Current and emerging screening methods to identify post-head-emergence frost adaptation in wheat and barley

T.M. Frederiks¹²,*, J.T. Christopher³, G.L. Harvey¹, M.W. Sutherland² and A.K. Borrell⁴

¹ Queensland Department of Agriculture, Fisheries and Forestry (DAFFQ), Leslie Research Facility, PO Box 2282 Toowoomba, QLD, 4350, Australia
² The University of Southern Queensland, Centre for Systems Biology, Toowoomba, QLD, 4350, Australia
³ The University of Queensland, Queensland Alliance for Agriculture and Food Innovation, Leslie Research Facility, PO Box 2282 Toowoomba, QLD, 4350, Australia
⁴ The University of Queensland, Queensland Alliance for Agriculture and Food Innovation, Hermitage Research Facility, 604 Yangan Road, via Warwick, QLD, 4370, Australia

* To whom correspondence should be addressed. E-mail: troy.frederiks@qld.gov.au

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Abstract

Cereal crops can suffer substantial damage if frosts occur at heading. Identification of post-head-emergence frost (PHEF) resistance in cereals poses a number of unique and difficult challenges. Many decades of research have failed to identify genotypes with PHEF resistance that could offer economically significant benefit to growers. Research and breeding gains have been limited by the available screening systems. Using traditional frost screening systems, genotypes that escape frost injury in trials due to spatial temperature differences and/or small differences in phenology can be misidentified as resistant. We believe that by improving techniques to minimize frost escapes, such ‘false-positive’ results can be confidently identified and eliminated. Artificial freezing chambers or manipulated natural frost treatments offer many potential advantages but are not yet at the stage where they can be reliably used for frost screening in breeding programmes. Here we describe the development of a novel photoperiod gradient method (PGM) that facilitates screening of genotypes of different phenology under natural field frosts at matched developmental stages. By identifying frost escapes and increasing the efficiency of field screening, the PGM ensures that research effort can be focused on finding genotypes with improved PHEF resistance. To maximize the likelihood of identifying PHEF resistance, we propose that the PGM form part of an integrated strategy to (i) source germplasm; (ii) facilitate high throughput screening; and (iii) permit detailed validation. PGM may also be useful in other studies where either a range of developmental stages and/or synchronized development are desired.

Key words: Barley, in-head frost, reproductive frost, spring radiant frost, wheat.

Introduction

Reproductive frost is a major constraint to increasing cereal production. Post-head-emergence frost (PHEF) damage and crop adaptation has been the focus of sustained research over several decades, being recognized as a major problem for more than a century (Farrer, 1900). Yield gains have been made through better management of frost risk, particularly by optimizing the combination of planting dates and varieties (Woodruff, 1992). However, there has been little progress in identifying true genetic PHEF resistance. There have been a number of instances where lines with putative PHEF resistance have been identified opportunistically when breeding and frost trials have been affected by natural frosts (Maes et al., 2001; Reinheimer et al., 2004; Fuller...
Freezing of crop tissues is a physical process moderated by factors such as plant development stage (Single, 1964), ice-nucleating bacteria (Lindow, 1983), and temperature. The actual temperature experienced by crops can vary widely in a given region due to the interactions of topographical, meteorological, environmental, and plant physiological factors (Marcellos and Single, 1975; Woodruff et al., 1997).

Radiant frosts occur when still cold air, clear skies, and a dry atmosphere combine, allowing rapid radiation of heat to the night sky (Foley, 1945; Hovevar and Martsoff, 1971; Willcocks and Stone, 2000). These radiant frosts are a particular problem in spring, as crops develop quickly to susceptible post-heading stages. The term ‘spring radiant frost’ is used here to describe such frost events. To enhance understanding of PHEF resistance mechanisms, it is helpful to use a framework similar to that proposed by Levitt (1972) for drought. Frost adaptation will be discussed in terms of frost escape (e.g. escaping frost due to phenology) and frost resistance (facing the challenge of frost post-head-emergence, Frederiks et al., 2011a). The latter can be further partitioned into freezing avoidance and freezing tolerance.

Techniques, technical limitations, and challenges involved in the study of PHEF resistance

Resistant cultivars with useful PHEF resistance have not, so far, been developed from materials identified opportunistically in frosted breeding trials (Maes et al., 2001; Fuller et al., 2007; Frederiks et al., 2009; Frederiks, 2010). This may be due to small differences in phenology determining whether a particular line is exposed to a frost event and/or due to spatial variation in temperatures across a trial. A number of attempts have been made to develop methods that minimize these confounding effects.

To date, methods used to screen for PHEF resistance fall into two general categories: (i) artificial screening methods; and (ii) field screening methods. When considering the most effective strategies for PHEF screening, it is useful to discuss each of these strategies in turn. A novel method of screening for PHEF resistance will be presented in the section following field screening methods.

Artificial screening methods for post-head-emergence frost resistance

Screening in the field using natural frosts can be problematic due to the unpredictability of natural frost events, in terms of both timing and severity. Freezing chambers could enable more efficient research and screening by allowing out-of-season screening and/or by providing more precise control of the timing and intensity of frost treatment (Ivory, 1978; Marcellos, 1981; Newton, 1988; Fuller and Le Grice, 1998; Chen et al., 2009a, b). Hence, there has been significant interest and effort put into developing artificial screening environments to simulate radiant frost damage for use as a research and breeding tool. Given the regulatory requirements to field trial genetically modified (GM) plants, it is also highly desirable to be able to test GM material for PHEF resistance in contained controlled environments prior
to field testing (Vickers et al., 2006). There have been a number of efforts to develop frost chambers (Ivory, 1978; Marcelllos, 1981; Newton, 1988; Fuller and Le Grice, 1998; Chen et al., 2009b), which can be subdivided into two main categories on the basis of their cooling method: convective or radiant. In practice, many ‘radiant’ chambers also use convective cooling to a greater or lesser extent.

Convective freezing chambers

Convection or forced-draft cooling, as found in commercial freezers, is effective at reproducing field ranking for wheat and barley in the vegetative stages and adequate to allow breeding selections (Marcelllos and Single, 1975; Limin and Fowler, 1988; Single, 1991). This forced-draft method is routinely used when selecting winter wheat for vegetative freezing tolerance (Fowler et al., 1981; Šâulescu and Braun, 2001). Convective cooling also appears to work satisfactorily when screening for vegetative tolerance in non-cereals including broad-leafed and coniferous species (Arakeri and Schmid, 1949; Burr et al., 1990; Meyer and Badaruddin, 2001). Unfortunately, this does not hold true for wheat and barley plants as they progress into the more susceptible reproductive stages. Frost resistance in the vegetative stages does not necessarily confer superior resistance in reproductive stages (Livingston and Swinbank, 1950; Fuller et al., 2007; Frederiks et al., 2011a). Thus, selecting for vegetative frost tolerance cannot be used to select for resistance in reproductive stages. Several convective freezing chambers have been developed with the aim of testing cereals post-head-emergence (D. Woodruff, personal communication; Newton, 1988; Single, 1991; Vickers et al., 2006).

Unfortunately, convective chambers do not simulate the radiant heat loss occurring during natural spring radiant frost. More importantly, to date, these convective chambers fail to reproduce consistently plant damage or screening results as observed in the field under natural radiant frost conditions (Single, 1991; Frederiks et al., 2004a). Crystalline frost, observed on the plant surface under radiant frost conditions, is absent from plants cooled convectively. The absence of this surface ice may allow excessive supercooling to occur (Aston and Paton, 1973). In field conditions, surface water (dew) affects ice nucleation, the spread of the ice front on the plant surface, and the nucleation of ice into plant tissues (Gusta et al., 2003; Frederiks et al., 2004a). In most freezing chambers, dew and surface ice deposit on the cooling surface of the chamber and not on the plant, as occurs during natural radiant frosts (Fig. 1). While a significant role for dew in frost damage has been recognized for some time (Single, 1964), a focused study is required to understand the mechanisms involved.

Radiant freezing chambers

As convective chambers have not proved successful for screening of PHEF resistance, a number of attempts have been made to build radiantly cooled chambers that better simulate spring radiant frost conditions (Aston and Paton, 1973; Marcelllos and Single, 1975; Fuller and Le Grice, 1998; Long et al., 2005; Chen et al., 2009b). Chambers require careful testing to establish that they (i) provide significant radiant cooling and (ii) reproduce field damage better than convective or forced-draft chambers. Ideally, chambers should be of adequate dimensions to allow for the larger scale and higher throughput screening necessary for breeding selections (Frederiks et al., 2004a; Fuller et al., 2009).

To date, possibly the best designed cabinet for the simulation of radiant frost in cereals was developed by Marcelllos (1981). This design was modified more recently by Fuller and Le Grice (1998). The ‘Marcellos’ chamber produced true dendritic crystalline frost on the surface of test plants, as observed under field conditions (Fig. 1), suggesting that this chamber may avoid some of the problems of deep supercooling and better mimic damage observed following natural spring radiant frosts. The small Marcelllos test chamber used a refrigerated blackened radiant heat sink in the ceiling separated from the chamber by a Mylar film. The walls of the chamber were temperature controlled and provided convective heat exchange. The chamber was humidified using chipped ice. Also notable was a larger scale radiant frost room used for studies of Eucalyptus (Aston and Paton, 1973). This frost room employed a liquid nitrogen-cooled ceiling acting as a heat sink. Good precision in temperature control was achieved across the 9 m² test area. More recently at the Australian Genome Research Facility (AGRF), University of Adelaide, South Australia, a radiative frost simulator was developed (Long et al., 2005; Vickers et al., 2006; Chen et al., 2009a, b). The design, however, could, in practice, more closely resemble a convective chamber with passive air movement, rather than a true radiatively cooled chamber. The AGRF frost simulator uses high surface area, reflective condensers operating at a small relative temperature difference to the target chamber air temperature. Thermal infrared imaging reveals that wheat plants in the AGRF frost simulator can supercool, escaping ice formation and damage, at temperatures that result in severe damage under field frost conditions (Fuller et al., 2009).

Use of nucleators in artificial freezing chambers

A particular problem of freezing chamber studies aimed at studying PHEF damage in both convective and radiant type chambers...
is the apparently random occurrence of escapes due to supercooling (Fuller and Le Grice, 1998; Fuller et al., 2009). Heading wheat plants can, in fact, cool to temperatures as low as –15 °C without freezing or damage (Fuller et al., 2007, 2009). This is well below the –6 °C to –8 °C plant canopy minimum temperature range for wheat and barley, respectively, that results in freezing and universal severe damage to spikes and stems under field conditions (Frederiks, 2010, Frederiks et al., 2011a). In chambers, the efficiency of plant freezing can be improved by spraying plants with water, or water and an ice nucleator such as the commercial Pseudomonas bacterial extract Snomax® (Chen et al., 2009b; Fuller et al., 2009). Alternatively, ice nucleation can be precipitated by using a spray of CO₂ under pressure applied to wet plants (Single, 1991). Whether or not such treatments are used to initiate freezing of water droplets, deep supercooling can still be observed in plant tissues even when droplets freeze on the plant surface (Single, 1991; Frederiks et al., 2004b; Fuller et al., 2009).

Optimization of frost simulation chambers

It may not be necessary to faithfully reproduce the conditions of a natural frost to identify plants with increased PHEF resistance. However, frost chambers do need to produce genotype rankings that are repeatable and consistent with field scores. Reproducibility of results remains a problem when assessing material in freezing chambers (Single, 1991; Frederiks et al., 2004b). Ice nucleation appears to be a key difference between artificial and field studies. A better understanding of how ice forms and is propagated through plant tissue during radiant field frost conditions would greatly assist the development of future artificial freezing tests. Similarly, improved quantification of meteorological conditions during natural radiant frosts will be informative in future chamber design.

The potential to control the timing and severity of the frost treatment in an artificial screening system would seem to justify further research effort to overcome the current technical difficulties. However, given the limitations of current frost chambers, field screening remains essential to study reproductive frost resistance in cereals. Regardless of future improvements in artificial screening methods, field screening will be necessary to validate the field performance of any genotypes identified in chambers.

Field screening methods for post-head-emergence frost resistance

Field screening strategies remain the most reliable method to screen for PHEF resistance, although a number of potential difficulties and many potential sources of error must be considered when devising a field trial strategy. The greatest challenge arises from the unpredictability and high variability of natural frost events. It is necessary to select sites where damaging frosts occur regularly. However, such sites may experience periods where a number of damaging events occur within a few days of each other, confounding data collection. A period of 7–14 frost-free days between damaging events is ideal, allowing symptoms to develop and damage to be assessed. If frosts are too frequent, insufficient newly developed, undamaged material is available to be assessed following subsequent frost events. During natural frost events the entire field is exposed to frost. It is therefore difficult to design a control treatment where plants are grown in similar field conditions to the test plants, but are not exposed to the frost treatment. It is also possible for small differences in plant height, plant canopy development, and field topography to cause differences in the minimum temperatures experienced by individual test lines in the field (Woodruff et al., 1997; Frederiks et al., 2004b). Sources of spatial variation include depressions in the field resulting in cold pockets, cross-site slopes affecting cold air drainage, trial design configurations resulting in gaps in the canopy, and canopy effects of test lines and neighbouring genotypes. It is, therefore, important to maintain uniform test plots, crop density, and canopy development. It is also important that spikes are compared at similar developmental stages and susceptibility.

History of development of field screening methods

Rigorous field screening methods, including methods to minimize frost escapes, have been developed over 50 years of research in Australia (Single, 1961, 1964, 1966). Dr W. Single, in collaboration with H. Marcellus, undertook significant fundamental research into PHEF damage in wheat throughout the 1960s, 1970s, and 1980s at Tamworth, NSW. While much of the work was initially undertaken in refrigerated chambers, Single stressed the need to look carefully at results from natural field frosts (Single, 1991). Practical difficulties in field frost screening include the comparison of germplasm with gross morphological and phenological differences. The unpredictability of natural frosts in terms of both timing and severity also complicates field screening. To help overcome these difficulties, screening was largely restricted to genotypes of similar phenology, mainly midseason or early maturing. Genotypes were grouped according to time of ear emergence and stem length. Early serial sowings of small plots were made at multiple sites. The relative performance of test lines was scored in terms of grain set per spike or, where possible, yields of plots. For yield assessments, the development of plots needed to be synchronized at the time of the frost. The practical difficulties in achieving synchronized development meant that useful yield comparisons were rarely achieved in practice (Single, 1991).

Other methods based on mechanized sowing of small plot breeding trials have been used to screen larger numbers of genotypes. However, it is inherently difficult to eliminate frost escapes in these trials (Frederiks et al., 2011a). Using plots requires larger land areas than single rows and inevitably leads to a greater likelihood of spatial and minimum temperature variations across the trial area. Differences in canopy development and leaf area dynamics of individual genotypes can affect the minimum temperatures observed within individual test plots. For example, a dense canopy may trap cold air near the heads more effectively than a sparse canopy which may allow more cold air drainage and back radiation of heat from the soil (Marcellus and Single, 1975; Woodruff et al., 1997). Similarly, the use of small plots makes it difficult to develop a uniform canopy across the trial. Gaps between plots can produce edge effects due to the moderating effect of heat radiated from exposed soil between plots and due to cold air drainage. The logistics of mechanical planting may also make full randomization for sowing dates more difficult.
In field screening, it is necessary to identify those heads which have been exposed to frost. To mark heads, spikes were sprayed with a coarse mist of paint without delay after each damaging frost (Single, 1991). A different colour was used for each frost event. This system of painting spikes produced a flecking effect rather than a solid spray, allowing periodic marking with more than one colour without obscuring previous markings. By noting the combination of paint droplet colours, and correlating this to the marking dates, it was possible to determine the frost events to which a particular spike had been exposed. In addition, Single proposed that future screening methods use carefully selected sites, artificial lighting to modify plant phenology, and a frost control shelter (Single, 1991).

Can a frost shelter provide a milder treatment or control in field screens?

A movable frost shelter was constructed for deployment in the 2000 season at the Hermitage Research Facility in north-eastern Australia (Fig. 2). The shelter was designed to cover part of the trial so as to attenuate the effects of frosts. Thus, some plots were partially protected from frost, leading to two different frost treatments for each frost event: a less severe sheltered treatment and a more severe unprotected treatment. The shelter was constructed of shade-cloth, 5 m in width (plus a 1 m curtain on each side) and 15 m in length. The shade-cloth was stiffened laterally with steel rods and fastened to three wire cables. The cables were attached to pulleys mounted on axles, such that the shelter could be positioned over the trial or removed to the south (to avoid shading) of the trial plots. The shelter was automated in 2001. A motor was activated to cover and uncover the plots at a pre-set trigger air temperature of approximately +2 °C to +4 °C.

Unfortunately, the frost screen used at the Hermitage Research Facility proved to be difficult to operate and unreliable when automated. The shelter was found to moderate the plant minimum temperature during a frost. However, damage observed from sheltered plots appeared atypical. In two of the damaging frost events, occurring on 10 and 18 August 2001, four marked plants, showing no visual signs of damage, were identified. At the minimum plant temperatures of –6 °C and –8 °C recorded under the frost shelter for 10 and 18 August, respectively, severe damage to all affected wheat plants would be expected. In all trials where the frost shelter was not used, these temperatures resulted in severe universal damage to exposed wheat heads. Supercooled liquid water was more often observed under the frost shelter than in the unprotected treatment. The undamaged plants and the increased observation of supercooled water under the shelter could not be explained by the small moderating effect on temperature alone. This suggested that the shelter was not providing results which fully mimicked milder field frosts nor unfrosted conditions. The increased likelihood of supercooling under the frost shelter invites comparisons with the increased supercooling that occurs in artificial screening chambers. The reason for this potential similarity remains to be investigated. The frost shelter appeared to offer greater protection than would be expected by the moderation of the temperature alone. These concerns, combined with the problems in operation and reliability of the field screen mechanism, led to the discontinuation of this system after 2002. Much of the mechanical complexity of this frost shelter could be eliminated by using heaters or fans to mitigate the effects of frost once a pre-set temperature is reached in the field. However, it might be difficult to avoid affecting unprotected test plots within small areas using such a method.

Manipulating plants to manage natural frost treatments

Another approach to managed frost treatment during natural frosts is to use plants grown in portable containers. Such plants can be grown in a glasshouse or similar structure to protect against frost until they have reached the required developmental stage. The plants can then be moved outside when a frost is predicted. Once the plants have been exposed to the desired minimum temperature, they can be moved back into the protection of a heated glasshouse, or the frost conditions ameliorated in situ, allowing plants to develop in a frost-free environment until the frost damage can be assessed. Such a system allows plants grown under uniform conditions to be selected at the desired developmental stage and tested at a range of different minimum temperatures during a single frost event. With increasing numbers of test genotypes, serial sowings to generate a range of heading dates, frost treatments, and glasshouse control, the number of potted plants required for statistically valid experiments multiplies rapidly. Should the results of such a system prove reproducible, it may be possible to develop an automated handling system to move plants at the desired times and temperatures between the external frost and the growing environment. Encouraging preliminary results from a manual system of this type have been observed (Tshewang, 2011), indicating that further development of managed natural frost systems should be pursued.

Serial sowing and managing phenology in natural field screening methods

D. Woodruff conducted frost trials from the 1970s until his retirement in 2001, both in the field, and using freezing chambers (Newton, 1988) at the then Queensland Wheat Research Institute...
(QWRJ), in north-eastern Australia. Towards the end of this time, field plots were discontinued in favour of serially sown single rows and, in the 2000 field season, serial sowings were coupled with photoperiod extension, producing two heading times for each time of sowing.

After the winter of 2000, the photoperiod extension method was further refined. Initially a string of lights was placed mid-row, running perpendicular to, and in the centre of, the 5 m rows. Re-positioning the lights to the end of the test rows provided a gradient of decreasing light intensity and photoperiod extension effect along the rows with increased distance from the lights. Moving the light string also doubled the number of rows that could be grown using a single light string, as one light string could have rows growing perpendicular on either side.

It is difficult to bring genotypes of different phenology to the same developmental stage during natural frost events. Small differences in developmental stage can dramatically affect frost susceptibility (Single, 1964; Frederiks et al., 2011b). Previously, serial plantings have been used in an attempt to synchronize plant development. However, fine differences in phenology and statistically significant effects of planting date on frost damage have been observed, suggesting that genotypic comparisons across planting dates may be confounded (Frederiks et al., 2004b, 2011a).

Thus, to prevent genotypes that escape frost injury in trials due to spatial temperature differences and/or small differences in phenology being misidentified as resistant, we developed a method that uses an artificial photoperiod extension gradient to bring genotypes of different phenology to common developmental stages for frost testing. We refer to this as the photoperiod gradient method (PGM). This method is the culmination of ideas developed by earlier frost researchers in north-eastern Australia over the past 50 years (Single, 1991).

A novel method for field screening: the photoperiod gradient method (PGM)

Over the last decade, field trials were established at multiple sites found to vary in minimum temperature on a given frost night, to quantify levels of PHEF resistance. Genotypes were established in randomized single rows at each sowing date, with two or three sowing dates at each site, and a minimum of two replicates, configured in a complete randomized block design.

Trials were fully irrigated and fertilized to provide non-limiting levels of moisture and nutrients. As the method developed, row length, artificial photoperiod extension, and the number of replicates and serial sowing dates varied with year. From 2006 onwards, few changes to the trial layout were made. Seed was hand-sown in 5 m rows (various row lengths including 1, 3, or 6 m rows have previously been used) with row spacing of 300–400 mm (consistent row spacing used in each trial) with a target population density of 130 plants m⁻² (Fig. 3). The sowing dates at each site were chosen to ensure heading of test lines throughout the peak frost risk period. In north-eastern Australia, the first sowing is typically established in early April, resulting in heading material from late May for screening during natural frost events, with subsequent sowings established at intervals of ~17–21 d. At one end of each test row, supplemental artificial lighting was provided by 100 W incandescent bulbs or comparable 18 W compact fluorescent bulbs at 800 mm spacing and ~1 m height above ground level, so that daylength was increased to 18 h in the vicinity of the lights. A 1 m buffer strip was planted directly below the lights, with test rows starting 0.5 m from, and running 5 m perpendicular to, the line of lights (see Fig. 3) so that artificial light intensity, and consequently, the effect of daylength extension, reduced with distance along the length of each test row. In subtropical north-eastern Australia (Toowoomba ~27 °S) the difference between the shortest (~10 h 20 min) and longest day (~13 h 50 min) is relatively small. The photoperiod extension to 18 h is intended to saturate any photoperiod requirement in the plants closest to the lights, resulting in more rapid progression from vegetative to reproductive development. The artificial photoperiod extension effect diminishes with diminishing light intensity for 3–4 m along the test rows, with little effect on phenology observed beyond that distance. Thus, a 5 m row sufficiently expresses the full range of the photoperiod gradient effect.

Serial sowings, in combination with the photoperiod extension gradient along the test rows, generates a wide spread of flowering times for each genotype through the season. Spikes of similar developmental stage can then be selected among different genotypes by selecting spikes at various distances from the lights. This spread in flowering time allows comparison between genotypes of varying phenology to be made at a similar developmental stage when natural frost events occur. Direct comparisons of plants sown at different times are made with caution, as differences in frost damage can be observed between plots with different times of sowing (Frederiks et al., 2004b, 2011a). Trials are planted with the aim of establishing a uniform, closed canopy. Guard rows are closely planted around the test rows to ensure a standardized canopy to the edges of the trial.

Selecting appropriate field screening trial sites

Field trials are established at multiple sites. These sites should: (i) be subject to a number of crop-damaging radiant frosts during the growing season, and routinely vary in minimum temperatures between sites on a given night; to vary the intensity of frost experienced on any given night, it may be possible to use multiple sites at various elevations along a natural slope; (ii) experience relatively high daytime temperatures, even mid-winter, allowing the continuous development of previously undamaged plant material throughout the season; (iii) exhibit minimal spatial variation within a site (e.g. have little variation in soil type, topography, or cropping history); (iv) produce low baseline levels of sterile florets (e.g. have high light intensities and non-limiting growing conditions to reduce the occurrence of confounding pollen sterility); and (v) preferably be located near research stations, allowing easy daily inspection of sites.

Characterizing frosts: temperature measurements

In radiant frost studies, it is useful to measure the actual plant temperature. Plant canopy temperature, although typically milder, also gives a good guide. However, air temperatures
measured in a Stevenson screen are not ideal for characterizing radiant frost, tending to record higher temperatures than those measured at the top of the crop canopy. This is because the lack of air movement during a radiant frost allows relatively warmer air to be retained in the screen. The screen also prevents radiant heat loss to the night sky. The magnitude of this temperature difference can vary depending on the severity of the radiant frost events (Hayman et al., 2007).

During a radiant frost, the temperature within the canopy can vary by several degrees (Marcellos and Single, 1975). For crops with a closed canopy, the coldest conditions are typically observed near the top of the canopy.

In the PGM trials, plant minimum temperatures at the top of the canopy are measured using fine thermistor probes as described in Frederiks et al. (2011a). Probes are attached, with adhesive tape, to the leaf blade of the uppermost expanded leaf, exposed to the night sky. Calibrated minimum/maximum thermometers are also used in the trials, to provide a convenient measure of minimum nightly temperatures at crop canopy height (Frederiks et al., 2011a). The minimum temperatures recorded by the thermometers at canopy height are generally slightly less extreme than those recorded by the small thermocouples attached to leaves which have considerably less thermal mass. When the minimum thermometer temperature has reached approximately −4 °C or lower, it is likely that plant minimum temperatures may have reached damaging levels. In such circumstances, trials are carefully inspected and spikes marked for later scoring of damage. Characterization of field conditions is now also augmented using a meteorological station recording Stevenson screen and open air temperatures, humidity, rainfall, leaf wetness, incoming solar radiation, net radiant exchange, soil temperature, soil moisture, soil heat flux, and wind speed and direction.

**Characterizing plant development: marking methods**

It is important for the genotypes that are being compared to be at a matching developmental stage during a particular frost event. It is also important to mark individual spikes as soon as possible following the occurrence of a measured frost event. This is important as spikes remaining protected by the flag leaf sheath inside the ‘boot’ may have escaped exposure to the full effect of the frost, but become difficult to distinguish from test heads as they continue to emerge from the flag leaf during subsequent days. The inclusion of these spikes in the experimental analysis would result in them being misidentified as resistant, leading to a ‘false-positive’ result.

Key to this methodology is the careful marking and monitoring of individual spikes immediately following well-characterized frost events. On the morning of a frost event, individual spikes are marked with spray paint. A minimal spray of paint is applied to the awns, head, or peduncle, just above the flag leaf sheath. Paint colours should give good contrast with foliage and should be pre-tested for potential plant toxicity. Different colours are used to indicate different dates. This method ensures that only spikes that are known to have been exposed to a particular frost event are assessed for damage.

To minimize false negatives, it is very important to avoid marking frost-damaged spikes from previous frosts as minor levels of damage may not be easily observable for several weeks. This is particularly problematic when minor levels of damage
such as individual floret sterility are being measured and because some heads inevitably evade paint marking.

It is suggested that investigators visit field sites often throughout the season, particularly before anticipated frost events. This is especially important in the first few seasons, as the expertise of researchers develops. This will allow the researchers to gain experience detecting the subtle differences in crop appearance due to frost damage, such as small changes in colour, surface lustre, and texture, and physical damage to floral structures that differentiate spikes already damaged prior to a particular frost event. Damaged spikes will remain at a stilted developmental stage, but may continue to look superficially green and viable for many days during subsequent mild winter conditions. Thus, experience in detecting previously damaged spikes on the days prior to a frost will greatly assist in avoiding previously damaged material when marking.

Following a frost, all screening material is marked after awn peep (Zadok’s stage 49; Zadoks et al., 1974). Prior to this stage, it is not possible to assess spikes non-destructively for previous damage. Also, before awn peep, full susceptibility to frost is not reached (Livingston and Swinbank, 1950; Single, 1964; Frederiks et al., 2011b). It is ideal if, before rating for damage, the spikes can be allowed to develop in the field for a minimum of 7–14 d following a frost event. However, if allowed to mature fully in the field, it is likely that later frost events will further damage plants. Therefore, it is important to rate damage to developing spikes before any subsequent frosts make collection and interpretation of data difficult. While floret symptoms develop slowly over several days, damage to the stem supporting the spike may be detected just above the highest node in the days immediately following the frost event.

Individual rows are used in this study in preference to plots. This reduces the size and potential spatial variability of the trial but also canopy effects of any individual line. Canopy effects that act to moderate the temperature at spike height may have potential to reduce frost damage in commercial crops. However, these trials aim to keep the treatment frost severity similar for all genotypes to allow comparisons between test genotypes and with elite cultivars. Small differences in temperature resulting from canopy variation can lead to large differences in plant damage and confound genotypic comparisons (discussed in detail in Frederiks et al., 2004b, 2011a). By randomizing row position and testing cultivars of similar height and growth habit in the same trial, the canopy effect of individual lines on themselves and their neighbouring plots is minimized. Randomized replication of test lines is necessary. However, improved statistical power of increased replication must be balanced with the logistical challenges of marking and rating more plant material.

Potential limitations of the photoperiod gradient method

The possibility that variation in the effect of photoperiod along the row could introduce some unanticipated effects on frost resistance needs to be considered. However, genotypic rankings for grain set following frosts were repeatable, regardless of differences in the position of spikes of particular barley genotypes up or down the photoperiod gradient (Frederiks, 2010; Frederiks et al., 2011a). Similarly, distance from the lights, along the photoperiod gradient, did not show a significant effect on grain set (P = 0.57 tested using a linear mixed model, two sites, and three frosts in 2011 for the barley cultivar Kaputar).

After many decades of research, there are no confirmed reports of wheat or barley varieties with adequately improved PHEF resistance, suggesting that resistance is at best, rare (Singh, 1984, 1991; Frederiks, 2010; Frederiks et al., 2011a). To maximize the likelihood of identifying rare or low levels of increased resistance, test lines should be assessed under a range of frost intensities. For severe or mild frost events, no difference between test lines would be expected. Useful screening frost events are those that result in moderate, or moderate to severe, frost damage to control genotypes. Genotypes should not be eliminated as susceptible following severe frost events only; as this increases the risk of false negatives. Similarly, mild frost events, resulting in little damage to controls, are likely to result in poor discrimination between control and potentially resistant genotypes. Unless at least moderate damage is routinely observed in controls, the efficiency of screening for improved resistance will be poor.

In order to improve the efficiency of research and breeding, stringent testing of lines is required. The conservative PGM minimizes the effect of small differences in phenology and temperature across the trial. However, given the intensive nature of the method, the number of lines that have been assessed remains low.

Wider applicability of the photoperiod gradient method

The PGM has been developed to allow diverse wheat and barley test lines to be compared when natural frosts occur. A similar technique using artificial photoperiod gradients could be applied in other crop research situations where matched phenology is desirable. For example, a PGM approach could be useful for studies of other abiotic stresses where the effect varies substantially with developmental stage. It would also be useful for synchronizing flowering in lines to be crossed.

An integrated approach to identify PHEF resistance

The current PGM is not ideally suited for screening large numbers due to the need for a uniform trial area and the requirement for intensive marking, scoring, and monitoring. In order to maximize the likelihood of identifying PHEF resistance, we propose a three-staged approach. Firstly, use an informed method of selecting the most prospective germplasm from international germplasm collections. Secondly, screen as many candidates as possible using a field method with high throughput. Even though a high throughput method is likely to identify a number of frost escapes or false positives, a well developed and validated high throughput screen should eliminate large numbers of sensitive genotypes. Techniques described in the PGM could be employed to improve the efficiency of high throughput screening. Thirdly, to eliminate frost escapes, we propose a final stage to retest and validate all promising genotypes using the more rigorous PGM approach described here. Given the success of a
similar multistage approach with drought research (Borrell et al., 2006), we believe such a three-tiered strategy could maximize the chances of identifying rare sources of PHEF.

Informed selection of germplasm for screening

To maximize the chance of identifying in-head frost resistance in cereals, it is important to identify material that is likely to exhibit variation for this trait. There is a limit to the number of genotypes that can be rigorously screened. Thus, prioritizing candidate lines is important.

Over 10 seasons, ~100 diverse wheat and barley genotypes, many of which have been reported to offer superior PHEF adaptation, have been tested while developing the PGM. Yet none exhibited superior PHEF resistance compared with current cultivars (Frederiks et al., 2008, 2011a; Frederiks, 2010). Lines tested using the PGM included genotypes with variation for morphological traits such as shortened rachis internode, reduced head size, and glume pubescence (Frederiks, 2010).

Testing has focused on wheat and barley cultivars, elite breeding lines, and types showing encouraging preliminary results (Single, 1968, 1991; Woodruff, 1992; Frederiks, 2010). As resistance has not been identified, it is necessary to widen the search, perhaps to include landraces and wild relatives of wheat and barley. But how can the most likely candidates be identified from the many hundreds of thousands of accessions held in international germplasm collections? Methods to identify accessions based on the collection locations, where climate variables may maximize the chances of variation for target traits, have been developed. The Focused Identification of Germplasm Strategy (FIGS) technique is one example (Mackay et al., 2004). Choosing climatic criteria that select for reproductive frost resistance may at first appear fairly obvious. However, more careful consideration suggests otherwise. Selecting genotypes collected from cold environments at high latitude and/or high altitude, for example, is likely to be too simplistic an approach. Wheat and barley are highly plastic in their phenology and have adapted to environments requiring extreme differences in flowering date. The most likely adaptation in the face of these cold environments is the modification of the flowering habit so that heading occurs outside the main frost-risk period. Hence these plants may rarely face the challenge of frost post-head-emergence, instead utilizing a frost escape strategy. Current frost management strategies used by growers employ a similar principle by delaying sowing so that head-emergence is delayed, thus reducing the frost risk. However, we are seeking genotypes that have developed adaptation, allowing flowering during frost periods. Finding environments where plants are constrained, for example by severe terminal heat or drought, to head during the frost-risk period is more challenging but potentially more rewarding in identifying PHEF adaptation.

In parallel with a more informed selection of germplasm, further collection of material should be considered. New accessions could be sourced to complement existing collections from the regions identified as likely target environments. It is possible that collection methods may have inadvertently selected against early flowering genotypes that are the most likely to have faced the challenge of frost when heading. This may come about if collectors, seeking to collect the maximum number of genotypes in a single trip, visit areas towards the end of the growing season after the late flowering genotypes have ripened.

High throughput field screening

The success of any screen will be dependent on accurate trial monitoring, marking, and rating. Care needs to be taken to ensure test genotypes are at matching developmental stage and susceptibility, and that previously damaged heads are excluded to minimize false-negative results. More work is required to develop techniques that are able to rapidly eliminate large numbers of frost-susceptible lines while maximizing the likelihood of identifying resistant types. The PGM could be modified to improve the efficiency of high throughput screening by reducing the number of serial sowings required to generate heading material of matched developmental stage among test lines of differing phenology. The PGM generates a continuum of flowering dates for each test row rather than a single flowering date achieved by serial sowing. If a similar spread of flowering dates was to be achieved with serial sowing alone, many more serial sowing dates would be required. To maximize trial plan flexibility, trial test rows are currently hand planted. Mechanizing the planting of the PGM to increase the number of lines assessed could be one way to achieve higher throughput. For example, mechanization could increase the number of genotypes assessed by ~4-fold from ~50 per year to 200 per year. While the overall size of trials could be increased with mechanised planting, care would be needed to retain canopy uniformity.

Validation using the PGM

A higher throughput field screening method would increase the likelihood of misidentified frost escapes. To eliminate these frost escapes, it would remain necessary to validate the smaller numbers of genotypes identified as promising using the detailed PGM.

Conclusions

Decades of effort have failed to identify useful PHEF resistance in cereals (Single, 1984, 1991; Frederiks, 2010; Frederiks et al., 2011a). Screening has proved particularly challenging. We believe that the PGM described here provides the most robust method reported to date for the testing of PHEF while minimizing both false positives and false negatives. The use of serial plantings and photoperiod extension to bring genotypes of different phenology to a similar growth stage at the time of naturally occurring frosts is a major advantage. Detailed monitoring both before and after frost events with careful marking of individual spikes is important. Although a degree of skill and experience needs to be developed, this method is transferable and will be pivotal to validating any potential sources of PHEF that may be identified.

Methods using artificial freezing chambers and managed natural field environments show promise as future screening options. Unfortunately, neither method has been developed and validated.
to a point where they could currently be relied upon for routine PHEF analysis.

Given the major economic impact of PHEF in many cereal cropping regions globally, we believe that the search for PHEF resistance must continue, and that an integrated strategy incorporating the PGM as described here can maximize the likelihood of success. Exchange of germplasm between research groups should be encouraged to ensure the accuracy of screening techniques and the verification of any positive results.

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A novel method for post-head-emergence frost screening in cereals


