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Overexpression of the acidic dehydrin WCOR410 improves freezing tolerance in transgenic strawberry leaves

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Summary

Progress in freezing tolerance (FT) improvement through plant breeding approaches has met with little success in the last 50 years. Engineering plants for greater FT through plant transformation is one possible way to reduce the damage caused by freezing. Here, we report an improvement of the selection procedure and the transfer of the wheat *Wcor410a* acidic dehydrin gene in strawberry. The encoded protein has previously been shown to be associated with the plasma membrane, and its level of accumulation has been correlated with the degree of FT in different wheat genotypes. The WCOR410 protein was expressed in transgenic strawberry at a level comparable with that in cold-acclimated wheat. Freezing tests showed that cold-acclimated transgenic strawberry leaves had a 5 °C improvement of FT over wild-type or transformed leaves not expressing the WCOR410 protein. However, no difference in FT was found between the different plants under non-acclimated conditions, suggesting that the WCOR410 protein needs to be activated by another factor induced during cold acclimation. These data demonstrate that the WCOR410 protein prevents membrane injury and greatly improves FT in leaves of transgenic strawberry. A better understanding of the limiting factors allowing its activation may open up the way for engineering FT in different plant organs, and may find applications for the cryopreservation of human tissues and organs.

Keywords: acidic dehydrin, cold acclimation, freezing tolerance, strawberry, transformation, transgenic.

Introduction

Low temperature (LT) tolerance has restricted the cropping alternatives available to farmers in northern countries and, each year, the world loses millions of dollars in crop production and market quality due to LT damage. Cold acclimation (CA) allows hardy plants to develop efficient tolerance mechanisms needed for winter survival. During this period, numerous biochemical, physiological and metabolic processes are altered in plants. These changes are regulated by LT at the gene expression level. To understand the molecular basis of adaptation to LT, efforts have been focused on the identification of LT-responsive genes. Cold-induced genes and their products have been isolated and characterized in numerous species (Breton *et al*., 2000; Hughes and Dunn, 1996; Thomashow, 1999). In wheat and other cereals, the level of expression of several genes during CA is a heritable trait that correlates with the capacity of each genotype to develop freezing tolerance (FT) (Houde *et al*., 1995; Limin *et al*., 1997; Sarhan *et al*., 1997).

Of the many LT-responsive genes characterized to date, several have been predicted to encode proteins with the characteristics of the D-11 or dehydrin class of late embryogenesis-abundant (LEA) proteins (Hughes and Dunn, 1996). The induction of this class of protein has been observed in more than 100 independent studies on drought stress, CA, salinity stress, embryo development and responses to abscisic acid (ABA) (Close, 1996). Dehydrin proteins have a wide size range, no similarity with any enzymes or proteins of known function and accumulate to high levels. They are largely hydrophilic proteins that contain different amounts of the K segment (lysine-rich repeat), S segment (tract of serine residues)

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and Y segment (conserved N-terminal sequence). Using these segments, Close (1996) classified dehydrins into at least five distinct subtypes.

In previous studies, we have identified a new dehydrin gene (*wcor410*) from wheat. Molecular analyses showed that its expression, at both the transcript and protein level, was associated with the development of FT (Danyluk *et al*., 1994, 1998). Due to its acidic nature, WCOR410 is thought to belong to a different subtype of the D-11 protein family, the so-called acidic dehydrins (Danyluk *et al*., 1994). Molecular and immunocytochemical analyses have revealed that, in hexaploid wheat, *wcor410* is part of a multigene family that encodes proteins accumulating to very high levels in the vicinity of the plasma membrane of cold-acclimated cells in the vascular transition area (Danyluk *et al*., 1998). Biochemical fractionation experiments have indicated that WCOR410 is a peripheral protein and not an integral membrane protein. The properties, abundance and localization of these proteins suggest that they are involved in the cryoprotection of the plasma membrane against freezing. It has been proposed that WCOR410 plays a role in preventing the destabilization of the plasma membrane that occurs during the dehydrating conditions associated with freezing, and could be a determining factor for increased cell resistance to freezing. However, to date, no evidence has been obtained on the impact of the overexpression of this type of protein on FT and membrane stability *in vivo*.

In order to gain further knowledge about the capacity of this protein to increase membrane stability and FT in plants, we transferred the wheat *Wcor410a* cDNA into an elite strawberry cultivar (*Fragaria* × *anassassa*, cv Chambly) (Khanizadeh *et al*., 1990). The standard procedure using low kanamycin selection pressure (James *et al*., 1990; Mathews *et al*., 1995; Nehra *et al*., 1990) resulted in chimeras with this elite cultivar, and was also a limiting factor for the cultivar totem (Mathews *et al*., 1998). We have thus used an improved selection procedure to obtain pure and stable transformed lines of strawberry. Improvement of FT in strawberry and related species with low levels of FT is an important economic issue, as these species need physical protection in northern regions to protect them during the winter (Turner *et al*., 1992). Three independent transgenic lines that expressed the WCOR410 protein to a level comparable with that in cold-acclimated wheat were analysed. The overexpression of this gene had no apparent deleterious effect on the growth and development of the transgenic plants under both non-acclimated and cold-acclimated conditions. Freezing tests showed an improvement of 5 °C in the cold-acclimated transgenic strawberry leaves.

Results and discussion

Production of transgenic strawberry

The efficient production of transgenic strawberry containing the *Wcor410a* cDNA (Figure 1A) was obtained using thidiazuron at 2 mg/L to induce abundant organogenesis after infection with *Agrobacterium* (Figure 1B). After the second subculture of the transformed explants, the concentration of thidiazuron was reduced to 1 mg/L to stimulate growth and differentiation (Figure 1C). The first series of transgenic strawberry plants was produced using the kanamycin selection

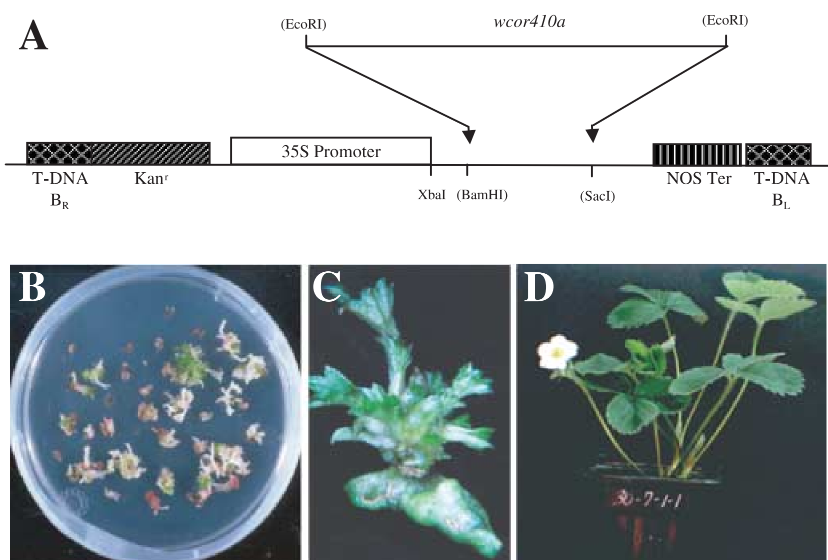

Figure 1 Transformation and regeneration of the strawberry elite cultivar Chambly. The freezing tolerance-associated gene *Wcor410a*, cloned in the sense orientation, was inserted in the expression vector pBI121 (Clontech) as described in 'Experimental procedures'. The recombinant vector pBI121-*Wcor410a* was introduced into *Agrobacterium tumefaciens* strain GV3101 and used to transform 4-week-old shoot cultures of the strawberry cultivar Chambly (*Fragaria* × *anassassa* cv. Chambly). (A) pBI121-*Wcor410a* construct. The binary vector pBI121 was modified to harbour the *Wcor410a* cDNA in the sense orientation after removal of the *gus* gene (*Sac*I, *Bam*HI sites destroyed by fill-in). B_L, left border; B_R, right border; Kan^r, kanamycin resistance gene; NOS Ter, NOS terminator. (B) Selection of transgenic explants on 450 mg/L kanamycin. (C) Transgenic shoot regeneration. (D) Regenerated transgenic strawberry plant with normal phenotype.

pressure recommended for strawberry (James *et al*., 1990; Mathews *et al*., 1995, 1998; Nehra *et al*., 1990). However, we found that the lines obtained using 50–200 mg/L kanamycin were still unstable and produced chimeras where new runners did not always express the WCOR410a protein (revertant line). To increase the genetic stability of transgenic plants, the selection pressure was rapidly increased to 450 mg/L kanamycin. This higher concentration of kanamycin had no deleterious effects on strawberry and produced healthy plants that expressed high levels of the transgene (Figure 1D).

Three transgenic strawberry lines that expressed the WCOR410a protein to a level equivalent to that of cold-acclimated wheat and one revertant line were selected in order to demonstrate that any improvement in FT was due to the transgene rather than to the transformation process. The results of LT_{50} (defined as the temperature at which 50% of electrolytes were released from the tissues due to cell and death membrane leakage) of non-acclimated (–7 °C) and cold-acclimated (–13 °C) plants were identical for the wild-type and the revertant line not expressing WCOR410. The selected lines expressing WCOR410, the non-transformed strawberry and the revertant line not expressing WCOR410 were all multiplied by *in vitro* culture as normally performed for strawberry. The transgenic lines expressing WCOR410 were stable through several generations of new plants derived from the runners after *in vitro* multiplication. It should be pointed out that, except for variety development, strawberry cultivar reproduction is normally performed by vegetative multiplication. This is due to the octaploid nature of strawberry where agronomic traits are very difficult to maintain through crossing.

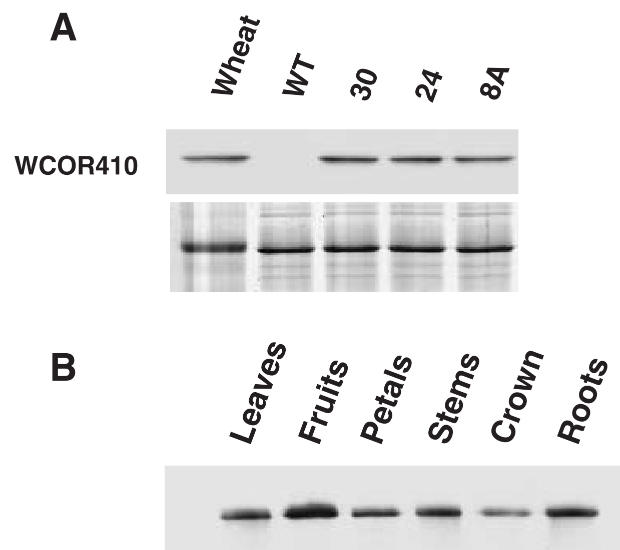

Figure 2 (A) WCOR410 level in transgenic strawberry. The WCOR410 protein level was determined in the regenerated transgenic strawberry plants using the anti-WCOR410 antibody as described in 'Experimental procedures'. A load control stained with Coomassie Blue R-250 is shown under the immunoblot. Wheat, 7-day-old winter wheat *Triticum aestivum* cv. Norstar seedling, cold acclimated at 4 °C for 2 weeks (Danyluk *et al*., 1998); WT, wild-type *Fragaria* × *anassassa* cv. Chambly; 30, 24 and 8A represent three independent transgenic lines transformed with the wheat *Wcor410a* gene. Strawberry plants were grown at 20 ± 2 °C with a 16 h light period (200 μmol/m²/s) for 2 months and were then cold acclimated for 3 weeks at 4 °C with a 12 h light period. (B) WCOR410 protein level in various plant organs of transgenic strawberry. The WCOR410 protein level was determined in various organs of the regenerated transgenic strawberry line 8A grown as in (A), using the anti-WCOR410 antibody as described in 'Experimental procedures'.

Expression of the transgene and effect on freezing tolerance

The level of WCOR410 protein expression was measured by immunoblot in the three transgenic lines and in the wild-type strawberry before and after CA. The data in Figure 2A show that no protein homologous to WCOR410 was present in wild-type strawberry plants acclimated for 3 weeks at 4 °C. On the other hand, the transgenic lines accumulated the wheat protein at a very high level, similar to that of tolerant winter wheat (Figure 2A). There was no difference in the level of WCOR410 protein expression in the transgenic lines before and after CA, suggesting that protein stability is not increased by CA. The WCOR410a protein was found to be expressed in all tissues examined, indicating that the cauliflower mosaic virus (CaMV) 35S promoter allowed strong constitutive expression of this gene in the different organs (leaves, fruits, petals, stems, crown, roots) of transgenic strawberry (Figure 2B).

The growth characteristics and FT of the wild-type strawberry cultivar Chambly were compared with those of the transgenic lines. No apparent difference in growth behaviour (leaf area, biomass, flowering time, number of flowers and fruits per plant) was observed between the transgenic and non-transformed cultivars, indicating that constitutive over-expression of the gene did not cause significant deleterious effects.

Freezing tests of the different transgenic lines grown under non-acclimated conditions (20 °C) did not show a significant improvement of FT compared with the wild-type or with the revertant line not expressing WCOR410, as the LT_{50} was –7 °C in all cases. Time course studies indicated that transgenic strawberry leaves increased their FT tolerance faster than the wild-type plants, and a clear difference was observed after 2 weeks of CA (Figure 3A). The transgenic leaves reached their maximal level of FT after 3 weeks of CA. Electrolyte leakage was 20% for cold-acclimated transgenic plants, compared with 60% for the wild-type (Figure 3A). This difference

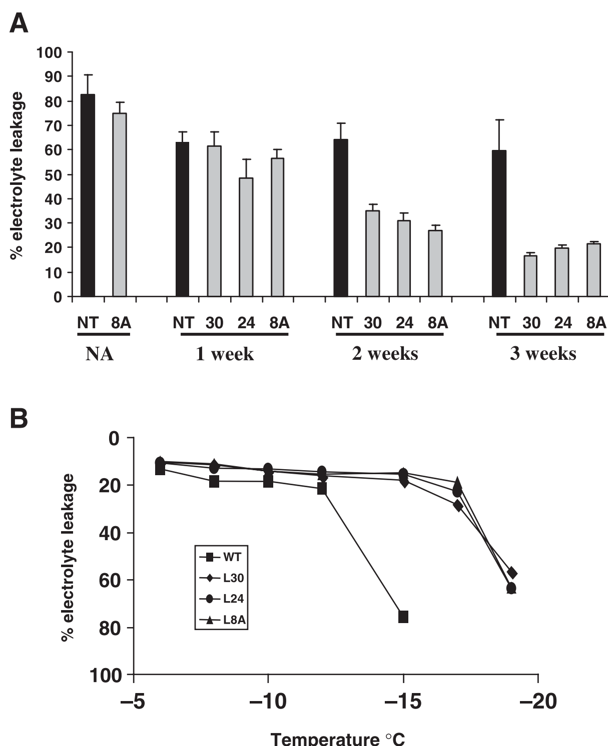

Figure 3 (A) Effect of time of cold acclimation on strawberry freezing tolerance. Wild-type and transformed strawberry plants were grown at 20 ± 2 °C with a 16 h light period (200 μmol/m^2/s) for 2 months and were then cold acclimated for different periods at 4 °C with a 12 h light period. The freezing tolerance of intact leaves from wild-type (WT) and three independent strawberry transgenic lines (30, 24 and 8A) was measured using the electrolyte leakage test after freezing at –15 °C as described in 'Experimental procedures'. (B) Freezing tolerance of wild-type and transgenic strawberry. Wild-type and three transgenic strawberry lines were grown and cold acclimated for 3 weeks as in (A). The freezing tolerance of excised leaves was measured using the electrolyte leakage test after freezing at various temperatures as described in 'Experimental procedures'. Each data point represents the mean of five independent experiments with three leaves used for each test. The standard deviation did not exceed 10%.

Figure 4 Survival of wild-type and transformed strawberry after freezing. Intact leaves were frozen at –15 °C as described in 'Experimental procedures'. Wild-type leaves (top row) are clearly dead and discoloured after the freezing test, whereas transgenic leaves (lines 30, 24 and 8A from left to right) appear to be intact and remain healthy and green (bottom row).

demonstrates that the three transgenic lines were more tolerant than the wild-type plants. To accurately measure the degree of FT, the LT_{50} was determined in the wild-type and in the three transgenic lines after 3 weeks of CA using the electrolyte leakage method. This freezing test provides a direct measurement of ions released from the cells due to plasma membrane leakiness (Danyluk *et al*., 1998). The data in Figure 3B show that 3-week cold-acclimated leaves from both the wild-type and transgenic strawberry lines were not affected by temperatures down to –12 °C. The wild-type plants, however, were sensitive to a further decrease in temperature, as more than 70% of ions leached out of the cells at –15 °C. On the other hand, the three transgenic lines did not show any signs of visible damage at –15 °C and were minimally affected at –17 °C. Significant ion leakage was observed in transgenic lines only when the temperature was lowered to –19 °C. The LT_{50} of leaves from the wild-type cultivar was –13 °C, whereas it was –18 °C for the three transformed lines (Figure 3B). These data demonstrate that the phenotypic expression of FT in the transgenic plants increased by 5 °C compared with the wild-type.

This result is illustrated in Figure 4, where it can clearly be seen that leaves from the three transgenic lines remained healthy and very green after freezing at –15 °C, whereas the wild-type leaves were dark, indicating irreversible injury and rapid degradation of chlorophyll due to cell death. When we conducted similar experiments with whole plants, the leaves looked healthy after the freezing test in the transgenic strawberry, but the plants began to collapse during the recovery period at 20 °C due to damage to other organs, presumably the root system. This result suggests that other important organs are not able to benefit from the overexpression of WCOR410, possibly due to their inability to produce the factors needed for WCOR410 activation. It is known that roots do not cold acclimate efficiently when compared with leaves that are more directly exposed to ambient temperature (Perras and Sarhan, 1989). The LT_{50} of roots in both wild-type and transgenic strawberry was estimated to be –7 °C before and after CA, indicating that this is also true for strawberry.

In nature, roots are normally protected by the soil and by the snow cover during overwintering. Furthermore, it has recently been shown that freezing tests conducted on strawberry plants that are artificially submitted to CA cannot accurately predict the relative winter hardiness of the genotypes, and that the best approach is to conduct freezing tests on plants that are cold acclimated in the field (Linden *et al*., 2002). In order to better evaluate the impact of WCOR410

overexpression on whole plant survival, it will be necessary to evaluate the transgenic plants under field conditions, which is difficult to implement at the present time due to the complex regulatory process. When compared with wild-type plants, abscisic acid treatments (0.1 mM for 4 days) did not have any effect on FT of either non-acclimated or cold-acclimated transgenic strawberry, indicating that the improvement of FT in transgenic plants is cold specific. Similarly, transgenic plants were not more tolerant to water stress, indicating that the effect of the transgene is cold specific.

Based on the ion leakage experiments, we conclude that WCOR410 from wheat plays an important role in protecting the plasma membrane from freezing injury and confers greater FT. Our results clearly indicate that the WCOR410a protein, which is constitutively expressed, needs to interact with other factors, such as kinases induced by LT exposure, to achieve maximal FT. These LT-induced factors may be needed to create the microenvironment that allows the WCOR410 protein to interact with the membrane.

Recent findings have shown that the *Arabidopsis* acidic dehydrin ERD14, a homologue of the wheat WCOR410, has an increased capacity to bind calcium ions after *in vitro* phosphorylation with casein kinase (Alsheikh *et al*., 2003). A similar effect was observed when ERD14 was phosphorylated using extracts from cold-treated tissues. It was concluded that the polyserine (S) domain was most probably the site of phosphorylation in ERD14 responsible for the activation of calcium binding (Alsheikh *et al*., 2003). This phosphorylation may change the protein conformation, allow its association with calcium (Alsheikh *et al*., 2003) and promote binding to the membrane bilayer (Danyluk *et al*., 1998). The absence of improved tolerance previously observed in roots may be explained, in part, by the lack of induction of appropriate factors, including kinases. Although the WCOR410 proteins are expressed throughout all organs of transgenic strawberry, they may not be activated by the appropriate factors in all organs.

The highly charged nature of WCOR410 may also suggest that it could retain or replace water for the 'solvation' of the membrane. During dehydration, loss of water from membranes can alter their transition temperature from the liquid crystalline to gel phase and lead to irreversible damage (Crowe *et al*., 1993). However, if sugars are added before dehydration begins, the physical properties of dry membranes are altered and resemble those of fully hydrated biomolecules. This stabilization is achieved through hydrogen bonding between hydroxyl groups on the sugars and polar residues in phospholipids. The hydrophilic nature of WCOR410 means that it is well suited to replace water and stabilize membranes through polar interactions. In addition, proteins such as WCOR410 contain a high content of acidic, basic and hydroxylated amino acids that may interact with membrane lipids, such as the different phospholipids, cerebrosides and sterols. If one WCOR410 molecule is able to bind to several lipid species at the same time, this could be the basis for preventing the lipid demixing that occurs during dehydration. This hypothesis is plausible because proteins known as annexins have been shown to bind to acidic and neutral phospholipids in a calcium-dependent manner (Raynal and Pollard, 1994), and to affect the lateral mobility of the phospholipids (Swairjo and Seaton, 1994). Furthermore, it has recently been shown that the DHN1 dehydrin can bind to lipid vesicles containing acidic phospholipids, possibly through the K segment, which resembles a class A2-amphipathic α-helical lipid binding domain found in other proteins such as apolipoproteins (Koag *et al*., 2003). It has been proposed by these authors that this type of protein may undergo function-related conformational changes at the water–membrane interface and play a role in the stabilization of membrane structures under stress conditions. *In vitro* cryoprotective assays have indicated that the recombinant WCOR410 protein (PD_{50} (concentration of added substance required to give 50% residual activity after freezing and thawing) of 10 μg/mL or 0.2 μM) is as effective as bovine serum albumin or 250 mM sucrose against freezing denaturation of lactate dehydrogenase. These results provide further evidence of the cryoprotective function of this protein.

The enhancement of FT by as much as 5 °C demonstrates that this gene is a key factor for the development of FT in strawberry. This may explain the abundance and strong correlation between the level of expression of WCOR410 and the degree of FT found in the highly tolerant species wheat and rye (Danyluk *et al*., 1994, 1998). The overexpression of this gene may thus have a major impact in several plants possessing the capacity to cold acclimate.

The high accumulation of WCOR410a proteins in flowers may also have an impact in protecting these reproductive organs during the spring when plants are vulnerable to LT damage. However, it remains to be established whether the period of LT before freezing is sufficient to allow significant CA and interaction with the WCOR410 proteins to improve FT in this organ. Expression in berries may also enhance membrane stability and preserve food nutrient quality during storage at LT. Increasing fruit shelf life could have an important economic impact for producers and increase the market value of their product. The WCOR410 protein needs to be activated by other factors during CA. Our results show that the WCOR410 protein from wheat has an important impact

on FT of the distant species strawberry, suggesting that this protein may be used in other plant species which possess the capacity to cold acclimate. The isolation of the specific kinases responsible for the phosphorylation of WCOR410 will improve our understanding of the mechanism involved in its activation, and may help to establish a strategy to improve FT in crops of agronomical importance. In addition, it may allow its efficient use as a cryoprotectant for frozen food and sensitive biological material, such as molecules, blood, cells, tissues and organs.

Experimental procedures

Strawberry transformation

The FT-associated cDNA *Wcor410a* (Danyluk *et al*., 1998), cloned in the sense orientation, was inserted in the expression vector pBI121 (Clontech, Palo Alto, CA, USA) by replacing the *uidA* (*gus*) gene (Figure 1A). The sense orientation was determined by restriction mapping. The recombinant vector pBI121-*Wcor410a* was introduced into *Agrobacterium tumefaciens* strain GV3101. Three- to four-week-old shoot cultures of the strawberry cultivar Chambly (*Fragaria* × *anassassa* cv. Chambly) (Khanizadeh *et al*., 1990) were maintained *in vitro* on Murashige–Skoog propagation medium containing 1 mg/L benzylaminopurine (BA), 0.1 mg/L indolbutyric acid (IBA), 0.01 mg/L gibberellic acid (GA3), 30 g/L sucrose, pH 5.8, and 0.7% agar. The cultured shoots (5–10 mm) were cut into 2–3 mm explants that were incubated in *A. tumefaciens* suspensions for 90 min in the liquid induction medium (MS basal salts (Murashige and Skoog, 1962) containing B5 vitamins (Gamborg *et al*., 1968), 3% sucrose supplemented with 2 mg/L thidiazuron and 50 μM acetosyringone, at pH 5.8). The explants were then blotted onto sterile filter paper and cultured with the abaxial surface in contact with the solid induction medium. After 2 days of cocultivation, the explants were transferred for 4 weeks to the same medium supplemented with 500 mg/L carbenicellin, 500 mg/L cefotaxime and 50 mg/L kanamycin. The first subculture was transferred for another 8 weeks onto the same medium containing 1 mg/L thidiazuron. The kanamycin selection pressure was then increased by steps of 100 mg/L at intervals of 4–6 weeks up to a kanamycin concentration of 450 mg/L in the propagation medium. For shoot induction and regeneration, the cultures were incubated at 24 °C with a 16 h photoperiod (50 $\mu mol/m^2/s$) on the propagation medium. Rooting was promoted in the same medium at half strength containing 75 mg/L of kanamycin without hormones.

WCOR410 protein expression

Antibodies directed against the WCOR410 protein were used to determine the level of expression in the regenerated transgenic strawberry plants and to select the lines with the strongest expression. Total proteins were extracted from frozen plant tissues, as described previously (Danyluk *et al*., 1998). Proteins, solubilized in sample buffer (60 mM Tris-HCl, pH 6.8, 10% w/v glycerol and 2% w/v sodium dodecylsulphate (SDS)), were quantified using the Bio-Rad Dc Protein Assay. Equal amounts of protein were separated by 12% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically for 1 h to a nitrocellulose membrane (HYBOND-C, Amersham Biosciences, Baie d'Urfe, Canada) without SDS in the transfer buffer. The membranes were blocked in a 4% (w/v) solution of reconstituted skimmed milk powder prepared in phosphate-buffered saline (PBS) containing 0.2% (v/v) Tween-20, and then probed with the anti-WCOR410 antibody at a 1 : 10 000 dilution for 1 h. After washing with PBS–Tween, the proteins recognized by the primary antibody were revealed with a horseradish peroxidase-coupled anti-rabbit immunoglobulin G (IgG) (Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA) at a 1 : 20 000 dilution. The complexes were visualized using the ECL™ chemiluminescent detection system (Amersham Biosciences, Baie d'Urfe, Canada) and X-OMAT-RP film (Eastman-Kodak, Rochester, NY, USA).

Evaluation of freezing tolerance

The FT of transgenic and wild-type strawberry was assessed by both survival and the electrolyte leakage method. Intact leaves from each line were washed in distilled water, blotted onto filter paper, placed in a Petri dish and covered with humid sand. The samples were placed in a programmable freezer at –2 °C for 3 h for temperature equilibration and ice nucleation. At the end of this period, the sand and the leaves were frozen. The temperature was then lowered at a rate of 1 °C/h. Samples were removed at temperatures from –6 to –19 °C. Leaves were allowed to thaw overnight at 4 °C. The thawed leaves were visually examined for damage and then incubated in test-tubes containing 25 mL of distilled water and shaken at room temperature overnight. After shaking, the conductivity of the solution was measured with a conductivity meter (VWR International, Ville Mont-Royal, Canada). The solutions were then autoclaved at 122 °C for 20 min to completely lyse the plant cell walls. The electrolyte conductivities of autoclaved solutions were recorded as the absolute conductivity. The percentage of electrolyte leakage was

calculated by dividing the initial conductivity by the absolute conductivity. The level of FT was expressed as LT_{50}, defined as the temperature at which 50% of electrolytes were released from the tissues due to cell death and membrane leakage.

Lactate dehydrogenase cryoprotection assay

The assay and PD_{50} calculation were essentially performed as described by Kazuoka and Oeda (1994), except that siliconized tubes were used to avoid the loss of enzyme activity due to adsorption on the tube wall. PD_{50} was calculated as the concentration of added substance required to give 50% residual activity after freezing and thawing.

Acknowledgements

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