Testing for frost tolerance in wheat (*Triticum aestivum* L.) transformed with a gene for antifreeze protein

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Abstract. Spring frosts in wheat growing areas of Australia can result in significant damage during the heading stage, resulting in losses millions of dollars each year due to decreased yields. To address this issue, wheat has been co-transformed via micro-projectile bombardment with an antifreeze protein (AFP) gene targeted to the apoplast or endoplasmic reticulum. T0 transgenic plants expressed AFP and showed the potential for frost tolerance in *in vitro* tests on plant extracts (Khanna and Daggard 2005). Additional transgenic wheat plants carrying the AFP gene targeted to both apoplast and ER have subsequently been produced. DNA extracted from plants surviving selection on phosphinothricin revealed 63.8% were PCR positive for presence of the AFP gene, indicating a wheat transformation frequency of 3.97%. Co-transformation of AFP and *bar* genes occurred in 53% of samples tested. PCR and gene expression analysis (via RT-PCR) have been carried out on plants that were subsequently tested in a controlled frost environment at the Australian Genome Research Facility (AGRF Adelaide). Analysis of frost induced sterility (FIS) showed that frosted control plants were significantly higher in FIS compared to unfrosted controls, and that FIS of plants transformed with one of the constructs was not significantly different to that of the unfrosted controls. This result suggests that the introduced AFP may protect the transgenic wheat plants against frost.

Introduction

There are now many reports in the literature describing successful production of transgenic wheat plants. However the important question is whether or not transgenic plants perform at the whole plant level in accordance with the intended role of the introduced transgene, and whether that performance persists in subsequent generations. This project aimed at answering these questions for plants transformed with a synthetic AFP gene. We have tested the plants in replicated trials in a frost chamber and initial results suggest that the introduced AFP transgene offers protection against mild frosts.

Materials and methods

Plasmids, plant material and plant transformation

Plasmids used for transformation contained a wheat codon optimised synthetic AFPI gene cloned into a plant expression vector containing the rice actin promoter and first intron, and *nos* terminator, and were targeted either to the endoplasmic reticulum (pAFP1) (Khanna and Daggard 2005), or to both to the apoplast and endoplasmic

reticulum (pAFP3). Plasmids containing the AFP gene were co-bombarded with pAHC20 (Christensen and Quail 1996), containing the selectable marker gene *bar*, using microprojectile bombardment as described (Gatford *et al.* 2001) but with callus incubation times in the dark extending to 6 weeks (Vickers *et al.* 2004). Most bombardments with pAFP3 included pCvGFPT (Schenk *et al.* 2001) as a screenable marker. Wheat varieties Seri 82 (pAFP1) and BW26 (pAFP3) were used for transformation.

PCR and RT-PCR analysis

High throughput DNA extraction was undertaken at AGRF using Machery Nagel "Nucleospin 96" system according to the manufacturer's instructions, and PCR using Qiagen "HotStar" polymerase, whilst RNA extraction was carried out using Concert 96 System (Invitrogen), and RNA quality assessed using an eGene RNA Analyser (eGene Inc., USA). DNA was removed via DNA Free kit (Ambion), and then reverse transcription carried out using SuperScript III "First Strand Synthesis" system (Invitrogen). PCR with gene specific primers and actin control primers was conducted with HotStar Polymerase (Qiagen). PCR primer sequences were as reported (Vickers *et al.* 2004).

Frost trials in AGRF frost chamber (Adelaide)

Plants tested in frost trials were T2 descendants of one original T0 transgenic plant transformed with pAFP1, and T1 descendants of 28 lines transformed with pAFP3. Twelve plants of each variety of origin were reserved as unfrosted controls. Plants (comprising 137 pots, 4 plants each, 12 plants per line) were grown in PC2 Controlled Environment Rooms in 12 h day, with temperatures of 12˚C for night and 17˚C day, light intensity 600 micromolar, approx 50% humidity, and high pressure sodium lighting until the majority were at Zadoks stage 59, ie. inflorescence fully emerged (Zadoks JC 1974). Stages ranged from early boot (stage 41) to end of flowering (stage 69). The AGRF frost chamber was programmed to hold at 4˚C for 4 h, decrease 1˚C/h to 0˚C, drop 2˚/h to the minimum temperature, hold for 3h then increase at 2˚C/h to 4˚C. Three pots of each variety, each of which contained 4 plants were placed into the frost chamber randomly. Three trials (Trial 1, 42 pots; Trial 2, 51pots; and Trial 3, 15 pots, all 8" diameter) were run at a minimum temperature of -4˚C. Plants in the first trial were then subjected to a slightly more severe frost event (-4.5˚C) approximately 4 weeks later at Zadoks stages 75 to 83, fruit development and ripening (Zadoks JC 1974).

Frost induced sterility (FIS) and frost induced grain damage (FIGD)

FIS (%) was calculated as: ((number of sterile (empty) florets) / (total number of florets with grain filling potential)) * 100 (Reinheimer *et al.* 2004), averaged for 2 rows per head (4 rows for Trial 3). FIGD was calculated as % of grains in a sample that showed blistering on the surface of the grain and/or pre-harvest sprouting (germination). Statistical analysis of FIS was carried out using GenStat.

Results and Discussion

Genome analysis, and expression

Of a total of 336 T1 plants derived from 28 T0 pAFP3 lines from different shots, 170 pAFP3 plants tested PCR positive. Numbers of PCR positive plants out of 12 for pAFP3 lines are shown in Table 1.

Table 1. Distribution of T1 PCR positive plants (out of 12), for 28 pAFP3 lines

PCR +ve plants out of 12	0	1	2	3	4	5	6	7	8	9	10	11	12
Number of T1 pAFP3 lines	4	1	1	0	1	2	5	6	2	3	2	0	1

Interestingly, the distribution peaks close to the 2/3 point (8 plants out of 12), which is the theoretical proportion of T1 generation PCR positive plants for selfed hexaploid wheat. Two-thirds of the T2/T3 pAFP1 plants were also PCR positive.

To date, expression has been shown in 13 plants from 11 lines (including 1 pAFP1 descendent) by RT-PCR. Experiments are currently underway on next generation plants using RNA extraction procedures that are optimised for isolation of small RNAs such as our synthetic AFP (183nt), as some conventional RNA isolation procedures have a lower size limit close to this size. Expression is often reduced or eliminated in generations beyond the T0 generation in transgenic plants, including wheat. For example, transgene expression was completely silenced in most T1 and T2 Bobwhite derived lines expressing pathogenesis related genes, although some stably expressing lines were found (Anand *et al.* 2003). Extracts from transgenic plants are currently being assessed for in-vitro AFP activity, via observation of inhibition of ice re-crystallisation and thermal hysteresis, using an optical re-crystallometer and nanolitre osmometer (Otago Osmometers, NZ).

FIS and FIGD

In the replicated frost trial, FIS was significantly greater for frosted control Seri 82 plants (12.52%) compared to unfrosted Seri 82 (8.68%). Critically, FIS for the frosted transgenic pAFP1 transformed plants (7.02%) was not significantly different from the unfrosted controls ($p=0.042$). This was the case for tillers at stages 59 through 65, from fully emerged to full flowering, which are stages considered from field trial data to be the most susceptible to frost damage (Fredericks, 2003). In addition, 4 of the pAFP3 transformed lines were significantly lower in FIS than untransformed bombarded and tissue cultured control lines, and 2 of these were among the lines shown by RT-PCR to contain expressing plants. These results suggest that the presence of the AFP transgene may be protecting the transgenic plants to some extent from the damaging effects of frost. Whilst a frost of -4˚C is considered to be a relatively minor event for wheat (Single, 2003), transient temperatures down to -6˚C were recorded in the frost chamber, during the 3 h minimum temperature hold.

The second frosting of Trial 1 at -4.5˚C produced frost induced grain damage as the appearance of translucent blistering and wrinkling on the dorsal surface of mature wheat grains, similar to that reported previously (Cromley *et al.* 1998), with pre-harvest sprouting also evident in some of the damaged grains. This is different from FIGD resulting from heavy frosting at pre-anthesis and anthesis, where the grains are only partially filled and have a pinched appearance. Initial analysis of this slightly more severe second frost event shows significantly less damage in some of the pAFP1 transformed plants than in frosted Seri 82 controls ($p=0.005$), suggesting that protection from frost mediated by the AFP transgene may extend into grain filling stages.

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