Chicory (*Cichorium intybus* L.) expressing the *lol1* gene exhibits inhibition of ice recrystallisation

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Abstract: A gene (*lol1*) from over-wintering rye grass (*Lolium perenne* L.) encodes a protein that inhibits the recrystallisation of ice. *Lol1* was introduced via *Agrobacterium*-mediated transformation into cotyledons excised from 17d-old seedlings of chicory (*Cichorium intybus* L.) cv. Brussels Witloof. Shoots were regenerated on medium containing kanamycin sulphate; 25 kanamycin resistant plants were transferred to compost. Molecular analysis confirmed the integration and transcription of the *lol1* gene in 8 of the kanamycin-resistant plants. Extracts from leaves of different ages excised from 140 d-old transgenic plants inhibited ice recrystallisation; inhibition was greater with extracts from younger leaves compared with extracts from older leaves. Leaf extracts from non-transformed plants failed to inhibit ice recrystallisation. The use of genes, such as *lol1*, in transformation-mediated genetic manipulation may enable the generation of plants more tolerant to low temperatures, both in the field and during storage.

Keywords: Antifreeze protein, *Cichorium intybus* L., chicory, freezing tolerance, ice recrystallisation.

Introduction

Injury caused during freeze-thaw processes is a major aspect of crop loss and may be a significant factor influencing plant distribution (Burke et al. 1976). Chicory (*Cichorium intybus* L.) is an important vegetable exploited for leaves, roots and forced shoots (chicons). During the forcing process, roots and chicons are exposed to low temperatures, which affect the quality of the edible product. Plants may also be exposed to prolonged periods of cold (-10°C) under field conditions. Exposure to low temperatures may result in cellular damage. The latter is believed to be caused by the withdrawal of water from the cytoplasm by the growth of ice crystals in the extracellular spaces (Steponkus et al. 1990) and by the physical damage caused by ice crystals to organelles. Several classes of proteins have been found to be associated with cold tolerance in plants and animals, some of which are implicated in other key stress responses, such as tolerances to drought and salinity.

Antifreeze proteins (AFPs) have been described in animals, fish and insects (Griffith and Ewart 1995). These proteins modify the thermal hysteresis of a solution *i.e.* they depress its freezing point, but do not affect the melting point. They also inhibit, to a lesser extent, ice recrystallisation, the latter involving the growth of large ice crystals at the expense of smaller crystals. AFPs with similar properties have also been isolated from cold acclimated plants. Although plant AFPs exhibit weak thermal hysteresis, they are efficient at inhibiting ice recrystallisation. A gene, *lol1*, which encodes a protein shown to inhibit the recrystallisation of ice, has been isolated from over-wintering rye grass (*Lolium perenne* L.) (Sidebottom et al. 2000).

Previously, AFPs have been introduced into plants with the aim of reducing freezing damage. For example, Hightower et al. (1991) transformed tomato with Type I AFP from the Artic Flounder. Inhibition of ice recrystallisation was observed in leaves, but not in fruit

tissues. In another investigation using Type I AFP, the protein was detected only when transgenic tobacco plants were grown at 4°C (Kenward et al. 1993), since protein breakdown was believed to occur at higher temperatures. Wallis et al. (1997) introduced a synthetic AFP into potato, based on a 37 amino acid sequence of Type I AFP from Winter Flounder. Leaf segments of transformed plants showed less damage after freezing, as demonstrated by reduced electrolyte release.

Genes encoding tolerance to freezing have not yet been identified in chicory or other leafy vegetables. Consequently, the introduction of genes resulting in increased tolerance of plants to low temperatures may also be beneficial in reducing damage, particularly during low temperature storage.

Materials and Methods

Transformation of chicory

Seeds of chicory cv. Brussels Witloof (E.W. King Ltd., Kelvedon, UK) were surface sterilised in 0.1% (w/v) mercuric chloride for 15min, followed by 20% (v/v) "Domestos" bleach (Diversey Lever, Northampton, UK) (30min), and washed 3 times in sterile purified water. Seeds were placed on 25ml aliquots of half-strength MS (Murashige and Skoog 1962) based medium (Duchefa, Haarlem, The Netherlands), with $15gl^{-1}$ sucrose, semi-solidified with 0.8% (w/v) agar (Sigma-Aldrich, Poole, UK) at pH 5.8 in 9cm diameter Petri dishes (Bibby Sterilin, Stone, UK; 15 seeds/dish). Seeds were incubated in the light [16h photoperiod, Daylight fluorescent tubes (Sylvania, Erlangen, Germany), 50µmol m^{-2} sec^{-1}] at 24°C±1°C.

Cotyledons were excised from 17d-old seedlings, scored (wounded) on their abaxial surfaces and immersed (5sec) in an overnight suspension (OD A_{600nm} = 0.6-1.0) of *Agrobacterium tumefaciens* strain LB4404. The latter carried a binary vector with *lol1* under the control of a double CaMV 35S promoter with a PRIa signal sequence and a *nos* terminator. The neomycin phosphotransferase gene (CaMV 35S-*npt*II-*nos*) permitted selection of transformed shoots. Cotyledons were blotted dry on sterile filter paper and transferred to full-strength MS-based co-cultivation medium with $30gl^{-1}$ sucrose, $1.0mgl^{-1}$ 6-benzylamino purine (6-BAP), $0.1mgl^{-1}$ indole acetic acid (IAA) and semi-solidified with 0.8% (w/v) agar (8 cotyledons/9cm diam. Petri dish).

After 3d, cotyledons were transferred to the same medium with $75mgl^{-1}$ kanamycin sulphate, $500mgl^{-1}$ carbenicillin (Melford Laboratories, Ipswich, UK) and $100mgl^{-1}$ cefotaxime (CP Pharmaceuticals Ltd., Wrexham, UK). Following 2 transfers to the same medium (each after 28d), cotyledons with regenerating shoots were transferred individually to 175ml glass jars (Beatson Clarke, Rotherham, UK) containing 50ml aliquots of the same medium.

Shoots (<2cm in height) were excised and inserted individually into 50ml aliquots of half-strength MS-based medium containing $15gl^{-1}$ sucrose, $1.0mgl^{-1}$ indole butyric acid (IBA) and 0.8% (w/v) agar to induce adventitious root formation. Rooted shoots were acclimatised in compost and maintained in a growth cabinet [22°C±2°C, 16h photoperiod, metal halide bulbs (Sylvania, Erlangen, Germany), 80µmol m^{-2} sec^{-1}]. Selected, kanamycin-resistant plants were designated Lol1.1-Lol1.25. Plant BWC1 was a non-transformed individual regenerated from an uninoculated cotyledon (tissue culture control).

Molecular analysis

Discs were removed from leaves of the selected 90 d-old kanamycin-resistant plants using lids of 1.5ml microfuge tubes, and DNA extracted (Edwards et al. 1991). Extracts were subjected to PCR analysis using primers targeting the *lol1* gene (Forward: 5' TCTTGCTGG

GAATGACAACA 3', Reverse: 5' ACGTGGTTGCTCCCAGTAC 3') to generate a 301bp product, and for the *npt*II gene (Forward: 5' ACAAGATGGATTGCACGCAGG 3' Reverse: 5' AACTCGTCAAGAAGGCGATAG 3') to give a 701bp product.

Total RNA was extracted from young leaves using a Plant RNeasy Extraction Kit (Qiagen, Crawley, UK); contaminating DNA was digested using RNase free DNase (Sigma-Aldrich) following the manufacturer's protocol. Purified RNA was used in a reverse-transcriptase-mediated PCR (RT-PCR) assay.

Ice recrystallisation assay

Initial screening of leaf extracts involved a modified "splat" method to assay inhibition of ice recrystallisation (Smallwood et al. 1999). Plants were maintained in a growth cabinet at 22°C for 140d before assay. Leaf discs (3 from each leaf) were removed from outer (oldest), middle and inner (youngest) leaves, using the lids of 1.5ml microfuge tubes and flash frozen in liquid nitrogen. Samples were ground to a fine powder, 50µl ice-cold water was added and the samples centrifuged 10,000 x *g* (10min) at 4°C. Supernatants were transferred to new microfuge tubes and stored on ice. Samples were centrifuged (10,000 x *g*, 5min) and each diluted with 50µl water. An equal volume of 60% (w/v) sucrose solution was added to give a final sucrose concentration of 30% (w/v).

A 5µl aliquot of sample was sandwiched between 1cm diam. circular cover slips and flattened with a 20g weight to ensure uniform sample thickness. Samples were placed in a bath containing trimethyl pentene cooled on a bed of dry ice to ensure rapid freezing, before being moved rapidly to a bath of trimethyl pentene circulating at –6°C to allow ice recrystallisation. Ice crystal formation was also assessed in 30% (w/v) sucrose solution and in sucrose solution containing Type III AFP purified from Ocean Pout [5µl in 50µl of 30% (w/v) sucrose solution]. Ice crystal formation was observed using light microscopy. Semi-quantitative analysis of extracts from selected plants was performed by placing samples on a cooled microscope stage and freezing to -40°C (cooling rate -100°C min^{-1}). This ensured the formation of a uniform layer of small ice crystals. The temperature was raised to -6°C, with a heating rate of 100°C min^{-1}, and held for 30min at this temperature. The degree of ice recrystallisation was assessed. Video images of extracts were recorded at T=0min and T=30min.

Results and discussion

Confirmation of the transgenic status of kanamycin-resistant chicory plants

PCR analysis for the *npt*II gene generated a fragment of the expected size (701bp) in DNA samples of 8 of the 25 kanamycin-resistant plants (Fig. 1a). A PCR product was not observed in DNA extracted from a non-transformed plant. DNA extracts from *npt*II positive plants also produced a fragment of the expected size (301 bp) when analysed for the *lol1* gene (Fig. 1b). RNA extracts from *lol1* positive plants produced a 301bp fragment following RT- PCR analysis of the *lol1* gene (Fig. 1c). RNA extracts from RT-PCR positive plants subjected to PCR, but without the RT step, failed to give a 301bp fragment (data not shown), confirming that the bands seen in Fig. 1c were not the result of DNA contamination of extracts.

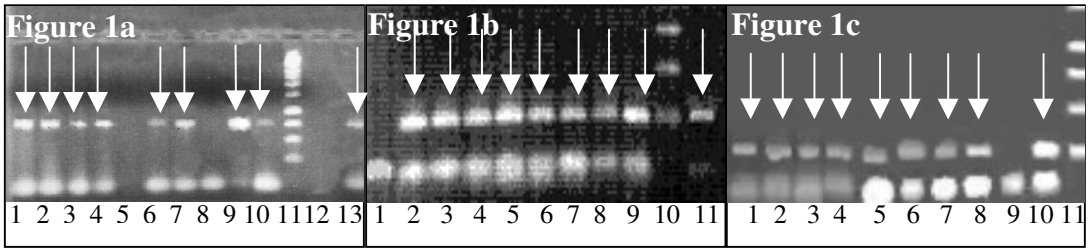

Figure 1a. PCR analysis for the *npt*II gene. Lanes 1-4, 6, 7, 10, 13 = DNA from kanamycin resistant plants showing amplification of a 701 bp fragment of the expected size; lane 12 = DNA from a non-transformed plant; lanes 5 and 8 = DNA from kanamycin resistant plants which failed to amplify a 701bp fragment; lane 9 = plasmid DNA; lane 11 = 1 kb DNA marker.
Figure 1b. PCR analysis for the *lol1* gene. Lane 1 = DNA from a non-transformed plant; lanes 2-9 = DNA from *npt*II-positive plants showing amplification of a 301 bp fragment; lane 10 = 1 kb DNA marker; lane 11 = plasmid DNA.
Figure 1c. RT-PCR analysis for the *lol1* gene. Lanes 1-8 = extracts from *lol1* positive kanamycin resistant plants showing amplification of a 301 bp fragment; lane 9 = extract from a non-transformed plant; lane 10 = plasmid DNA; lane 11 = 1 kb DNA marker.

Table 1. Inhibition of ice recrystallisation for protein extracts of leaves of different ages from 8 transgenic chicory plants (Lol1.14 - Lol1.24) expressing the *lol1* gene and the non-transgenic (control) plant BWC1. Inhibition of ice recrystallisation: - (large ice crystals), + (large and medium ice crystals), ++ (medium ice crystals), +++ (medium and small ice crystals), ++++ (small ice crystals), +++++ (no ice recrystallisation, as with purified Type III AFP), n/a (not assayed). See Fig. 2 for appearance of ice crystals.

Plant	Outer leaf extract	Middle leaf extract	Inner leaf extract
Lol1.14	++	+	++++
Lol1.15	++	++	++++
Lol1.17	+	+	++
Lol1.18	++	+++	+++
Lol1.19	n/a	+	+++
Lol1.20	n/a	+	n/a
Lol1.21	n/a	+	+++
Lol1.24	n/a	+	n/a
BWC1	n/a	-	n/a

Inhibition of ice recrystallisation
Purified Type III AFP resulted in total inhibition of ice recrystallisation over the assay time and was scored '+++++'. In contrast, ice crystal growth in 30% (w/v) sucrose solution without Type III AFP was rapid and extensive, resulting in a small number of large ice crystals and was scored '–' *i.e.* no inhibition. Leaf extracts from a non-transformed plant also failed to inhibit ice recrystallisation. Leaf extracts from the 8 PCR and RT-PCR positive plants inhibited ice recrystallisation (Table 1), with extracts from the inner leaves inhibiting ice-crystal growth more strongly than extracts from middle or outer leaves.

Video images showed an inhibition of ice recrystallisation in leaf extracts from transformed chicory plants. In contrast, this inhibition was not observed in extracts from a non-transformed plant. At T=0min, extracts from transformed and the non-transformed plant, BWC1, showed similar ice crystal formation, both in size and number (Fig. 2). At 30min, ice crystals were smaller in extracts from the middle leaves of the transgenic plant Lol1.15, as an example, than in extracts from BWC1. A greater number of ice crystals were observed in samples from plant Lol1.15 than in the extract from plant BWC1. Extracts from younger (inner) leaves of transgenic plants retarded the growth of ice crystals more than extracts from older leaves. After 30min, little further change was seen in either the size or number of ice crystals in extracts of inner leaves from plant Lol1.15.

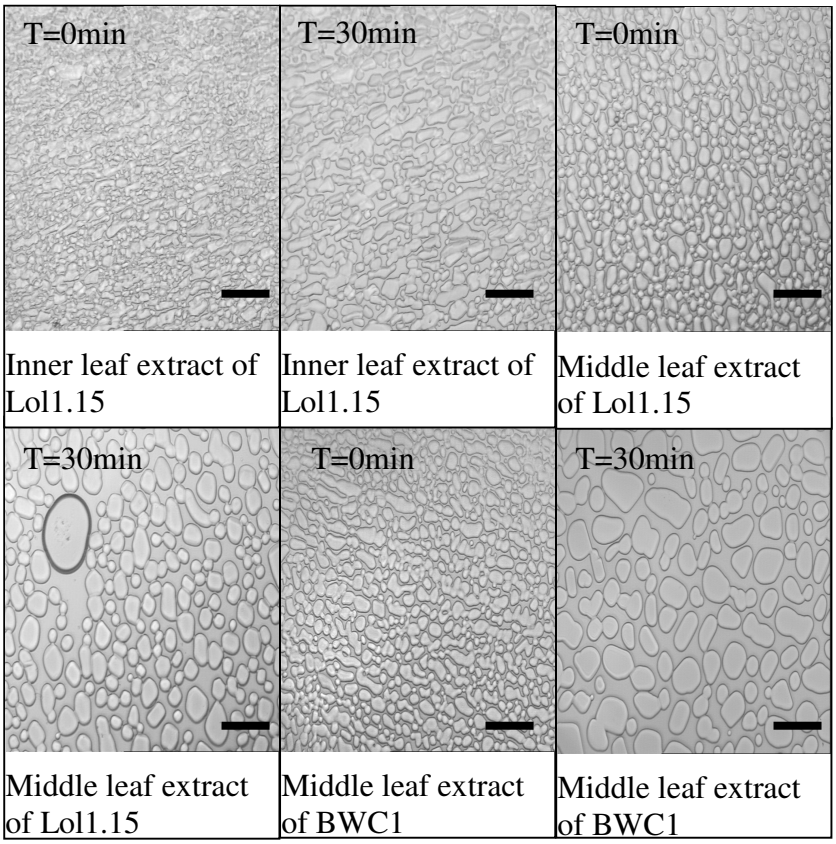

Figure 2. Video images of ice-crystals in frozen leaf extracts of transformed (Lol1.15) and non-transformed (BWC1) chicory plants at the beginning of the assay and after 30min incubation at -6°C. Bar = 55µm.

The data presented here demonstrate the integration, transcription of the *lol1* gene and the presence of active protein(s) in leaf extracts from transgenic chicory plants grown at 24°C. Leaf extracts from such transgenic plants inhibited ice crystal growth. In contrast, inhibition of ice recrystallisation was not seen in leaf extracts of a non-transformed plant, suggesting that the expression of the *lol1* gene was directly responsible for the alteration in ice recrystallisation properties in leaf extracts of transgenic plants. Reasons for the increased inhibition in youngest leaves have not been investigated. Future studies will now be performed on the influence of *lol1* gene expression on survival of cold-treated chicory leaves, and will investigate the inheritance of the *lol1* gene in subsequent seed generations.

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