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Isolation and characterization of cold inducible genes in carrot by suppression subtractive hybridization

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Abstract

Daucus carota is cultivated widely but grows best in cool climates. Suppression subtractive hybridization (SSH) is a PCR based method used to selectively amplify differentially expressed cDNAs and simultaneously suppress non-target cDNA. A subtraction forward library was constructed using RNA isolated from the leaves of unstressed and cold stressed carrot plants to determine the genes upregulated during cold stress. Out of the hundreds of clones obtained, sequences of 41 promising clones were submitted to the *NCBI EST* database. Sequence analyses revealed that these genes have significant roles in signal transduction, osmolyte synthesis and transport, regulation of transcription, translation, and protein folding. Semiquantitative real-time polymerase chain reaction analysis (sqRT-PCR) of *Dc cyclin, Dc WD*, and *Dc profilin* shows that the first two genes were upregulated whereas *Dc profilin* was constitutively expressed, but the analyses of the same with SSH, a much more sensitive technique, showed an upregulation of all three genes.

Additional key words: cyclin, *Daucus carota,* low temperature, profilin, sqRT-PCR, upregulated genes.

Introduction

Low temperature is one of the major abiotic stresses, which limit growth, geographical distribution, and productivity of various crop plants (Thapa *et al.* 2011). Plants that grow in high altitude as well as in arctic climates are adapted to very low or even freezing temperatures by modifications in their metabolism. The change in adaptability of plants is mainly due to the difference in gene expression patterns. The cold responsive genes encode proteins involved in plant metabolism or membrane integrity, several stress-protective proteins (dehydrins, antifreeze proteins, *etc*.), and low molecular mass compounds (sugars, proline, mannitol, *etc*.), which act as osmolytes.

Daucus carota L. (carrot) is tolerant to cold stress and it can withstand cold weather and mild frost. Also, the plant flowers only after vernalization (Samuoliene *et al*. 2008). This gives indirect support that the low temperature tolerance may be due to the expression of various cold-responsive genes in plant. The traditional breeding strategy for improvement of abiotic stress tolerance is limited (Rodrigues *et al*. 2006). A wide range of modern molecular techniques and strategies such as serial analysis of gene expression (SAGE), suppression subtractive hybridization (SSH), microarray, quantitative real-time PCR (qRT-PCR), and cDNA microarray enables identification of different genes having high potential to achieve tolerant phenotypes (Sreenivasulu *et al*. 2007). SSH is one of the popular methods for generating cDNA libraries to study differential gene expression (Lukyanov *et al.* 1994), especially to characterize differentially expressed genes in response to various stresses (Kang *et al*. 2010).

In this study, we used SSH to isolate cold responsive genes from leaves of cold-treated carrot plants. Several potential genes were identified and the putative functions were correlated.

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Abbreviations: DREBs - dehydration-responsive element-binding proteins; EST - expressed sequence tag; MS - Murashige and Skoog; qRT-PCR - quantitative real-time polymerase chain reaction; RACE - rapid amplification of cDNA ends; SAGE - serial analysis of gene expression; SSH - suppression subtractive hybridization; SUMO - small ubiquitin like modifier.
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Materials and methods

Daucus carota L. cv. Kuroda seeds were germinated in commercially available soil mix supplemented with 1/10 strength sterile Murashige and Skoog (MS) medium and the plants were maintained in controlled conditions in greenhouse (temperature of 23 °C, 16-h photoperiod, irradiance of 200 ± 20 μmol m^{-2} s^{-1}, air humidity of 70 - 75 %). One month old plants were exposed to cold stress at 4 °C for 24 h in a plant growth chamber. Plants maintained at 23 °C were used as controls.

Young leaves from the control and cold stressed plants were harvested at the same time and immediately frozen in liquid nitrogen untill further use. Total RNA was isolated from control and test samples using *Qiagen* (Valencia, CA, USA) *RNeasy* RNA isolation kit and on column DNase digestion was performed as per the manufacturer's instruction. The mRNA was purified from the total RNA using *PolyATtract*® mRNA isolation system I (*Promega*, Madison, USA).

SSH was carried out using PCR-select cDNA subtraction kit (*Clontech Laboratories*, Palo Alto, CA, USA) according to the manufacturer's instructions. Double stranded cDNA (ds cDNA) was synthesized from 4 μg of poly A^+ purified mRNA. The cDNA of control sample (driver population) and the stressed sample (tester population) were quantified and digested with *Rsa*I (*Fermentas*, Maryland, USA). The tester cDNA was ligated to adapter 1 and adapter 2R separately in 2 ligation reactions at 16 ºC overnight. The first hybridization reaction was carried out at 68 ºC for 8 h after adding excess of driver cDNA (4 - 5 times) to the adapter ligated tester cDNA 1 and 2R separately. The second hybridization reaction was carried out at 68 ºC for 12 h after mixing the products of first hybridization reaction 1 and 2 in presence of excess of fresh driver cDNA (4 - 5 times).

The resultant subtractive product was amplified by PCR using primers which were complementary to sequence of adapters 1 and 2R. The PCR was carried out in *Mycycler*™ (*Bio-Rad*, Hercules, CA, USA) using the following conditions: initial denaturation at 94 °C for 5 min followed by 27 cycles of 94 °C for 30 s, 66 °C for 30 s, and 72 °C for 90 s. The first PCR was followed by a nested PCR of 12 cycles at 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 90 s. The final PCR product was the subtracted forward cDNAs which were enriched with genes over expressed during cold stress in carrot.

The subtracted cDNA library product was purified using *Genei Pure*™ quick PCR purification kit (*Bangalore Genei*, Bangalore, India). The purified product was digested with *Rsa*I and ligated in the *Sma*I site of pBluescript KS (+) vector for blunt end cloning. The ligated product was transformed to *Escherichia coli* DH5α cells. Transformed colonies were selected in Luria Bertani agar supplemented with ampicillin, isopropyl β-D-1-thiogalactopyranoside (IPTG), and X-gal.

The putative white recombinant colonies from the subtracted library were screened for the size of insert by colony PCR using M13 primers. All the reagents for the PCR reaction were from *Fermentas*. The reaction mix contained 10 mM Tris HCl (pH 8.8), $MgCl_2$ (2.5 mM), dNTP mix (1 mM each), M13 forward primer and M13 reverse primer (10 pM), Taq DNA polymerase (5 U mm^{-3}) and the volume was made with PCR grade water. PCR was carried out with an initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 60 s, 72 °C for 90 s, and a final extension of 5 min at 72 °C. The PCR products were resolved in 2 % (m/v) agarose gel and stained with ethidium bromide.

Plasmid DNA was isolated from the randomly selected recombinant clones using *Hipura* plasmid DNA isolation kit, (*Himedia Laboratories*, Mumbai, India). The plasmid DNA was sequenced in 3730 DNA analyzer (*Applied Biosystems*, Foster City, CA, USA) using M13 forward primer. The vector sequences were removed from the obtained sequence using the *VecScreen* online software (www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). The EST sequences were analyzed using *BLASTN* from the *NCBI* database (www.ncbi.nlm.nih.gov/blast/). Homology searches were performed against non-redundant nucleotide sequence using *BLASTN*. The unique EST sequences were submitted to the *NCBI* GenBank EST database.

The transcript accumulation of the 3 genes were studied after application of cold stress on carrot plants. One month old carrot plants were exposed to 4 °C for 5 d. Samples were harvested every day at the same time and were immediately frozen in liquid nitrogen and stored in -80 °C until further processing. RNA was isolated from all the samples using *Qiagen* RNeasy RNA isolation kit and on column DNase digestion was performed to remove the genomic DNA from the RNA sample. The RNA was quantified in *Nanodrop* (*Thermo Scientific*, Pittsburgh, PA, USA) and the integrity was checked in formamide gel. RNA (2 μg) was used for the synthesis of cDNA using *RETROscript*® kit for RT-PCR (*Ambion*, Austin, TX, USA) according to manufacturer's instructions. Primers were designed using *Primer Express* software (*Applied Biosystems*) from the already available sequence from GenBank for *Daucus carota* (*Dc*) WD, *Dc* profilin 4 and *Dc* cyclin 2b. The first strand cDNA was

Table 1. Primer used for the semiquantitative RT-PCR analysis.

Gene	Primer sequence
Dc WD	F: 5'- TGTCAATGGCCTCCACAAATT-3'
	R: 5'- TTTACAGCTGAAGTGTGTTCTTCCA-3'
Dc Profilin 4	F: 5'- AAGCTCTGGTGTTTGGAGT-3'
	R: 5'- TAATCTCCAAGCCTCTCAAC-3'
Dc Cyclin 2b	F: 5'- ACAATTCGAGTGCTGTTTCT-3'
	R: 5'-CTGTGGGTCATCAAATTTCT-3'
Dc EF1α	F: 5′- TGGTGATGCTGGTTTCGTTAAG -3′
	R: 5'-ATGGGAGGGTAGGACATGAAGGT -3′

diluted 10 times using nuclease free water and used as template for PCR. PCR was performed using *Ready to go* PCR beads (*GE Healthcare*, Little Chalfont, UK). The primer sequences are given in Table 1. *Dc* eukaryotic elongation factor 1 *α* (eEF1α) was used as the internal control for semiquantitative (sq) RT-PCR. The PCR cycle consists of 28 cycles with an initial denaturation at 94 °C for 5 min followed by denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. A final extension at 72 °C for 10 min was performed. The sqRT-PCR was repeated twice to verify the results.

Results and discussion

The reliability of the SSH method is dependent on the quality and quantity of RNA used. The RNA used in this study was isolated from young rosettes and two clear bands of 18S and 28S rRNA were observed in the formamide gel indicating RNA integrity (data not shown). High quality mRNA was separated from the total RNA and used for the synthesis of cDNA. The forward library was constructed using the stress-induced sample as the tester population and the control sample as the driver population to determine the genes upregulated during the cold stress. The cDNA samples, which were differentially expressed, were obtained after two rounds of hybridization followed by two rounds of suppression PCR. These cDNAs were enriched with genes upregulated during cold stress or using the forward cDNA library.

The PCR product of the second round of PCR, representing the subtracted cDNA library, was ligated to *pBluescript KS* (+) vector and was used to transform *E. coli* cells. The white colonies were screened using M13 primers and selected clones were used for the sequencing.

Around 75 randomly selected clones were sequenced. Among that, 62 clones gave readable sequences. After removing the redundant or vector backbone sequences, 41 sequences were submitted to the EST database (Table 2).

The sqRT-PCR indicated accumulation of cyclin and WD repeat protein after stress whereas there was no change in content of profilin (Fig. 1). Cyclin is one of the major proteins that regulate different stages of the cell cycle by its concerted expression and degradation. Cyclins along with cyclin dependent protein kinases (CDKs) achieve cell cycle regulation by phosphorylating different targets. Interaction of *Arabidopsis* cyclin D2 expressed in transgenic rice with endogenous CDK enhanced seedling growth (Oh *et al*. 2008). Cell cycle activities involved in stress responses are mediated by transcription factors (Morano *et al*. 1999). Transgenic rice expressing *OsMYB3R-2* enhanced low temperature tolerance that has been shown to be mediated by alteration in cell cycle (Ma *et al*. 2009). The transcript level of cyclin D2, cyclin B2-2, and CDK were upregulated during drought stress in *Arabidopsis*, wheat, and rice (Kamal *et al*. 2010). In the present study, the cyclin2b was upregulated in carrot during cold stress.

The proteins with WD repeats play a key role in signal transduction, cytoskeletal dynamics, ribosomal RNA biogenesis (Neer *et al*. 1994, Smith *et al*. 1999), cytokinesis, apoptosis, floral development, and meristem organisation (Nocker and Ludwig 2003). They have been classified based on sequence similarity (Nocker and Ludwig 2003). Recently, a WD 40 in *Brassica napus* (*BnSWD1*) was reported to play a major role during salt stress (Lee *et al*. 2010). Sucrose non-fermenting 1 (SNF1) kinase is a key enzyme in plant steroid biosynthesis and it phosphorylates the 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR). Its activity is regulated by

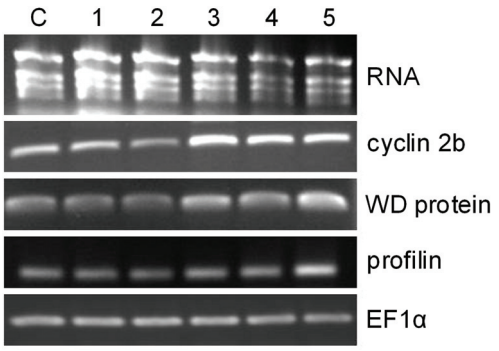

Fig. 1. Expression profile of three genes of *D. carota* by sqRT-PCR coding cyclin, WD protein and profilin. C - control plants, 1 to 5 corresponds to the samples from plants after 1 to 5 d of cold stress.

pleiotropic regulatory locus 1 (PRL1), a conserved nuclear WD-protein that is implicated in cold tolerance in *Arabidopsis* (Bhalerao *et al*. 1999). It was 2- to 16-fold repressed in leaves or shoots of cold and high-salinity stressed chickpea (Mantri *et al.* 2010). On the other hand, it was upregulated in carrot during low temperature stress suggesting it may have a role in cold stress tolerance.

The expression of *profilin* was unaffected under cold stress as seen from the sqRT-PCR results (Fig. 1) although SSH analyses show upregulation (Table 2). SSH is a powerful technique that can identify even minute changes in transcript levels as in the present case. Profilins are a group of low molecular mass ubiquitous actin binding proteins which are involved in the remodelling of actin cytoskeleton (Huang *et al.* 1996) and also involved in various signaling cascades in yeast and plants (Vojtek *et al*. 1991, Machesky and Poland 1993). Swoboda *et al*. (2001) reported that remodelling architecture of cytoplasm is a normal process in cells to maintain the membrane integrity during various environmental stresses and in carrot, profilins might also be contributing to the cold tolerance.

The unique expressed sequence tags (ESTs) were annotated based on their similarities with existing sequences in GenBank using *BLAST* and categorized in to six groups according to their functions. They are cold/salt/drought/UV stress responsive genes, genes involved in transcription/translation, genes involved in sugar/protein/lipid metabolism, genes involved in maintaining structural integrity, genes involved in signal transduction, and genes of unknown function.

A comparative analysis of the cold upregulated transcriptome was carried out to correlate and understand the mechanism of low temperature tolerance in plants. The upregulation of several genes of varied functions suggests that the unique ESTs may play role in complex biological processes. Wang *et al*. (2007) have reported that all the upregulated genes may not have a role in stress tolerance but could be induced in response to damages caused by the stress. The largest number of genes belongs to the group that is upregulated during cold, drought, or salinity stress. Apart from these, the upregulation of genes of unknown functions and those involved in transcription or translation suggests that the responses to cold stress are rather complex and multigenic as was reported earlier by Sun *et al*. (2007a), Zhang *et al*. (2009), and Kang *et al*. (2010).

There are several classes of transcription factors such as AP2/ERF, DREB, YABBY, and Trihelix families, which are unique to plants and act as molecular switches (Xie *et al*. 2009). Ramamoorthy *et al.* (2008) have identified that some of these transcriptional factors are

Table 2. The cold stress responsive genes isolated from carrot.

Acc. No.	Predicted gene or protein family	Putative function	E-value
GW316731	*A. thaliana* cyclin2b	regulator of cyclin-dependent kinases	3e-33
GW342890	*R. communis* WD protein	signal transduction/cell cycle regulation	5e-04
GW314857	*Dc* 4 profilin	actin-binding protein	3e-24
GW343024/ GW343026	*Solanum lycopersicum* - FAS protein	encodes transcription factor	4e-12
GW315340	*Chrysanthemum vestitum* - DREB	cold tolerance	1e-15
GW342888	*A. thaliana* - Zinc finger CCCH protein	cold/salt stress	2e-06
GW343025	*Talaromyces stipitatus* - Rop GTPase activator	cold/salt/drought stress	9e-06
GW316484	*Dc* RNA polymerase	transcription	7e-14
GW316488	*Hypochaeris megapotamica* - maturase K	intron splicing	6e -19
GW316489	*Odontorrhynchus variabilis* ribosomal rRNA	translation	1e-13
GW342893	*Peltandra virginica* - tRNA-Lys (trnK) gene	translation	2e-14
GW276092	*Dc* tRNA Leu trnL - trnF IGS	translation	9e 75
GW342886	*Beta macrocarpa* - mitochondrial genome	DNA synthesis/transcription and translation	1e-06
GW316482	*A. thaliana* - sec 61 beta-subunit	protein translocation to ER	3e-09
GW342894	*Aspergillus clavatus* GABA permease	translocation of GABA	8e-10
GW276089	*O. sativa* - E3 ubiquitin ligase	UV B response/low temperature tolerance	9e-48
GW343027	*Populus trichocarpa* - shikimate kinase	cold stress	9e-07
GW316730	*Candida albicans* - choline kinase	salinity stress	0.070
GW276087	*Bacillus subtilis* - levan sucrase	osmotic stress	2e-74
GW314859	*Chlamydia trachomatis* - phosphoglucoisomerase gene	involved in synthesis of galactomanan	6e-43
GW276091	*Talaromyces stipitatus* - sugar transporter protein	transport of sugar across the membranes	1e- 24
GW343023	*Cucumis melo* - catalase 2	oxidative stress	6e-08
GW342891	*A. thaliana* - lipid associated protein (fibrillins)	cold/photooxidative stress	0.003
GW342892	*Neosartorya fischeri* - integral membrane protein	cell structure maintenance	1e - 05
GW314854	*A. thaliana* - 60 α chaperonin subunit CCT family	cold tolerance	6e-43
GW316481	*Ricinus communis* - ATP binding protein	osmotic stress	1e-22
GW342889	*Penicillium marneffei* cyanate hydratase	nitrogen metabolism	1e-12
GW316732	*E.coli* - serine deaminase activator gene	aminoacid metabolism	2e-21
GW316733	*E.coli* - BioH gene	lipid metabolism	6e-11
GW316485	*Brassica cretica* S receptor kinases	self incompatibility	1e-18
GW276090	*Populus sp* - drought stress related protein	drought stress tolerance	1e-38
GW314853	*Populus trichocarpa* - stress protein	stress tolerance	0.002
GW342887	*Hydra magnipapillata* - putative signal tranduction	signal transduction	0.014
GW315344	*A. thaliana* similar to part of genome	unknown	
GW316487	*B. rapa* genomic DNA clone	unknown	
GW314858	*Oryza sativa* genomic DNA - chromosome 4, BAC clone	unknown	
GW314852	*Vitis vinifera* clone	unknown	
GW315342	*Populus trichocarpa* clone	unknown	
GW316483	*Oryza sativa* japonica BAC clone	unknown	
GW316728	*Populus trichocarpa* - predicted protein, mRNA	unknown	

involved in transcriptional regulation during environmental stresses. Liu *et al.* (2011) reported that the low osmotically responsive gene 2 (*LOS2)* from *Poncirus trifoliate* acts as a transcriptional activator for various cold-responsive genes. In cold stressed carrot, expression of *fas* and *DREB* were found to be enhanced. *Fas* is involved in regulating flowering through *YABBY* in tomato (Cong *et al*. 2008). In carrot, vernalization is required for flowering and the cold treatment could have led to the expression of *fas* which would in turn initiate flowering. The dehydration-responsive element-binding proteins (DREBs) have been found to confer tolerance to cold/drought stress in *Arabidopsis* (Kasuga *et al*. 2004), wheat (Andeani *et al*. 2009), rice (Dubouzet *et al*. 2003), cotton (Shan *et al*. 2007), and could also be functioning similarly in carrot. Similarly, the zinc finger CCCH protein was seen to be enhanced in response to salt stress in *Arabidopsis* (Sun *et al*. 2007a). Other zinc finger proteins like Zat 12 and *Gh* ZFP 1 have been found to confer tolerance to oxidative stress, salinity, and biotic stress (Guo *et al*. 2009).

The G proteins function as molecular switches to regulate numerous cellular responses such as proliferation, differentiation, responding to external environmental signals, *etc*. Rho-related GTPase of plants (Rop) play an important role in plant growth and development as a signaling protein and also confer abiotic stress tolerance in *Arabidopsis* (Shin *et al*. 2009) and cold tolerance in rice (Hashimoto and Komatsu 2007). The enhanced levels of similar proteins in carrot may be a signaling mechanism in response to low temperature treatment.

Regulation of transcription and translation plays an important role in stress alleviation (Miranda *et al*. 2003). RNA polymerase and maturase K are not only involved in transcription and post-transcriptional modification but have also been implicated in regulation of gene expression through miRNA/siRNA formation in response to stress in plants (Sunkar *et al*. 2007b). There are reports of enhanced levels of RNA polymerases and maturase K and a corresponding increase in the miRNA levels in various plants exposed to biotic and abiotic stresses (Lee *et al*. 2005, Zhou *et al*. 2008, Garavaglia *et al*. 2010).

Translation is regulated at the level of stability of transcripts and initiation of translation (Prabu *et al*. 2011). Transcript stability under stress is enhanced by the formation of polysomes (Arendt and Weidner 2011) and upregulation of rRNA, as seen in carrot, could contribute to this stability. Translation initiation factor 4α also showed upregulation on exposure to cold stress and similar results were observed in other plants like pea (Pham *et al*. 2000, Vashisht *et al*. 2005), and wheat (Kamal *et al*. 2010). Sec β 61, which was also upregulated in cold stressed carrot, showed a similar response to wounding stress in *Arabidopsis* (Pnueli *et al*. 2003) and is also essential for translocation of proteins to endoplasmatic reticulum (ER) and also for the cell viability in many organisms (Leroux and Rokeach 2008). In carrot, it might also be responsible for the translocation of different cold tolerant proteins to ER. The expression of γ-aminobutyric acid (GABA) permeases, that translocate GABA to combat stresses, was also enhanced in cold stressed carrot. GABA, a non-protein amino acid, has been found to increase in response to various stresses (Cholewa *et al*. 1997, Serraj *et al*. 1998, Bouche and Fromm 2004).

Ubiquitin ligases are the next group whose expression was enhanced under cold stress in carrot. Similar E3 ubiquitin ligases (SIZ1) result in the accumulation of small ubiquitin-like modifier (SUMO) protein conjugates that provide cold tolerance in *Arabidopsis* (Chinnusamy *et al*. 2007). Sumoylation is a post-translational modification that protects the target protein from proteosomal degradation by preventing ubiqutination.

Shikimate derived compounds have a major role in plant response to biotic and abiotic stresses (Hamberger *et al*. 2006), mainly shikimate kinase, the first enzyme in the pathway, is reported to play a role in providing stress tolerance to *Arabidopsis* (Fucile *et al*. 2008) and maize (Zheng *et al*. 2006). Thus, the upregulation of shikimate kinase may probably increase the synthesis of metabolites which confer tolerance to cold stress in carrot. Cold stress mimics water deficit similarly to salinity stress. Multiple mechanisms are known to confer tolerance to osmotic stress. Choline kinase catalyses the synthesis of phosphatidylcholine responsible for main-taining the osmolarity of the plant cell (Tasseva *et al*. 2004) and an upregulation of this gene in carrot may have important implications for cold tolerance.

Cold stressed carrot showed upregulation of a transcript that had similarity with the bacterial levansucrase. Levansucrases are hexosyltransferases, mainly involved in the metabolism of starch and sucrose and their expression in tobacco enhanced the plant osmotic tolerance (Park *et al*. 1999). We presume that the levansucrase might play similar role during low temperature stress.

Phosphoglucoisomerase catalyses the biosynthesis of galactomannans in plants (Lee and Matheson 1984) which apart from their primary role as storage reserves in endosperm also provide osmoprotection during leguminous seed germination under drought and low temperature (Mulimani and Prashanth 2002). Low molecular mass sugars also play a role as osmolytes and cryoprotectants and sugar transporters are involved in distribution of sugars to various cells and tissues (Williams *et al*. 2000). In *Arabidopsis,* a putative sugar transporter protein ERD6 (Kiyosue *et al*. 1998) and tonoplast monosaccharide transport proteins (Eckardt 2006) have been shown to be expressed in response to water stress.

Low temperature induces the generation of reactive oxygen species (ROS) which have strong adverse effect on plants (Chaitanya *et al.* 2001). ROS are mitigated by the antioxidant enzymes like superoxide dismutase, catalase, and peroxidase (Yong *et al*. 2008, Mallik *et al.* 2011). The prominent role of catalase during low temperature stress has been already reported in wheat

(Apostolova *et al*. 2008) and maize (Prasad 1997). Fibrillins are a group of lipid binding proteins in plastids which are induced by various abiotic stresses like a combination of photooxidative and cold stresses in *Arabidopsis* (Youssef *et al*. 2010), or low temperature stress in rice (Lee *et al*. 2007).

Maintenance of membrane stability is important for stress tolerance (Gulen *et al*. 2008). The low temperature stress affects the normal functioning of membrane proteins and the fluidity of the cell membranes. *Xv* SAP1, an integral protein isolated from *Xerophyta viscosa,* show high homology to WCOR413, a cold responsive protein from wheat (Garwe *et al*. 2003). This shows that the membrane proteins are not only involved in maintaining the cell structure but they are also associated with low temperature stress.

Chaperonin 60 and other kind of chaperonins play a key role in folding proteins. They have been found to be cold-induced in yeast (Somer *et al*. 2002) or heat-induced in rice (Han *et al*. 2009). In the present study, the upregulation of chaperonins resulted in the correct folding of proteins expressed during cold stress in plants (Zhang and Guy 2006). The proper folding of the membrane proteins would be of paramount importance. ATP binding proteins are group of membrane proteins involved in the transport of solutes across the cell membranes. They have been found to have a role in cold stress tolerance in maize (Jian *et al*. 1999) and rice (Cui *et al*. 2005), as well as drought stress tolerance in *Arabidopsis* (Valliyodan and Nguyen 2006).

The role of enzymes like cyanases, serine deaminases, S receptor kinase, *etc*. during cold stress is not very clear yet. Though they have shown to have different roles in the plant metabolic pathways, the upregulation of these may have direct/indirect functions during the cold stress.

In conclusion, the comparative analysis of the transcriptome showed that most plants have a common mechanism to tolerate the low temperature stress. From the current study, it is clear that the cold regulated transcriptome is conserved in different plants species though the level of upregulation or down regulation varies among different plant species. We have identified around 50 genes which play a major role in diverse functions to combat cold stress in carrot. Thorough characterization of all these genes may throw a light on the role of these genes in overcoming the cold stress. Hence, this study will greatly help in the development of cold tolerant transgenic crops employing several newly characterised genes.

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