two with spring habit ("Gitane" and "Tidone").

2.2. Field experiments

A field trial with 1.36 m^2 individual plots was sown on 27 October (first sowing), 11 November (second sowing) and 18 November 1998 (third sowing) at Fiorenzuola d'Arda (Italy), organized as a randomised block design with three replications. About 10 g of green leaves were sampled over the three replications in two times (21 January and 15 February 1999) and frozen in liquid nitrogen. Immediately before sampling the degree of frost damage of the same plants collected for molecular analysis was visually estimated on a 0–9 scale (0: no damage; 1: slightly yellowed leaf tips; 2: half yellowed basal leaves; 3: fully yellowed basal leaves; 4: whole plants slightly yellowed; 5: whole plants yellowed and some plants withered; 6: whole plants yellowed and up to 10% plant mortality; 7: whole plants yellowed and between 10% and 20% plant mortality; 8: whole plants yellowed and 50% plant mortality; 9: all plants killed). The growth stage of the plants was recorded according to Zadoks et al. (1974).

2.3. Growth chamber experiments

Seeds were germinated in a peat medium mixed with soil in pots of 12 cm diameter (5 seeds each). The following experimental procedures were used:

(I) *hardening at first-leaf stage*: plants were grown at 20/15 °C (day/night) for 1 week, with a daily regime of 10 h light (250 μmol photons m^{-2} s^{-1})/14 h darkness till the plants reached the first-leaf stage; then they were hardened for 4 weeks at +3/+1 °C with a daily regime of 10 h light (150 μmol photons m^{-2} s^{-1})/14 h darkness.

(II) *hardening in plants of different growth stage*: plants were grown at 20/15 °C till the plants reached the first, two and three-leaf stage and, subsequently, hardened at 3/1 °C for 4 weeks, as in (I);

(III) *growth at suboptimal temperature*: plants were grown at suboptimal hardening temperature 11/7 °C (day/night) with a daily regime of 10 h light (250 μmol photons m^{-2} s^{-1})/14 h darkness till the plants reached the first, two, three-leaf stage;

(IV) *growth at suboptimal temperature followed by hardening*: plants were grown at 11/7 °C till the plants reached the first, two, three-leaf stage, as in (III), then hardened at 3/1 °C during 4 weeks.

Temperature and relative humidity were constantly monitored by an hygrothermograph installed in each growth chamber. Irradiance (cool white fluorescent lights) was measured at the tops of the plants by using a GaAsP sensor for photosynthetically active radiation (PAR) described by Pontailler (1990).

At the end of the treatments I–IV the youngest fully expanded leaf of each plant was collected for protein analysis. Thereafter, all the plants were subjected to a freezing treatment in the dark. Temperature was gradually (2 °C/h) reduced at −3 °C. Plants were held at this temperature for 16 h. Subsequently, temperature decreased gradually (2 °C/h) to the designed stress temperature depending on the acclimation level simulated in each experiment. Plants were kept at this temperature for 16 h, temperature was then gradually raised to +1 °C at 2 °C/h. Plants were stored for 1 h at 1 °C to enable assessment of leaf-tissue damage. Frost tolerance was quantified by measuring the extent of membrane injury as the rate increase in ion release, using the whole fully expanded leaf, excluding the tip, according to Rizza et al. (1994).

Sample for protein analysis were collected, at the end of the stress treatment, 1 h after return at +1 °C, only in the experiment I.

Each experiment was arranged as a randomised complete block design with 6–10 replications. An analysis of variance (ANOVA) was performed for each experiment using the software MSTATC. In statistical analysis a single factor ("cultivar")