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(54) **LOW TEMPERATURE RESPONSIVE  
NUCLEOTIDE SEQUENCES AND USES  
THEREOF**

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(57) **ABSTRACT**

This disclosure provides gene sequence of a novel gene DaRub1 of *Deschampsia antarctica* expressing in low temperatures. The promoter sequence of this gene is identified and characterized. The promoter is inducible by low temperatures, wounding and auxin treatment. Additionally the disclosure shows improved low temperature tolerance of transgenic *Eucalyptus* plants expressing DaRub1 gene.

A  
75  
GAAAGGATTTTGGTTTCTTCCCTCCGCTTCGTTCTCAGCGAGCTTGACGCTCTCTCCTTGACCCCCCTCC  
150  
CTCCACCCCTCCAGGCCCTCGCCGCCACACGGGAGCAGGAGGAGGATGCAGATCTTCGTCAGACCCCTGACGCGG  
H Q I F V K T L T G  
225  
AAGACCATCACCCCTCGAGGTGAGAGCAGCGACACCATCORTAACGTCAAGGCCAATCCAGGACAAAGAGG  
K T I T L E V E S S O T I O N V K A K I Q D K E G  
300  
ATTCTCCAGACCAACACGTTTGATATTTGCTGCCAGCAGTTGGAGATGACGACACTTGCTGATTACAT  
I P P O Q Q R L I F A G K Q L E D G R T L A D Y N  
375  
ATTCAAGAGGAGTCTACTCTTCACTTGGTCTCAGGCTCAGGGCGGTACTATGATCAAGGTTAAGACACTCACT  
I Q K E S T L H L V L R L R G G T M I K V K T L T  
450  
GGAAAGGAGATCGAATTGACATTGAGCCCACTGACACGATTGATAGGTCAGGAGCGTGTGAGGAGAAAGAA  
G K E I E I D I E P T D T I D R V K E R Y E E K E  
525  
GGCATTCTCCCGTGCAGCAAGGTTGATTTATGCTGGTAAAGCACTTGCAATGACAAAGCTGCCAAGCACTAC  
G I P P V Q Q R L I Y A G K Q L A D D K T A K D Y  
600  
AACATCGAGGTGCTCCGCTCCATCTTGTACTTGCTCTGAGGGGTGTTACTAGTAATCTAGCTAGTGACA  
N I E G G S V L H L V L R L R G G Y \*  
675  
CTACTGCCCTCATCTACTCTAATGGTTGAGAGAGTGACTATTGGTGGTATGGAAGTACATATACAAATAC  
750  
AACTAGCTTTTCTAGTGGGAGGGGCTGTCTTCATGTTATTATCTTGTATGATTTGATAAATCACTCCCTAG  
825  
TTTATCTGCTCTGTTGTTATGCTGCTGTGAGAAATCCATGATCATCTGAAAAAAAAAAAAAAAAAAAA  
AAAAAA

Fig 1A

|            |    |
|------------|----|
| DaRUB1     | 60 |
| AtRUB1     | 60 |
| AtRUB2     | 60 |
| AtRUB3     | 60 |
| MouseNEDD8 | 60 |
| CeRUB1     | 60 |
| SpUb11     | 60 |
| SCRUB1     | 60 |

|            |    |
|------------|----|
| DaRUB1     | 77 |
| AtRUB1     | 00 |
| AtRUB2     | 78 |
| AtRUB3     | 81 |
| MouseNEDD8 | 77 |
| CeRUB1     | 78 |
| SpUb11     | 77 |
| SCRUB1     | 77 |

Fig. 1B

## Vitrogen Low temperature non provisional

CTGTTTGGCTTTATTTATAAAGCGGAACGAAAGCCTGTTTCGAAAAGAGAGAAGTCACGACGGACCAAACC  
CATTTAGAGTAGCGACACTCGATGCCTCGCCATTTTCGTGAGGAATTTCCATAGTTGGCAAGTGATGTCAC  
CTACTTCATGGTGCATTTAGAGTTGAGGCAGAGCTTACCCGGCATGTGTAAGGGTGGCAATATTTCACTGTG  
GGGACGAGTACCCGCGGGGAGTTACCCATCATGAAGCGGAGATGGGGGACAAATTCACCCACGGGTATTG  
TCTCATGGGGCGGGGATGGGGAGCAAATCCTCCATGGATACCCGACGAACTATGCCATGTGAACTACAGAT  
GCACAATATATAGATTAACCTAGATAGTCCACTTAATTTTTTTTCTCCATCTCTCTCAGCCACCTTGTC  
CTTTCTAATCTCATTTATCACAGTCGACGCTGAGTTCCCATAGAGTCCCGACGGGTACCGGATACCCGTTA  
TTGACAGGGATGGGGACAGTTTTGTCCCGTGAAGGTTTATGGGGCTGGCGGGTATGAGGACTGGTGGGGT  
TCGAGGTGAGGACGGCGGAGTCATCACCGGTCGTCTCAGTCCCGTTGCCACCTGAGGCGTGAGCAGAAC  
TTAGGTCGCTCTTGCCAATATAGGTCAAACGGAAGCTAGAACTGATGCGTGGCTTCGTTTCAGGGCCAGCAA  
ACATACCTCAACTCTGTTTCGAGGATTGTAATCCGGTTGCTTTAAGTCCCATACCACCCCATTTCGCGAAAA  
GGAAAAACGAAAAAGGATTTTGTAGTAGACGAGTTTTTACATGCGACGCAAGCCGATGGGTGTCCAAACCAG  
TTCGTCTGTACGTCCGTGGCATTTCGTGTGCACAACAGCACGAGTATAACATTTTATACGTACCTGAAGAT  
TAGGATCAAGCGTCTTGCCATAGTCTCAGGAGAAGGAAAAAAAAAAGGTCCCAACCGACGTTACAGAACGGG  
TCGGAAGTCTGAGCCCCGAGCGGAAAACCGGCTCACGGGGTTCCCCGACTTGACGCCGCGCACCCCTCAATA  
AATCCACCGGGTCCCGAGGAAAAAGAAAGGAATTTTCGTTTGTTTCTGCCTCCGCTTCGTTCTCACGCAGA  
CTTGACGCTCTCTCCTTGACCCCCCTCCCTCCACCCCTCCAGGCCTCGCCGCCACACGGGAGCAGGAGGAGA  
AGATGCAGATCTTCGTCAAGACCCTGACGGGGAAGACCATCACCCCTCGAGGTGGAGAGCAGCGACACCATC  
GATAACGTCAAGGCCAAAATCCAGGACAAAGAAGGGATTCTCCAGACCAACAACGTTTGATATTTGCTGG  
CAAGCAGTTGGAAGATGGACGCACACTTGCTGATTACAATATTCAAAGGAGTCTACTCTTCACTTGGTCC  
TCAGGCTCAGGGGCGGTACTATGATCAAGGTTAAGACACTCACTGGAAGGAGATCGAAATTGACATTGAG  
CCCCTGACACGATTGATAGGGTCAAGGAGCGTGTGAGGAGAAAGAAGGCATTCTCCCGTGCAGCAAAG  
GTTGATTTATGCTGGTAAGCAGCTTGAGATGACAAGACTGCGAAGGACTACAACATCGAAGGTGGCTCCG  
TCCTCCATCTTGTAATTGCTCTGAGGGGTGGTTACTAGTAAATCTAGCTAGTGACACTACTTGCCCTCAATC  
TACTCTAATGGTTGAGAGAAGTGACTATTGGTGGTATGGAAGTAGATAATAACAAGTAACAACTAGCTTT  
TCCTAGTGGGAGGGGCTGTCTTCATGTTATTATCTTGTATGATTTGATAAAATCACTCCCTAGTTTATCT  
GCCTCTGTTGTTATGTCTGCTGTGTGAGAAAATCCATGATCATCTGAAAAAAAAAAAAAAAAAAAAAAAAA  
AAA

FIG 1. C

Vitrogen Low temperature non provisional

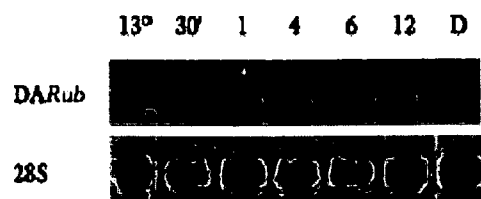


FIG 2.

Vitrogen Low temperature non provisional

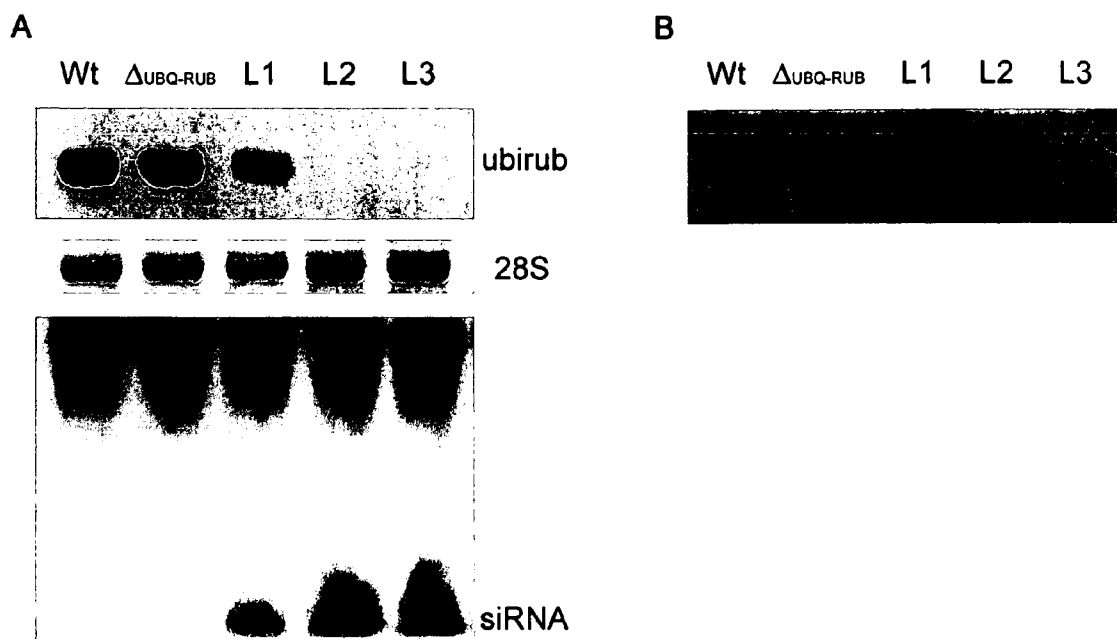


FIG 3

Vitrogen Low temperature non provisional

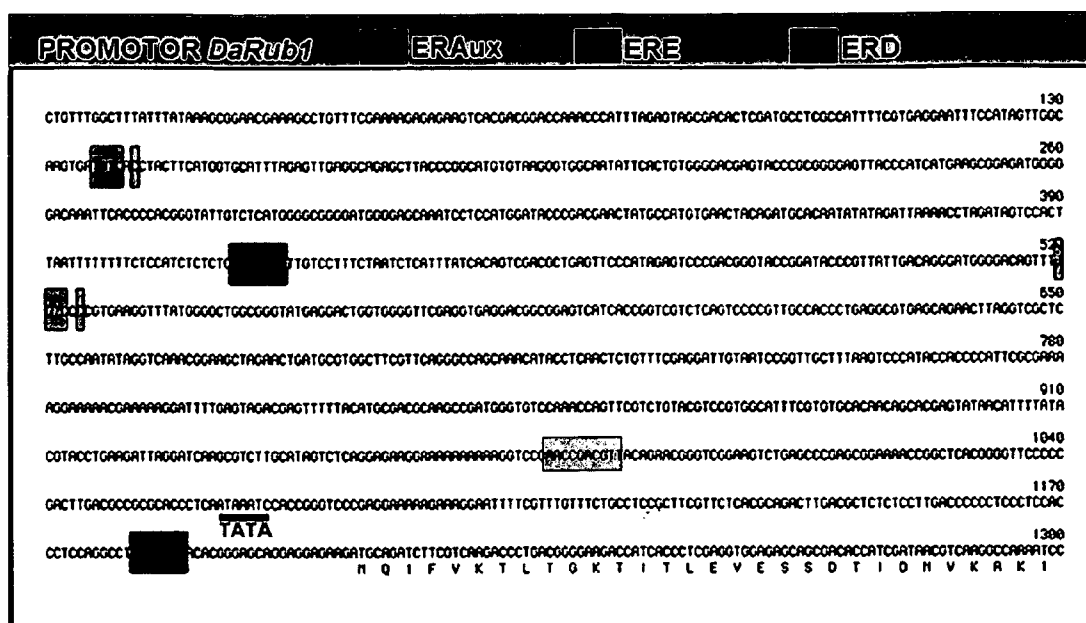


FIG 4.

Low temperature non provisional

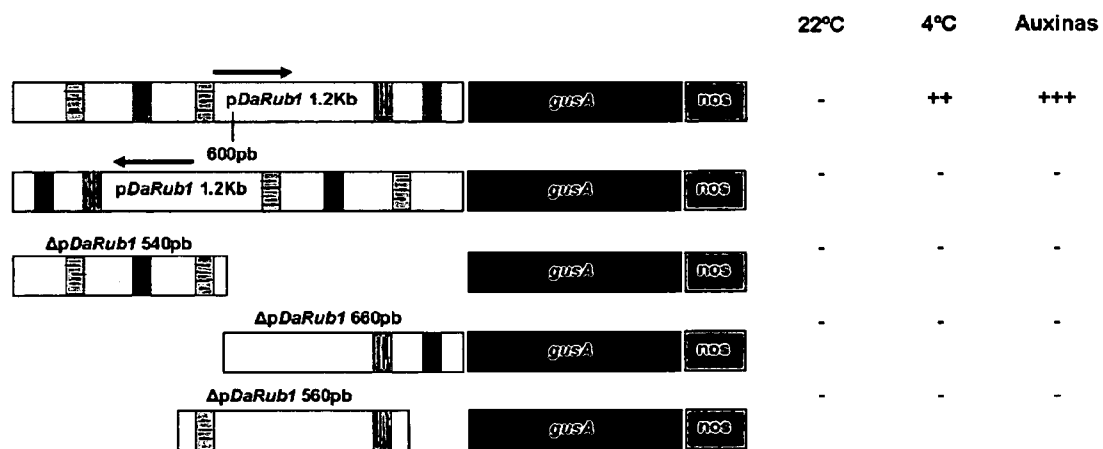


FIG. 5.



Vitrogen Low temperature non provisional

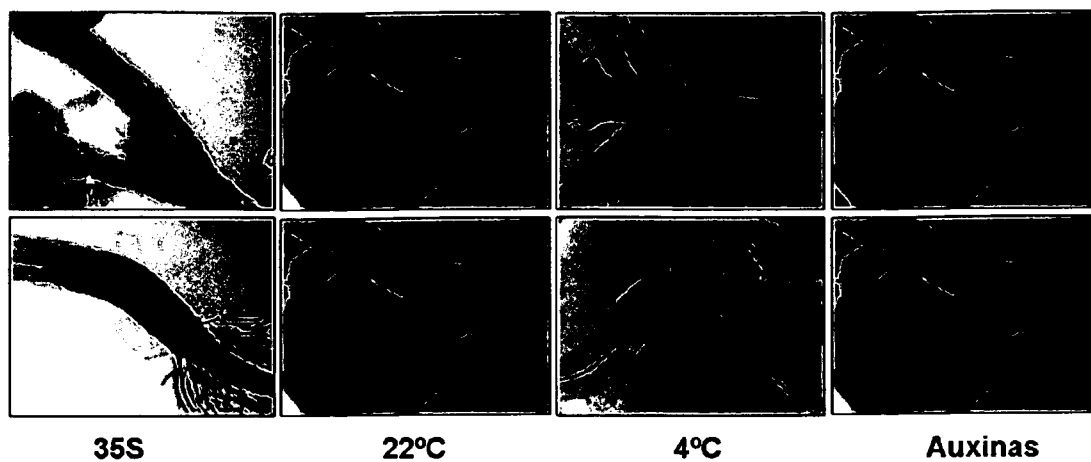


FIG. 6.

Vitrogen Low temperature non provisional

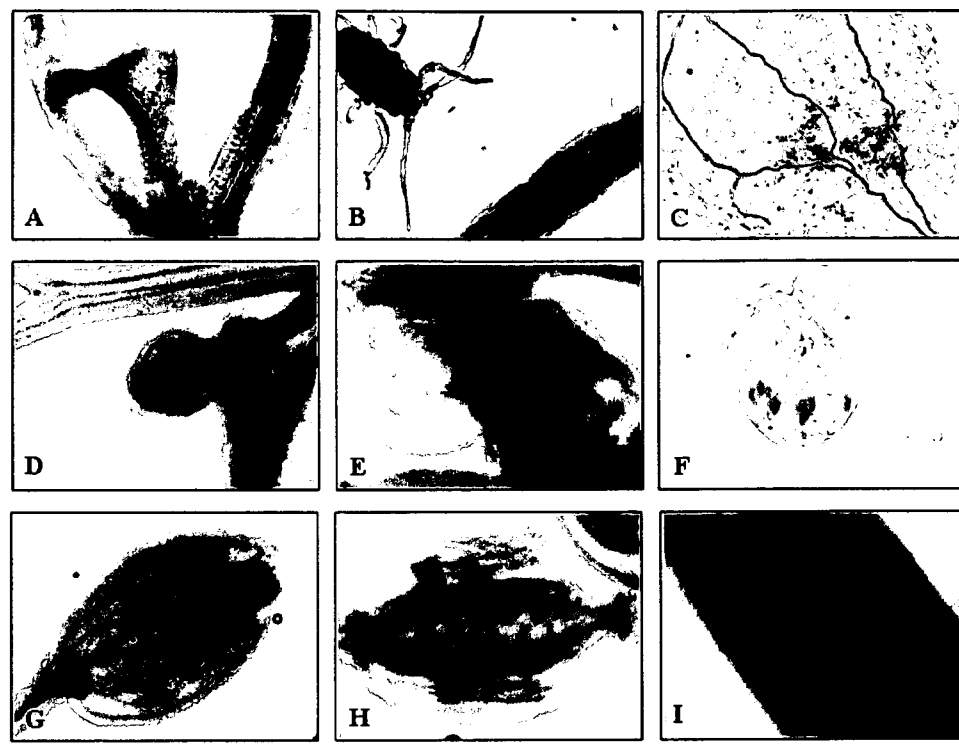


FIG. 7 A-I

Vitrogen Low temperature non provisional

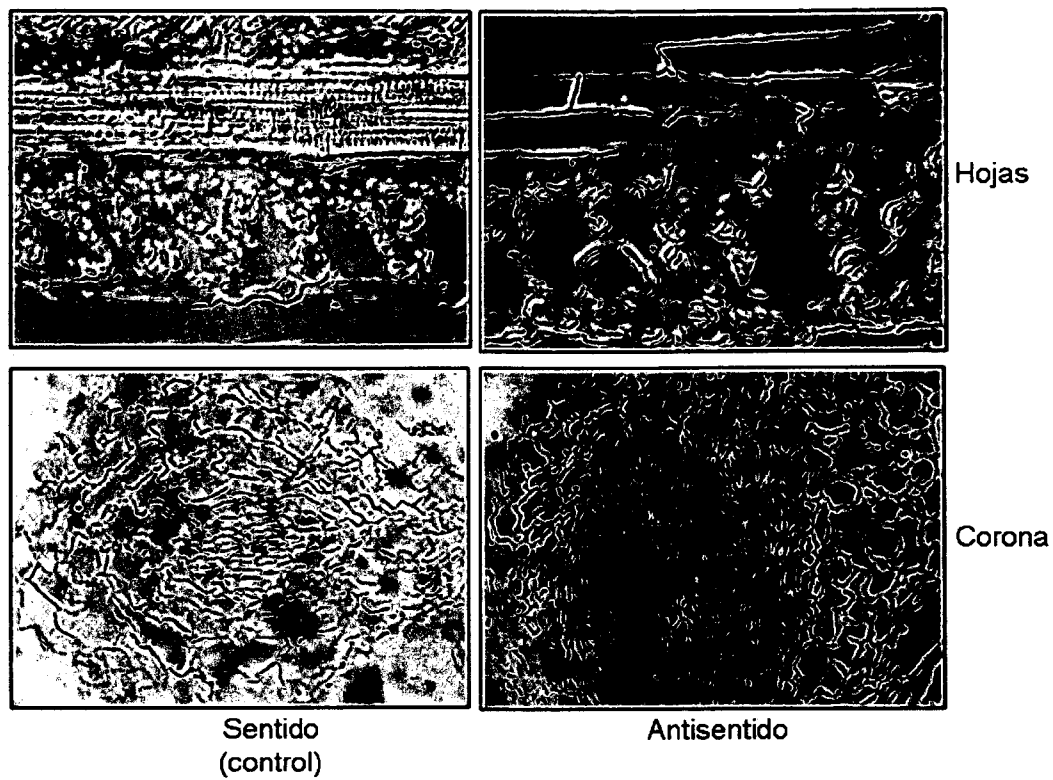


FIG. 8.

Vitrogen Low temperature non provisional

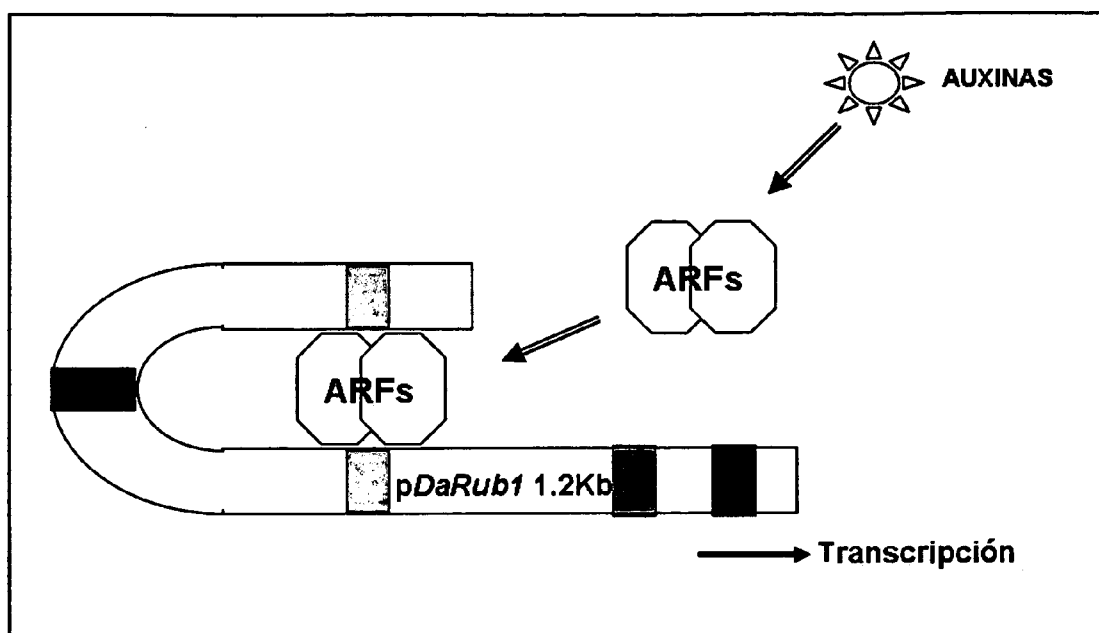


FIG. 9.

## LOW TEMPERATURE RESPONSIVE NUCLEOTIDE SEQUENCES AND USES THEREOF

### BACKGROUND OF THE INVENTION

#### [0001] 1. Field of the Invention

[0002] The present invention relates to the field of improved stress tolerance of plants, more specifically to genes and promoter sequences expressing at low temperatures and even more specifically to low temperature tolerance improved by means of gene transfer.

#### [0003] 2. Background

[0004] Low temperature is one of the major limiting factors of growth, development and geographical distribution of plants. Some plants are able to acclimate by increasing their freezing tolerance upon exposure to low non-freezing temperature. This process involves physiological and biochemical changes, many of which are regulated through qualitative and quantitative modification of gene expression leading to the accumulation of newly synthesized proteins and mRNAs.

[0005] A number of genes having expression induced by low temperature have been isolated and characterized in a wide range of species. Most of the studies of gene expression during cold acclimation have been performed in plants from temperate and semi-temperate climates. Differently to this approach, our approach has been to study gene expression of genes induced by cold acclimation in a highly freezing tolerant plant. *Deschampsia antarctica* Desv. (Poaceae) is a highly tolerant plant to the harsh freezing conditions and it is one of the two vascular plant species that have naturally colonized Maritime Antarctic Peninsula. A high accumulation of soluble carbohydrates, especially sucrose and furofurans has been found in leaves of *D. antarctica* during growth period in the Antarctic summer. Total protein extracts from leaves of *D. antarctica* growing in the Antarctica has been shown to have a high cryoprotective activity on barley chloroplasts. Membrane lipid contents and the degree of unsaturation of fatty acids in the leaves do not differ significantly from plants in temperate zones. The optimal photosynthetic activity of this species is at 13° C. and it can maintain up to 30% of this rate at 0° C. Cold acclimation experiments have shown that *D. antarctica* is able to acclimate from -14.8° C. (LT<sub>50</sub> at -14° C.) to -26.8° C. when growing at +2/-1.5° C. for 21 days in a solid substrate in the laboratory.

[0006] Due to the high capacity of the cold acclimation that this species possesses we were interested of its gene expression during cold acclimation. Not only is there a need to characterize genes that are responsible for low temperature tolerance of plants but there is also a need for characterization and identification of new plant promoters. Characterization and identification of such genes and promoters will be greatly useful in improvement of various crops. There is a clear need to improve low temperature tolerance of various sensitive plant species. Furthermore, in plant breeding and research there is a constant need for plant promoters inducible by various environmental factors such as low temperature.

[0007] Therefore, one object of the present disclosure is to increase cold resistance or tolerance of plants through gene transfer, especially of fruit trees such as, but not limited to

eucalyptus, avocado, orange and peach trees. The system can be applied both to monocots and to dicots.

[0008] Another object of this invention is to reduce photoinhibition caused by chilling temperatures.

[0009] Still another object of the present invention is to provide transgenic plants, plant cells, plant tissue, plant organs or plant components of plants such as, but not limited to *Arabidopsis thaliana* or *Eucalyptus globulus* carrying a recombinant transgene capable of expressing a modified transcript of a related-to-ubiquitin protein of *Deschampsia antarctica*.

[0010] An even further object of the present invention is to provide promoters inducible by low temperatures, wounding and auxin treatment.

[0011] *Eucalyptus globulus* has become one of the most important forest species for example in Chile due to its fast growth and good wood quality especially for the purposes of pulp manufacturing. Currently, it represents the second most planted species in Chile, amounting approximately along with other eucalyptus species to a total of 350,000 hectares. However, strong world market competition makes it necessary to develop better technologies for its cultivation. Despite of its excellent characteristic there are wide surface areas up to 2 million hectares where growth limiting restrictions are to be found. In particular areas under frost conditions along with this species' low cold tolerance have limited plantation expansion both to the south and to the foothills of Andean Mountains. Also Chile's Center Valley has undergone frequent losses in those areas affected by frost conditions.

[0012] The present disclosure resolves the problem of low freezing tolerance of trees such as *eucalyptus* by a method rendering transgenic plants to tolerate lower temperatures. In addition to eucalyptus this method is applicable to various low temperature susceptible plants for example peach.

### BRIEF DESCRIPTION OF THE DRAWINGS

[0013] **FIG. 1A.** Depicts the nucleotide sequence of the cDNA encoding DaRub1 and deduced amino acid sequence.

[0014] **FIG. 1B.** Depicts a multiple alignment of the RUB protein sequence derived from the cDNA sequence DACOR-1.0 with other RUB proteins from the GeneBank databases: *Arabidopsis thaliana* (AtRUB1, AtRUB2, AtRUB3), Mouse RUB1 (NEDD8), *C. elegans* (CeRUB1), *S. pombe* (SpUB11), *S. cerevisiae* (ScRUB1).

[0015] **FIG. 1C.** Depicts the nucleotide sequence of the full length of the gene DaRub1 (SEQ ID NO:1). The first 1089 nucleotides represent the promoter sequence (SEQ ID NO:2). The transcript initiation site is shown bold and underlined. The coding sequence (SEQ ID NO:3) is shown with bold letters.

[0016] **FIG. 2.** Northern blot analysis of DaRub1 transcript levels in leaves of *Deschampsia antarctica* cold acclimated plants at different times: 30 min, 1-12. Control plants were kept at 13° C. De acclimated plants were 24 h at 14° C. Total RNA (10 ug) was separated on formaldehyde/1% agarose gels and probed with the 3'UTR region of the corresponding cDNA clones. A 28S ribosomal RNA probe was used as loading control on the same membranes.

**[0017] FIGS. 3. A and B.** To test silencing of the UBQ-RUB gene Northern and Western analysis were performed. Briefly, total RNA was isolated with TRIZOL (Promega). dsRNA was obtained by digesting total RNA with RNase I (Promega) (0.5 Units/ $\mu$ g of total RNA) at 37° C. for 3 hours. For Northern blot analysis, total RNA was fractionated on 1.2% formaldehyde-agarose gels, transferred to Hybond membranes and hybridized with <sup>32</sup>P-labeled UBQ-RUB probes. siRNA analysis was performed according to Llave et al. The probe for UbiRub siRNA analysis was obtained by T7 RNA polymerase in vitro-transcription using <sup>32</sup>P-UTP and corresponded to the 350 nt UBQ-RUB 3'-UTR. For western analysis, plant tissue was homogenized in buffer Laemmli (5% glycerol, 1% SDS, 2.5%  $\beta$ -mercaptoethanol, Tris-HCl 60 mM pH 6.8). Total protein extracts (15  $\mu$ g) were separated by SDS-PAGE and electro-transferred to nitrocellulose membranes. An anti-UBQ-RUB polyclonal antibody was used (1:2500 dilution). The signal was visualized with an anti-rabbit antibody conjugated to phosphatase alkaline.

**[0018] (A)** Total RNA of *Arabidopsis* plants: wild type (wt), transgenic lines L1, L2, L3 and  $\Delta$ UBQ-RUB (vector alone) was extracted and hybridized with a UBQ-RUB probe (top panel). Sample loading (20 20  $\mu$ g) was monitored using a 28S RNA probe (central panel). siRNA (50  $\mu$ g) was hybridized with an anti-sense transcript corresponding to the UbiRub 3'-UTR (bottom panel)

**[0019] (B)** Total protein (15  $\mu$ g) of *A. thaliana* wild-type, transgenic lines L1, L2, L3 and AUBQ-RUB (vector alone) plants were separated by SDS-PAGE, blotted on nitrocellulose membranes, and probed with a UBQ-RUB polyclonal antibody

**[0020] FIG. 4:** DaRub1 gene putative promoter sequence (SEQ ID NO:2) and 5'-terminal cDNA sequence. Responding elements putative and TATA box are indicated. ERAux: auxin responding element, ERE: ethylen responding element, ERD: dehydration responding element.

**[0021] FIG. 5.** Schematic representation of the DaRub1 promoter region and its deletion. The constructions were named according pb length fragment. Blocks with horizontal lines: ERAux, Grey blocks without lines: ERE, blocks with vertical lines: ERD. Arrow indicates the cloning direction of complete promoter region (sense and antisense). To the right a summary of the transgenic *Arabidopsis* plant response of each one of the reporter fusions, during control temperature (22° C.), cold acclimation temperature (4° C.) and auxin treatment

**[0022] FIG. 6.** GUS expression in fusion p DaRub1/gusA *A. thaliana* transgenic lines, during cold acclimation and auxin treated. Plantlets cold acclimated (4° C.) and auxin treated were kept on X-Gluc substrate and GUS expression was observed. 35S: positive control plants (pCAMBIA 1303), 22° C.: plants maintained at 22° C., negative control; 4° C.: one hour cold acclimated plants; Auxin: 1.0  $\mu$ M of 2,4D treated plants. All the photographs represent 40 $\times$  magnification.

**[0023] FIG. 7.** GUS expression in fusion DaRub1/gusAA. *thaliana* transgenic lines, during wound and in different flower development phases. A and B: injuries by wounds, A: mechanical damage, B: damage by aphid infection; C: senescent leaves; D-H: different flower developments phases, F: 100 $\times$  approach of D; I: silique. All the photographs represent 40 $\times$  magnification, except F, that represents 100 $\times$  magnification.

**[0024] FIG. 8.** DaRub1 antisense RNA in situ hybridization in *D. antarctica*. RNA obtained from DaRub1 3' UTR cDNA sequence was utilized for the tissue in situ hybridization (leaves and crown) of *D. antarctica* plants cold acclimated for 24 hours. A sense RNA probe was utilized like control.

**[0025] FIG. 9:** Proposed model for transcription induction by AuxER of DaRub1 promoter region. ARFs: Auxin response factors.

#### DETAILED DESCRIPTION OF THE INVENTION

**[0026]** During the exposure of plants to low temperature many physiological and biochemical changes occur which lead to the development of freezing tolerance. The survival of tolerant plants at freezing temperatures depends on timely modulation of the gene sets of the species, the accumulation of both mRNAs and protein products of such genes correlates with the development of freezing tolerance. A major difficulty is in the fact that many low temperature responsive genes are also induced by other stimuli, such as drought, abscisic acid (ABA) and salinity. It is unclear, however, how such different stimuli converge to induce the same gene. For example, it has been reported that low temperature and ABA regulates gene expression through separate transduction pathways. An alternative explanation is that several cis-acting elements are present in the promoter regions of these genes that respond to multiple factors.

**[0027]** Ubiquitin (UBQ) is one of the most conserved proteins among eukaryotes. The covalent attachment of UBQ to other proteins targets them for degradation by the 26S proteasome. Attachment of the ubiquitin carboxyl terminus to  $\epsilon$ -amino lysyl-groups of substrate proteins requires ubiquitin-activating enzyme (E1), in an adenosine 5'triphosphate-(ATP)-dependent reaction in which a thiolester bond is formed between the COOH-terminus of UBQ and a cysteine within the E1 enzyme. The UBQ-moiety is transferred to a cysteine residue of ubiquitin-conjugating enzymes (E2). Finally, UBQ is covalently attached to a target protein by an isopeptide linkage directly from E2 or by UBQ-protein ligase (E3), such as the SCF (Skp1, Cdc53, F box protein, respectively). Recently, several families of ubiquitin-like proteins (RUB; related to Ubiquitin) have been described. These RUB-protein families include the mouse protein Nedd-8, *Arabidopsis thaliana* ubiquitin UBQ7, and the *Saccharomyces cerevisiae* protein ScRub1p. *Arabidopsis* ubiquitin-like protein has 62% identity to ubiquitin, 83% identity to Nedd-8, and 57% identity to ScRub1p. The RUB family includes the plant RUB1, RUB2, and RUB3 of *A. thaliana*, and RUB1 of *Brassica napus*, the mammal RUB of mouse, rat and human, an open reading frame with significant RUB identity from *C. elegans*, and the fungal RUBs. The *Arabidopsis* proteins RUB1 and RUB2 differ by only one amino acid at position 60. BnRUB1 and AtRUB1 encode identical proteins with the exception of the COOH-terminal additional amino acid.

**[0028]** Several studies in different models have shown that RUB proteins are conjugated to target proteins through the sequential action of RUB-activating and RUB-conjugating enzymes in a similar way as ubiquitin conjugation. In *Arabidopsis*, RUB1 is activated by an E1-like heterodimer AXR1/ECR1 and transferred, by an RUB-E2 enzyme called

RCE1, to a cullin protein of a SCF complex. The unique targets for RUB modification are members of cullin protein family. These cullins are subunit of E2 ligase protein of the SCFs complex. In *Arabidopsis*, an E3 complex called SCF<sup>TIR1</sup> cullin is required for auxin respond and it seems to be target for AUX/IAA proteins for degradation.

[0029] We have now isolated and characterized a gene expressing during cold acclimation of *Deschampsia antarctica*. The gene is called DaRub1, and it has an open reading frame (ORF) encoding for 153 amino acids polypeptide consisting of an ubiquitin monomer fused in the same ORF to a RUB protein. By Northern blot analysis we verified that this gene is expressed during cold acclimation during the first hours and being maintained with different expression levels up to 12 hours of acclimation. Furthermore, we isolated the promoter region of DaRub1 gene and identified and characterized the 5'-regulatory region of this gene.

[0030] The present invention is directed toward a novel gene of *Deschampsia antarctica* expressing during acclimation and a promoter region of the gene. The invention is also directed to transgenic plants containing a modified gene transcript of *Deschampsia antarctica*.

[0031] The present disclosure includes the steps of a) characterization and identification of a novel gene of *Deschampsia antarctica* expressing during cold acclimation of the plant; b) characterization of the promoter region of the novel gene and showing the functionality of the promoter by fusing it to a reporter gene and transforming into *Arabidopsis* plants c) introducing into plant cells a transgene including a DNA encoding a modified version of a related-to-ubiquitin protein from *Deschampsia antarctica* operably linked to a promoter functional in plant cells to yield transformed plant cells; and d) regenerating a transgenic plant from the transformed cell, wherein the transcript of the modified version of the related-to-ubiquitin protein (RUB) interacts with signal transduction machinery of the plant, thereby increasing the level of cold-resistance or—tolerance in the transgenic plant. As a result the ice-nucleation and freezing temperatures are significantly lower in the transgenic plant.

[0032] Furthermore, the invention features a method for reducing the photoinhibition caused by chilling temperatures. The method includes the steps of a) introducing into plant cells a transgene including a DNA encoding a modified version of a related-to-ubiquitin protein from *Deschampsia antarctica* operably linked to a promoter functional in plant cells to yield transformed plant cells; and b) regeneration a transgenic plant from the transformed cell, wherein the transcript of the modified version of the related-to-ubiquitin protein interacts with the photosynthetic machinery of the plant, thereby increasing the photochemical or non-photochemical quenching capacity in the transgenic plant. As a result the transgenic plant is more tolerant or resistant to adverse effects caused by photoinhibition.

[0033] Even further the invention according to this disclosure features a model explaining auxin dependent modification of RUBs.

[0034] The following examples are meant to be descriptive and by no means limiting of the various embodiments of the present invention.

#### EXAMPLE 1

##### Plant Material and Growth Conditions for Obtaining Cold Acclimated *Deschampsia antarctica* Plants

[0035] *Deschampsia antarctica* Desv. (Poaceae) plants were collected in the Coppermine Peninsula on Robert Island, Maritime Antarctica (62° 22'S; 59° 43'W) during the austral summer and transported in plastic bags to the laboratory. Plants were propagated vegetatively in plastic pots using a soil: peat mixture (3:1), fertilized with 0.12 g/l Phostrogen (Solaris, Buckinghamshire, UK) once every 2 weeks, and maintained at 13° C. in a growth chamber with a photon flux density of 180  $\mu\text{mol m}^{-2} \text{s}^{-1}$  at the top of the canopy and photoperiod of 21 h/3 h of light/dark. The light source consisted of cool-white florescent tubes (F40CW IGE, Charlotte, N.C., USA). Relative humidity was around 60-70%. Plants growing under these conditions were considered as controls. For cold-acclimation studies plants were maintained 4° C. at the same light and photoperiod conditions than control plants for different times (0-21 days). De-acclimated plants were obtained by returning cold-acclimated plants to normal growth conditions for 24 hours.

#### EXAMPLE 2

##### Construction of Cold-Acclimation cDNA Library and Identification of Cold-Acclimation-Responsive cDNAs by mRNA Differential Display

[0036] Total RNA isolated from 24- and 48 h cold-acclimated *D. antarctica* leaves were pooled and used for cDNA library construction by means of the SMART cDNA Library Construction Kit (Clontech, Palo Alto, Calif., USA) and Gigapack II Gold Packaging Extract (Stratagene, La Jolla, Calif., USA), following the manufacturer's instructions. The primary library contained an estimated  $5 \times 10^6$  independent clones with an average insert size of 1,200 bp. The library was amplified and stored in 7% DMSO at -80° C. The cDNA library was screened by in situ plaque hybridization with a random-primed <sup>32</sup>P-labeled probe of differential display clone D0-B1.

[0037] *D. antarctica* cold-acclimation gene expression was studied by mRNA differential display using leaf total RNA extracted from non-acclimated and cold-acclimated *Deschampsia antarctica* plants taken at different intervals of treatment. A total of 38 cDNA bands were found to exhibit differential expression. Fragments were cut out of the gels, reamplified by PCR, and sub cloned into the pGEM-Easy vector. Twenty three partial cDNAs were shown by Northern blot analysis to be up- or down-regulated upon cold acclimation. DNA inserts of seven clones, ranging in size from 200 to 500 bp, were sequenced and the resulting sequences were compared with the NCBO non-redundant sequence database and three clones were left with unknown functions. The seven clones and the found homology of four of them are shown in Table 1 below.

TABLE 1

| List of selected cold induced cDNAs |                  |   |                  |
|-------------------------------------|------------------|---|------------------|
| Clone identity                      | Insert size (bp) | Sequence homology                               | Accession number |
| DACOR-1.3                           | 316              | <i>Brassica napus</i> Galactinol synthase       | CA 748463        |
| DACOR-0.7                           | 614              | <i>Oryza sativa</i> Glutaredoxin                | AP374461         |
| DACOR-1.0                           | 831              | <i>Arabidopsis thaliana</i> UBQ15/UBQ7          | AY090541         |
| DACOR-2.0                           | 1730             | <i>A. thaliana</i> Piruvate kinase-like protein | AY090539         |
| DACOR-0.9                           | 184              | Unknown   | CA 748460        |
| DACOR-0.8                           | 191              | Unknown   | CA 748461        |
| DACOR-0.65                          | 346              | Unknown   | CA 748462        |

## EXAMPLE 3

## Sequence Analysis and Characterization of Cold Acclimation Responsive Gene DaRub1

[0038] A 260-bp differential clone, DO-BI, was isolated from total RNA from cold-acclimated *Deschampsia antarctica* plants and unveiled a ca. 1.0 kb transcript in Northern blots. In order to isolate a full-length cDNA, a cDNA library (95×10<sup>4</sup> pfu), prepared from total RNA isolated from cold-acclimated *Deschampsia antarctica* leaves, was screened

GSP1: 5'-GGGTCAAGGAGAGAGCGTCAAGTCTG-3' (SEQ ID NO:6)  
 GSP2: 5'-AAGTCTGCGTGAGAACGAAGCGGAGGC-3'; (SEQ ID NO:7)  
 and  
 GSP3: 5'-GAACGAAGCGGAGGCAGAAACAAACG-3' (SEQ ID NO:8)

with the 260 bp insert as the probe. An 831 bp cDNA, DACOR-1.0 was isolated. This cDNA has a putative open reading frame (ORF) of 462 bp, flanked by a 120 bp 5' untranslated region and a 3' UTR of 249 bp that includes a 28 bp poly-A tail (FIG. 1A). FIG. 1C shows the full length sequence (SEQ ID NO:1) of the gene. Database searches revealed that the putative ORF encodes a polypeptide of 153 amino acids (SEQ ID NO:4) that consists of an ubiquitin monomer fused in frame to an ubiquitin-like protein RUB (related to ubiquitin). BLAST analysis showed that the RUB moiety is almost identical to other plant members of the RUB family of proteins. Two of the three *Arabidopsis* RUB genes, AtRUB1 and AtRUB2, are also fusions of the RUB coding region with that of ubiquitin. Similarly as ubiquitin, RUB proteins are 76 amino acids in length, but share only 52-63% sequence identity with ubiquitin (FIG. 1B).

## EXAMPLE 4

## Regulation of Transcript Levels by Cold Acclimation

[0039] To investigate the regulation of DaRub1 during cold acclimation, total RNA was extracted from leaves of *Deschampsia antarctica* at various time points of cold acclimation treatment. There was no detectable signal for the gene in total RNA samples from young leaves, although rather low signal was found in older leaves. Due to the

presence of the ubiquitin encoding moiety of the gene DaRub1, which in other systems seems to be encoded by a family of genes, the Northern blots were probed with the 3'UTR of the cDNA clone DACOR-1.0. Cold-acclimated leaves accumulated mRNA of DaRub1 as early as 30 min (FIG. 2). Northern blots at other time points of the 21 days cold acclimation treatment indicated that DaRub 1 was up regulated during cold acclimation and messages were found through the cold treatment. The transcripts declined rapidly to control levels 24 hours after the plants were returned to 13° C.

## EXAMPLE 5

## Isolation and Analysis of the DaRub1 Promoter

[0040] For promoter isolation genomic DNA was isolated through a CsCl gradient. For DaRub1 primer extension analysis total RNA was extracted from cold-acclimated plants (30 min to 24 hours) using Concert™ Plant RNA Reagent (Invitrogen) following the instructions of the manufacturer. Quality of total RNA was checked by electrophoresis on 1×TAE agarose gels. Primer extension analysis was performed using the 5'-TCTTCTCCTCTGCTCCCGTGTGGC-3' (SEQ ID NO:5) primer.

[0041] GenomeWalker libraries were prepared from *D. antarctica* genomic DNA using the Universal GenomeWalker™ kit (Clontech, Palo Alto, Calif., USA) following the instructions of the manufacturer. Three gene specific primers (GSP) were designed from DaRub1 cDNA sequence:

[0042] In order to confirm the co-linearity of the putative promoter fragment and the previously isolated DaRub1 cDNA the following two primers were designed:

Pro-F: 5'-CGTCTCAGTCCCGTTGC-3'; (SEQ ID NO:9)  
 and  
 Pro-R: 5'-TCGCTGCTCTCCACCTCG-3'. (SEQ ID NO:10)

[0043] Resulting promoter fragments were cloned into pGEM T-Easy vector (Promega, Madison, Wis., USA).

[0044] Sequencing of the amplified fragments was performed in an automatic sequencer using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PROMEGA, Southampton, UK). The obtained sequences were analyzed for regulatory sequences and promoter-like elements using the MathInspector program.

[0045] The genomic DNA was digested with DraI, EcoRV, PvuI or StuI restriction enzymes, provided by the Kit, generating thus GW Bank (GWB). Subsequently, the DNA fragments generated were ligated to adapters in the kit. Finally, PCR reactions were executed utilizing the GSP primers and adapter primer, also supplied by the kit. Three PCR reactions were carried out following the instructions of the kit.



[0046] From third PCR two fragments were isolated; one being 1200 pb and originating from the third GWB (DL3:PvuI) and another of 566 pb originating from the fourth GWB (DL4:StuI). Both PCR fragments (1200 pb and 566 pb) were cloned in pGEM T Easy vector (Promega) and their adequate insertion in the vector MCS was analyzed by restriction.

[0047] The cloned fragments were sequenced and their sequences were compared. DL4 GWB fragments have high similarity with 3'-terminal sequence of the DL3 GWB. DL3 GWB (1200 pb) fragment sequence was compared at MatInspector program for searching regulatory sequences and promoter like elements. Several typical eukaryotic and plant regulatory sequences were found but only those directly related to DaRub1 gene characteristic or function were taken into account. The most important responding elements found were the ethylene response element (ERE), auxin response element (AuxRE) and dehydration response element (DRE) (FIG. 4)

[0048] Two primers were designed from promoter 3'-terminal sequence and cDNA 5'-terminal sequence, and used to isolate a 672 pb fragment from the genomic DNA. This 672 pb fragment was used to determine the transcription start site (TSS) and to verify promoter sequence (GW product) and cDNA sequence co-linearity. The fragment amplified by PCR was isolated and cloned in pGEM-T Easy. Its adequate insertion in the vector was checked by restriction analysis and by PCR amplification of an internal fragment, utilizing fragment internal primers. The sequence of the 672 pb fragment was compared with the sequence expected, about 296 bases from 3'-terminal sequence were read resulting to 99.3% identity with expected sequence.

[0049] TSS was determined by mean primer extension method. TSS resulted to be a G, which was found 50 pb downstream to putative TATA box. FIG. 4 shows the putative promoter sequence (SEQ ID NO:2) and the responding elements obtained with MatInspector Program analysis, as well as the putative TATA box.

#### EXAMPLE 6

##### Construction of Promoter-GUS Fusion and Study of DaRub1 Putative Functionality

[0050] To study the putative promoter functionality, different reporter fusions were carried out with the promoter and its deletions (FIG. 5). For reporter fusion construction, the putative promoter and its deletions were fused at pCAMBIA 1391 vector "Promoterless" (CAMBIA, Canberra, Australia), having the gusA reporter gene. Each one of the promoter or deletions -pCAMBIA1391 fusions was analyzed by restriction and/or Southern blot analysis. Once verified, each fusion was introduced at *Agrobacterium tumefaciens* by electroporation. Adequate introduction was verified by Southern blot analysis.

[0051] All fusions were introduced to *Arabidopsis thaliana* Columbia mediated Floral Dip method. pCAMBIA1303 vector having 5'GusA-mgfp5'-3' fusion having expression directed by 35S CaMV promoter region was used as a transformation control. Plants that contained pCAMBIA 1303 vector were utilized as GUS expression positive control (FIG. 6). For the selection of putative transgenic plants, seeds from T0 plants were germinated in MS medium

supplemented with 25 mg/l hygromycin. Survival plantlets were removed and transplanted to the soil and the cycle was repeated until T3 plants generation.

[0052] Promoter region functionality in transgenic plants obtained for each one of the fusions (FIG. 5) were studied for cold acclimation, de-acclimation and other related stress by random selection of several plantlets. Only those transgenic lines originating from the complete promoter region fusion were capable to induce the GUS expression (FIG. 6). The results prove that promoter region needs all its regulatory elements to induce the protein expression.

[0053] Analysis was carried out in different organs of adult transgenic plants, such as flowers, siliques leaves, senescent leaves and roots. We also analyzed GUS expression in whole in vitro plantlets. Adult plants were also analyzed after wounding (detachment of leaf or *aphis* infection).

[0054] FIG. 6 shows that during cold acclimation at 4° C. GUS expression was observed in roots as well as in apex, fundamentally in the zones with greater meristematic tissue. This result is in agreement with the Northern blot result of FIG. 2 showing accumulation of mRNA of DaRub1 gene as early as 30 minutes of acclimation. The expression was highest during the first hour of acclimation and the expression was maintained until second hour and subsequently was observed with a similar intensity after 6 and 12 hours.

[0055] We studied expression in relation different auxins (2,4D) concentrations as well. Auxin treatment was carried out by transferring plants for 18 hours to liquid MS medium supplemented with 10 nM, 1.0 μM and 10 mM of 2,4 D. GUS expression induced by auxin was little more intense than GUS expression induced by cold but less than GUS expression induced by 35S promoter region in pCAMBIA 1303 (FIG. 6). This expression was maintained in the main cellular growth and development zone, meristematic tissue, apex and root growth zone. No remarkable differences were detected with different auxin concentrations used ((10 nM, 1.0 μM, and 10 μM). This is a surprising result, because the DaRub1 promoter has to AuxRE-regions (FIG. 4).

[0056] FIG. 9 shows the model according to this disclosure which explains the importance of two AuxRE-regions. The AuxinRE-regions (TGTC(T)C) identified in the DaRub1 promoter region are recognized by the auxin response transcription (ARFs: auxin response factors). Synthetic palindromic or direct repeats of these six nucleotides are sufficient to bind ARFs and confer auxin regulation on the transcription of the reporter gene. The promoter deletions where either of the AuxER was lacking (FIG. 5) the GUS expression was not induced because both of the regions are required for folding of the promoter region so that ARFs can be recognized as shown in FIG. 9. Thus ARF mediates auxin-regulated gene expression through binding to AuxER, and AUX/IAA has the potential to alter the transcription of auxin-inducible genes by interaction with ARFs.

[0057] No synergic effect in GUS expression was observed when plantlets were cold acclimated and auxin treated simultaneously

[0058] When adult plants (approximately 2-3 weeks in greenhouse) were cold acclimated an equal GUS expression was observed as in the in vitro plantlets. Interestingly, a clear

GUS expression was observed in senescent leaves (**FIG. 7C**). This expression is probably related to the ERE present in the promoter region.

[0059] We studied the GUS expression in relation to other stress factors and could only find expression related to wounding. As seen from **FIGS. 7A** and **B** the expression was locating around the wounds. In **FIG. 7A** it can be seen that GUS expression is only around the injury tissue caused by detachment of the leaf and the expression is observed on both sides of the wound. This expression was seen only some minutes after the wound. In **FIG. 7B** the wound is made by an insect (*aphis*) and the GUS expression was detected for a much longer period than the expression induced by leaf detachment.

[0060] In **FIGS. 7D-H**, it can be seen that GUS expression changes during different flower development phases: the expression is strong in early flower development phase and is less intense in advanced phases. **FIGS. 7 D, E, F** and **G** show expression in pollen. **FIG. 7 H** shows an advanced phase after floral anthesis and GUS expression can be observed only in the growth and elongation zones of the meristematic tissue in flower base. In **FIG. 7 I** it can be seen that once the siligues are formed the GUS expression is limited to the seed funicular.

#### EXAMPLE 7

##### DaRub1 Expression in *D. antarctica* Studies by RNA in situ Hybridization

[0061] A fragment of 190 pb, which included the 3'UTR, was amplified for PCR using DaRub1 cDNA and the primers 5'-CCTGTGTGTAACATCAGACTCTCTCCAC-3' (SEQ ID NO: 11) and 5'TCCAATTCTGCCAATGCTAACTCC-3' (SEQ ID NO: 12.) The amplified product was cloned in pGEM® T-Easy vector (Promega). RNA probes Digoxigenin-11-UTP labeling was carried out by Digoxigenin RNA labeling kit (Roche), following manufacturer's instructions. Sense and antisense probes were used.

[0062] *D. antarctica* plants were acclimated at 4° C. for 24 hours. The hybridization was according to the protocol of Jackson (1991) with modifications (Vielle Woen et al, 1999). 400 to 800 ng of RNA probes were utilized for each reaction. The hybridization was carried out at 55° C. overnight. For the immune detection, slides were incubated two times (45 minutes and 30 minutes) in 0.5% blocking agent (Boehringer Mannheim) in TBS (100 mM Tris HCl at pH 7.45, 150 mM NaCl) followed by 45 minutes in 1% BSA, 0.5% Triton X-100 in TBS. Antibody conjugation was made with 1:1250 in 1% BSA antidigoxigenine dilution for 2 hours. For revealed, the slices were washed in buffer C (100 mM Tris at pH 9.5, 50 mM MgCl<sub>2</sub>, 100 mM NaCl) two times and incubated for 2 to 5 days in 0.34 mg/l of nitroblue tetrazolium salt (NBT) and 0.175 mg/ml 5-bromo-4-chloro-3-indylphosphate-toluidinium salt (BCIP) in buffer C plus 7.6 mM levamisole (SIGMA). The reaction was stopped with 10 mM Tris HCl, 1 mM EDTA and the slices were re-hydrated in degree of ethanol series and Cytochrome c mounted (Stephens Scientific). The analysis was carried out with a microscope Leica DMRB under brilliant field and optical Nomarski.

[0063] The antisense RNA in situ hybridization (**FIG. 8**) showed gene expression in meristematic tissues coinciding with the GUS expression induced by pDaRub1 in *A.*

*thaliana*. This shows that the promoter region isolated is complete and that it directs the expression.

#### EXAMPLE 8

##### Plant Transformation Process with *Eucalyptus globulus*

[0064] Genetic transformation of *Eucalyptus globulus* elite individuals via *Agrobacterium tumefaciens* was carried out according to the procedure described for recalcitrant species by Perez-Molphe & Ochoa-Alejo N. (1998) with some modifications. *Agrobacterium tumefaciens* hypervirulent strain AGL0 was used, a single colony of *Agrobacterium* was inoculated into 25 ml of YEP media (Yeast extract 20 g., peptone 20 g, NaCl 10 g.) together with the selection antibiotics, hygromycin 100 mg/L, rifampicin 100 mg/L, chloramphenicol 250 mg/L. The culture was shaken at constant 150 rpm. For 2-3 days until it reached an OD<sub>600</sub> of 0.5. The culture was centrifuged at 3000×g for 20 min., resuspended in diluted MS media 1:10 and 50 µl of acetosyringone. Before use the suspension was incubated for 5 hours at 28° C. and at 100 rpm.

[0065] The tissue was prepared for transformation by selecting shoots that correspond to the second and third internodes of the in vitro cultivated plants. Additional longitudinal wounds were performed at both sides of the stem after which the explants were transferred an Erlenmeyer containing the *Agrobacterium* suspension.

[0066] The explants were incubated in the *Agrobacterium* suspension for 10 minutes under vacuum. Thereafter they were dried with sterile filter paper and transferred to the cocultivation media (MS [1/2] without hormones) placing them horizontally for 2 days.

[0067] After this the explants were rinsed in liquid MS plus cefotaxime 250 mg/mL to prevent *Agrobacterium* overgrowth, dried with sterile filter paper and placed vertically on selection media (MS, sucrose 3%, BAP 0.1 mg/mL, NAA 0.01 mg/mL, cefotaxime 250 mg/mL, selection antibiotic kanamycin 100 mg/mL or hygromycin 15 mg/mL). The explants were transferred to fresh new media every 7 days for the first 4 weeks.

[0068] Putative transgenic explants were transferred to rooting media (MS [1/2], IBA 2 mg/L, sucrose 20 g/L, agar 8 g/L, pH 5.8) with selection antibiotics, after which roots develop after 2 weeks.

[0069] Five transgenic eucalyptus lines were regenerated, each of which expressed DaRub1 gene.

#### EXAMPLE 9

##### Physiological and Biochemical Characterization of DaRub1 Transgenic *Eucalyptus* Plants

[0070] Lethal Temperatures for 50% of Leaf Tissue (TL<sub>50</sub>) was analyzed from five transgenic *Eucalyptus* lines and two wild type lines in leaf tissue according to the methodology of Cloutier and Andrews (1984), with slight modifications (Alberdi et al., 1993). The leaves were placed in tight glass containers, AgI and H<sub>2</sub>O were added to prevent supercooling and to secure freezing of the samples. The glass containers were placed in a cryostat and exposed to freezing temperatures (approx. between -1 and -30° C.) for 120

minutes. After that the samples were thawed at 4° C. for many hours. Deionized water was added to the samples, stirred at 29° C. for 1 hour, and the ion leakage was measured by conductivity. TL<sub>50</sub> corresponds to the temperature at which 50% of the dead tissue conductivity in liquid nitrogen is reached. This analysis was carried out first with control plants (wild type lines), and according to the results the conditions for cold acclimation and freezing were determined and used for the evaluation of the transgenic plants.

[0071] Table 2 below shows the freezing temperatures (upper and lower limits) of the transgenic lines 1-5 and two wild type lines. The table also shows ice nucleation temperature of the plants. The lines have been ranked based on the freezing tolerance as deduced from the Lt50 value and the ice nucleation temperature. It can be clearly seen that all of the transgenic lines are more tolerant to freezing stress than the control lines and that lines 3 and 5 show the best freezing tolerance.

TABLE 2

| Line         | Ranking | Nucleation<br>temperature ° C. |                | Freezing<br>temperature ° C. |                |
|--------------|---------|--------------------------------|----------------|------------------------------|----------------|
|              |         | Lower<br>limit                 | Upper<br>limit | Lower<br>limit               | Upper<br>limit |
| 5 transgenic | 1       | -8.83                          | -7.77          | -5.73                        | -5.08          |
| 3 transgenic | 2       | -8.32                          | 7.43           | -4.70                        | -4.36          |
| 2 transgenic | 3       | -7.61                          | -7.2           | -5.22                        | -4.50          |
| 1 transgenic | 4       | -7.25                          | -6.73          | -5.24                        | -4.19          |
| 4 transgenic | 5       | -7.4                           | -6.66          | -4.55                        | -3.86          |
| 6 wild type  | 6       | -5.03                          | -4.43          | -3.50                        | -2.84          |
| 7 wild type  | 7       | -5.31                          | -3.97          | -3.66                        | -2.40          |

[0072] The transgenic plants and wild type plants were also evaluated based on the physiological state of photosys-

tem II (PSII) at low temperatures during cold acclimation and freezing by monitoring PSII fluorescence in the leaves. Fluorescence was measured with a pulse amplitude fluorimeter (Hansatech). Table 3 below shows the order of average percentage of photoinhibition of the leaves at different freezing temperatures. Clearly the transgenic lines (1 to 5) possessed less photoinhibition in low temperatures, thereby proving better low temperature tolerance of the transgenic lines as compared to the wild type lines (lines 6 and 7). Again transgenic lines 3 and 5 showed the best low temperature tolerance of the transgenic lines.

TABLE 3

|            | Temperature ° C. |    |    |    |     |
|------------|------------------|----|----|----|-----|
|            | -3               | -5 | -7 | -9 | -11 |
| Ranking of | 5                | 3  | 3  | 5  | 5   |
| the lines  | 3                | 5  | 5  | 3  | 3   |
| analyzed   | 2                | 2  | 4  | 4  | 2   |
|            | 4                | 1  | 4  | 4  | 4   |
|            | 1                | 1  | 4  | 1  | 4   |
|            | 7                | 7  | 6  | 6  | 6   |
|            | 6                | 6  | 7  | 7  | 7   |

[0073] The freezing damage of the transgenic and wild type *Eucalyptus* leaves were also evaluated visually. Freezing damage in *Eucalyptus* leaves can be detected by color change in the central vein that turns red. This experiment also showed that the transgenic plants were clearly more freezing tolerant than the wild type plants: central veins of wild type plants turned reddish at -3° C. and clearly red at -5° C.; while the leaves of all transgenic lines showed reddish central veins in temperatures clearly lower than the wild type plants.

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Ser Thr Leu His Leu Val Leu Arg Leu Arg Gly Gly Thr Met Ile Lys
65              70              75              80
Val Lys Thr Leu Thr Gly Lys Glu Ile Glu Ile Asp Ile Glu Pro Thr
              85              90              95
Asp Thr Ile Asp Arg Val Lys Glu Arg Val Glu Glu Lys Glu Gly Ile
              100             105             110
Pro Pro Val Gln Gln Arg Leu Ile Tyr Ala Gly Lys Gln Leu Ala Asp
              115             120             125
Asp Lys Thr Ala Lys Asp Tyr Asn Ile Glu Gly Gly Ser Val Leu His
130             135             140
Leu Val Leu Ala Leu Arg Gly Gly Tyr
145                      150

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<210> SEQ ID NO 5
<211> LENGTH: 153
<212> TYPE: PRT
<213> ORGANISM: Deschampsia antarctica

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<400> SEQUENCE: 5

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Met Gln Ile Phe Val Lys Thr Leu Thr Gly Lys Thr Ile Thr Leu Glu
 1              5              10              15
Val Glu Ser Ser Asp Thr Ile Asp Asn Val Lys Ala Lys Ile Gln Asp
              20              25              30
Lys Glu Gly Ile Pro Pro Asp Gln Gln Arg Leu Ile Phe Ala Gly Lys
              35              40              45
Gln Leu Glu Asp Gly Arg Thr Leu Ala Asp Tyr Asn Ile Gln Lys Glu
 50              55              60
Ser Thr Leu His Leu Val Leu Arg Leu Arg Gly Gly Thr Met Ile Lys
65              70              75              80
Val Lys Thr Leu Thr Gly Lys Glu Ile Glu Ile Asp Ile Glu Pro Thr
              85              90              95
Asp Thr Ile Asp Arg Val Lys Glu Arg Val Glu Glu Lys Glu Gly Ile
              100             105             110
Pro Pro Val Gln Gln Arg Leu Ile Tyr Ala Gly Lys Gln Leu Ala Asp
              115             120             125
Asp Lys Thr Ala Lys Asp Tyr Asn Ile Glu Gly Gly Ser Val Leu His
130             135             140

```

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-continued

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Leu Val Leu Ala Leu Arg Gly Gly Tyr  
145 150

<210> SEQ ID NO 6  
<211> LENGTH: 25  
<212> TYPE: DNA  
<213> ORGANISM: Deschampsia antractica  
<220> FEATURE:  
<221> NAME/KEY: misc\_feature  
<222> LOCATION: (1)..(25)  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 6

tcttctcctc ctgctcccggt gtggc

25

<210> SEQ ID NO 7  
<211> LENGTH: 26  
<212> TYPE: DNA  
<213> ORGANISM: Deschampsia antarctica  
<220> FEATURE:  
<221> NAME/KEY: misc\_feature  
<222> LOCATION: (1)..(26)  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 7

ggtcaaggag agaggcgtca agtctg

26

<210> SEQ ID NO 8  
<211> LENGTH: 26  
<212> TYPE: DNA  
<213> ORGANISM: Deschampsia antarctica  
<220> FEATURE:  
<221> NAME/KEY: misc\_feature  
<222> LOCATION: (1)..(26)  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 8

aagtctgcgt gagaacgaag cggagc

26

<210> SEQ ID NO 9  
<211> LENGTH: 26  
<212> TYPE: DNA  
<213> ORGANISM: Deschampsia antarctica  
<220> FEATURE:  
<221> NAME/KEY: misc\_feature  
<222> LOCATION: (1)..(26)  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 9

gaacgaagcg gaggcagaaa caaacg

26

<210> SEQ ID NO 10  
<211> LENGTH: 16  
<212> TYPE: DNA  
<213> ORGANISM: Deschampsia antarctica  
<220> FEATURE:  
<221> NAME/KEY: misc\_feature  
<222> LOCATION: (1)..(16)  
<223> OTHER INFORMATION: primer

<400> SEQUENCE: 10

cgtctgtccc cgttgc

16

<210> SEQ ID NO 11

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<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Deschampsia antarctica
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(18)
<223> OTHER INFORMATION: primer

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<400> SEQUENCE: 11

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tcgctgctct ccacctcg

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18

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<210> SEQ ID NO 12
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Deschampsia antarctica
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(28)
<223> OTHER INFORMATION: primer

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<400> SEQUENCE: 12

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cctgtgrgta acatcagact ctctccac

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28

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<210> SEQ ID NO 13
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Deschampsia antarctica
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (1)..(25)
<223> OTHER INFORMATION: primer

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<400> SEQUENCE: 13

```

```

tccacttctg cccaatgcta actcc

```

25

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What is claimed is:

1. A nucleotide sequence encoding for amino acid sequence essentially according to SEQ ID NO:4; said nucleotide sequence having coding sequence according to SEQ ID NO:3.

2. The nucleotide sequence according to claim 1, wherein the nucleotide sequence further comprises promoter sequence according to SEQ ID NO:2.

3. The nucleotide sequence according to claim 1, wherein the nucleotide sequence is essentially according to SEQ ID NO: 1.

4. An inducible promoter having nucleotide sequence according to SEQ ID NO:2.

5. The promoter according to claim 4, wherein the promoter is inducible by low temperature, wounding and auxin.

6. A method for conferring low temperature resistance in a plant, said method comprising a step of introducing into the plant a recombinant expression construct, said construct further comprising a plant promoter operably linked to DaRUB1 sequence essentially according to SEQ ID NO:3.

7. The method according to claim 6, wherein the promoter is essentially according to SEQ ID NO:2.

8. The method according to claim 6, wherein the sequence encodes a gene product having a amino acid sequence essentially according to SEQ ID NO:4.

9. The method according to claim 6, wherein the plant is *Euclayptus globulus*.

10. The method according to claim 6, wherein the plant is *Arabidopsis thaliana*.

11. The method according to claim 6, wherein the plant species is a fruit tree.

12. The method according to claim 11, wherein the plant species is selected from the group consisting of peach, avocado and orange.

13. A DNA construct for cloning and transforming plants, said construct comprising nucleotide sequence selected from a group consisting nucleotide sequences according to SEQ ID NO:2 and SEQ ID NO:3.

14. A transgenic plant comprising the DNA construct according to claim 13.

15. The transgenic plant according to claim 14, wherein the plant comprises DNA construct according to SEQ ID NO:3 and the plant further has an increased low temperature tolerance.

16. The transgenic plant according to claim 15, wherein the plant is an *Eucalyptus globulus* plant.

17. The transgenic plant according to claim 15, wherein the plant is an *Arabidopsis thaliana* plant.

18. The transgenic plant according to claim 15, wherein the plant is a fruit tree.

19. The transgenic plant according to claim 18 wherein the plant is selected from the group consisting of peach, avocado and orange.

\* \* \* \* \*