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ORIGINAL ARTICLE

Isolation and Characterization of Nuclear Localized Abiotic Stress Responsive Cold Regulated Gene 413 (*SsCor413*) from *Saccharum spontaneum*

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Abstract

Winter survival develops efficient tolerance mechanisms in plants by regulating cold-responsive and cold-regulated genes at the transcriptional level. Hence, an insight into the expression would provide the molecular function of these cold responsive genes. In this study, an uncharacterized gene encoding a cold-regulated (*Cor413*) protein identified from *Saccharum spontaneum* (wild relative species of sugarcane) low-temperature transcriptome with environmental adaptability is isolated and characterized. The full-length coding region possesses an open reading frame of 642 bp, which encodes a putative polypeptide of 213 amino acids of molecular weight 25.6 kDa and an isoelectric point (Pi) of 9.69. The *SsCor413* sequence showed a high similarity to monocot *Cor413* proteins comprising a *WCOR413* domain. Bioinformatics analysis revealed that *Cor413* protein has multispanning transmembrane helices along with highly conserved phosphorylation sites. String analysis suggested that *SsCor413* is grouped with *LEA* and *Rab* proteins that are involved in freezing tolerance. Gene ontology analysis assigned the protein to terms such as "plasma membrane," "cold acclimation," and "response to cold." Sub-cellular localization experiments of sugarcane callus and onion epidermal cells indicated the nuclear localized expression. Quantitative gene expression analysis indicated that the *SsCor413* gene is up-regulated in leaf and root tissues of *S. spontaneum* under low temperature, salinity, and water deficit stress conditions. These results highlight the potential role of *SsCor413* in abiotic stress tolerance, and this gene could be a new candidate for combating multiple stresses in sugarcane.

Keywords *Saccharum spontaneum* · *Cor413* · Cold stress · Sub-cellular localization · Onion cells · qRT-PCR

Introduction

Plants exhibit a maximum growth rate and development at an optimum temperature (Fitter and Hay 1981) and when this temperature gets altered, physiological and molecular changes occur. Low temperature (LT) is one of the major abiotic stress factors that disturb plant growth, regulation, and yield (Chinnusamy et al. 2007). LT stress is known to induce several abnormalities at various organizational levels of the cell and its symptoms include changes in the membrane system, ion homeostasis disturbance, inhibition of photosynthesis, reduction in water and mineral uptake, and induction of oxidative stress.

Tolerance mechanisms such as chilling tolerance and cold acclimation process will be in use during low-temperature stress as a defense response. The chilling tolerance is the ability of a plant to tolerate LT (0–15 °C) without injury or damage (Somerville 1995). Cold acclimation is an enhanced

Key Message *Cor413* is identified as abiotic stress responsive gene in *Saccharum spontaneum* and acts as potential gene for combating abiotic stresses in sugarcane.

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tolerance process (Guy 1990). Cold stress-induced genes are known as cold-regulated genes. The transcription of gene *Cor* is differentially regulated under LT conditions (Baker et al. 1994; Chinnusamy et al. 2006). The regulation of *Cor* genes results in cell wall modifications, cold signaling transduction, transcriptional regulation, and molecular changes under low-temperature stress conditions (Thomashow 1998).

There are different types of *Cor* genes, viz., *Cor6.6*, *Cor15A*, *Cor47*, *Cor78*, and *Cor413* that have been identified in plants. Among the *Cor* genes, *Cor413/Cor413*-like gene has been well studied from several plant species, including *Triticum aestivum* (Breton et al. 2003), *Gossypium barbadense* (Wang et al. 2007), *Phlox subulata* (Qu et al. 2015), and *Solanum lycopersicum* (Ma et al. 2017). The *Cor413* is a plant-specific protein family that is involved in the cold acclimation process (Breton et al. 2003). Amino acids of *GbCor413* protein exhibits hydrophobic nature (Wang et al. 2007) and is predicted to have many phosphorylation sites (Blom et al. 1999). The *Cor413* protein was also predicted to consist of a minimum of four transmembrane domains (Krogh et al. 2001) which are required for targeting the protein toward the plasma membrane and thylakoid membrane (Breton et al. 2003). The sequence of *Cor413* has not been predicted for the presence of signal sequence. However, its mechanism and sub-cellular localization studies are far from being fully understood.

Saccharum spontaneum (wild sugarcane) belongs to the *Poaceae* family and is a tall perennial grass with a deep root system (Amalraj et al. 2008). The genetic background of sugarcane is complex as *Saccharum* hybrids are highly polyploid and derived from interspecific hybridization between *S. officinarum* and *S. spontaneum*, suggesting that each gene has 8–10 copies (Premachandran et al. 2017). *S. spontanuem* is known to have developed tolerance to low-temperature stress conditions (Friesen et al. 2014; Dharshini et al. 2016, 2018). Studying the genetic nature of abiotic tolerance mechanisms would help to understand the gene networks that are employed for cold tolerance. Though several cold-responsive genes have been identified, their functions are still unknown. Hence, studying *SsCor413* is relatively necessary to understand its role in different abiotic stress response mechanisms and its likeliness to be selected as a candidate gene for the improvement of sugarcane (Sun et al. 2018).

So far, *Cor413* gene has not been studied in *Saccharum* complex. Hence, the present study was undertaken to isolate and characterize the *S. spontaneum* homolog of the *Cor* gene. Our earlier studies on transcriptome analysis of *S. spontaneum* revealed the *Cor413* gene family has an important role in the regulation of cold stress (Dharshini et al. 2016, 2018). Utilizing the *Cor413* gene sequence information from the transcriptome data, a coding region of *SsCor413* was isolated from *S. spontaneum*. In silico analyses, such as domain prediction, conserved blocks, protein structure prediction, string analysis, and gene ontology annotation were performed. The temporal and spatial expression of *SsCor413* was studied during different abiotic stress, such as low-temperature stress, water deficit, and salinity stress, and the transient expression of *SsCor413* protein was studied to identify the sub-cellular localization in sugarcane and onion epidermal cells.

Materials and Methods

Plant Material and Stress Treatments

Saccharum spontaneum IND 00–1037 clone from the high altitude regions of Arunachal Pradesh, North eastern India was previously used for the experiment to develop low-temperature transcriptome profiling (Dharshini et al. 2016). The same cultivar was raised under glasshouse conditions at Indian Council of Agricultural Research—Sugarcane Breeding Institute (ICAR-SBI), Coimbatore, Tamil Nadu, India. Ninety days grown *S. spontaneum* IND 00-1037 seedlings were selected and shifted to aerated hydroponics setup supplied with Hoagland solution and maintained at 26 ± 2 °C in a glass chamber. Leaf samples were collected from plants exposed to 10 °C for 24 h. As described above, the experiment was repeated and root samples excised at different time intervals (3 h, 6 h, 12 h, 24 h, and 48 h). Leaf and root samples collected from non-treated served as control. All samples collected were immediately frozen in liquid nitrogen and stored at − 80 °C until RNA extraction.

S. spontaneum plants were raised and planted in 16-inch pots (containing soil, sand, and farmyard manure in 1:1:1 ratio) and maintained with regular irrigation inside a glasshouse at ICAR-SBI. Water deficit stress was imposed on the plants at the tillering phase (90 days after planting) by withholding irrigation for 7 days and was released on the 8th day, and normal irrigation was continued (Augustine et al. 2015). Leaf samples were excised from the plant on the 0th day, 1st day, 2nd day, 3rd day, 5th day, and 7th day of drought induction. The collected samples were ground in liquid nitrogen and stored at − 80 °C for further analysis.

Sixty days old plantlets were treated with 200 mM of sodium chloride (NaCl) for salt treatment. Leaf samples were harvested during 1 h, 3 h, 12 h, and 24 h of salt stress along with the control samples under normal irrigation. The collected samples were frozen in liquid nitrogen and stored at − 80 °C until further use.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from frozen tissues using the RNeasy plant media kit (Qiagen, MD) and DNA contamination was removed using RNase free DNase I (Thermo Scientific, USA). The quality and quantity of total RNA were

analyzed by agarose gel and NanoDrop Spectrophotometer (Thermo Scientific, USA), respectively. RNA integrity was checked using Agilent RNA Bioanalyzer chip (Agilent Technologies Inc., Santa Clara, CA). The RNA samples with 260–280 ratios of more than 2.0 and RIN (RNA integrity number) of more than 9.0 were selected and used for the further experiment. First-strand cDNAs were synthesized from 100 ng of total RNA using RevertAid First strand cDNA Synthesis Kit (Thermo Fisher Scientific Company, USA) following the manufacture's instruction.

Gene Isolation and Cloning

The gene-specific primers were designed for *SsCor413* gene using the sequence information available from a previous cold transcriptome study. *Cor413*FP (5'ATGGGGAAGGGGTT CGCGTCGTACT-3′) and *Cor413*RP (5′- CTACAGGA TTTGCAGCACCCCGGTC-3′) primers were used for PCR amplification. Using leaf cDNA (LT-treated sample) as a template, PCR amplification was carried out as follows: initial denaturation of 4 min at 94 °C, 35 cycles (94 °C for 45 s, 63 °C for 45 s, and 72 °C for 45 s) and final extension of 10 min at 72 °C in a thermocycler (Eppendorf, Hamburg, Germany). The PCR product was analyzed on 1.2% agarose gel and gel purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific USA). The eluted DNA fragment was ligated into pTZ57R/T vector using the InsTAclone PCR Cloning Kit (Thermo Fisher Scientific, USA) and the ligated product was transformed into *E. coli* DH5α cells. Recombinant colonies were selected in media containing ampicillin (Amp^R). Positive colonies were confirmed using M13 and *Cor413* gene-specific primers. The recombinant plasmid was isolated using a Plasmid Isolation Kit (Qiagen, MD) and was Sanger sequenced using the facilities available at the University of Delhi, South Campus, New Delhi, India.

Bioinformatics Analysis

Database searches to identify *SsCor413* homologs were performed using the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST) Web implementation of BLAST (Altschul et al. 1990) against the GenBank non-redundant sequence database. The ORF of the *SsCor413* gene was analyzed using the ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). Simple modular architecture research tool (SMART) (http://smart.embl-heidelberg.de/) was used for conserved domain prediction (Schultz et al. 1998; Letunic et al. 2014). The conserved domains were also predicted using batch web, CD-search tool (http://www.ncbi.nlm.nih.gov/Structure/bwrpsb) for ten monocot *Cor413* protein sequences. Expasy's Protparam Server (http://web.expasy.org/protparam/) (Gasteiger et al. 2005) was used for physicochemical characterization including theoretical molecular weight (MW), isoelectric point (pI), instability index (II) (Guruprasad et al. 1990), and aliphatic index (AI) (Ikai 1980). Secondary structure was predicted using a PSIPRED server (McGuffin et al. 2000) (http://bioinf.cs.ucl.ac.uk/psipred/) and HNN (Hierarchical Neural Network) (Guermeur et al. 1999) (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_hnn.html). SignalP was used for detection of signal peptides (Nielsen and Krogh 1998) (http://www.cbs.dtu.dk/services/SignalP/). ProtScale program (http://web.expasy.org/protscale/) was used to generate Kyte and Doolittle hydropathy plot with the Kyte and Doolittle option and a window of nine amino acids (Kyte and Doolottle 1982). ngLOC, an n-gram-based 209Q7 Bayesian classifier that predicts subcellular localization of proteins both in prokaryotes and eukaryotes used to predict sub cellular localization (King and Guda 2007) (http://genome.unmc.edu/ngLOC/index.html). Importin α-dependent nuclear localization signals were predicted using cNLS mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi). LocSigDB server predicts with protein sorting signals using experimental and literature database (http://genome.unmc.edu/LocSigDB/index.html). TMHMM server v2.0 (http://www.cbs.dtu.dk/services/TMHMM/) was used for transmembrane domains (TMD) prediction (Krogh et al. 2001).

STRING is a database of known and predicted protein networks derived from genomic context, co-expression, high-throughput experiments, and previous knowledge. STRING database (http://string-db.org/) with default parameters was used to search the protein network for protein query sequence and provide protein networks with known functional links as well as from predicted molecular models. The *SsCor413* sequence was used as a query and searched against *Zea mays* database and the network was analyzed with different categories such as "Network," "Experiments," "Text mining," "Databases," "Co-occurrence," "Co-expression", and "Neighborhood," and Gene ontology components such as Molecular function, Biological process, and Cellular component using AmiGo server (http://amigo.geneontology.org/amigo) with default parameters against *Arabidopsis thaliana.*

Chromosomal Mapping of *Cor* Gene

Chromosomal location of *Cor* gene was identified by mapping the *Cor* sequence to mosaic monoploidy sugarcane sequence (Garsmeur et al. 2018) using CLC workbench version 11 with default parameters.

Phylogenetic Construction

A total of ten *Cor413* sequences of different plants were retrieved from the NCBI database, and multiple sequence

alignment was performed using ClustalX (Thompson et al. 1997) using default settings. For building up of the evolutionary tree, Maximum Parsimony (MP) analysis was performed with MEGA7 (Kumar et al. 2016) using the tree-bisection-regrafting (TBR) algorithm (Nei and Kumar 2000) where the random addition of sequences (10 replicates) with search level 1 was employed for obtaining the initial tree. Average pathway methods (Nei and Kumar 2000) were used to calculate the branch lengths and represented as units of the number of changes over the whole sequence. The resulting percentages of replicate trees (1000 replicates) where the associated taxa clustered together were indicated next to the branches (Felsenstein 1985).

Development of GFP Constructs

The sub-cellular localization of *SsCor413* protein was studied using both N′-terminal and C′-terminal GFP fused constructs. The *ZmUbi-GFP* vector was used as template. Fusion *GFP-SsCor413 (ZmUbi-GFP– SsCor413)* was constructed by amplifying the *SsCor413* using primers GFPCOR-F (5'-GATCACTAGTATGGGGAAGGGGTTCGCGT-3') and GFPCOR-R (5'-TAGCGGCCGCCTCAGGACTCG-3') with restriction sites for *SpeI* and *NotI*, respectively, and cloned into *ZmUbi-GFP* vector digested with same restriction enzymes according to Xue (2002) and Shivalingamurthy et al. (2018). Also, to generate C-terminal *SsCor413-GFP* fusion (*CaMV35S-SsCor413-GFP*), *SsCor413* without a stop codon was amplified with primers CORGFP-F (5'-ATACCATG GATATGGGGAAGGGGTTCGCG-3') and CORGFP-R (5'-GCGCCTAGTCAGGACTCGCAGCACCGC-3') with restriction sites for *NcoI* and *SpeI*, respectively, and cloned into a pCAMBIA1302 vector (*CaMV35S-GFP*) digested with the same set of restriction enzymes.

Particle Bombardment and GFP Localization

Sugarcane calli and young onion epidermal cells were used for sub-cellular localization experiments. The young shoot tips from 4 to 6 months old sugarcane variety Co 86032 was used as a starting material for raising the embryogenic calli. The surface sterilized sugarcane meristematic explants were selected, and young leaves surrounding the sub-apical meristematic portion were cut into pieces, placed on the MS + 2,4-D medium, and incubated in the dark at 25 °C. The proliferated calli were sub-cultured every 15 days in fresh MS + 2,4-D medium for the production of embryogenic calli for particle bombardment.

Sugarcane embryogenic calli and onion epidermal tissue were placed in concentric circles on MS + osmotic medium (MS + 50 g/L mannitol and 50 g/L sorbitol) for 3 h prior to bombardment. A mixture of 1.5–3 μm-sized gold particles were sterilized and used as a microcarrier for particle bombardment. The gold suspension was added to the *SsCor413*-GFP localization plasmids (6 μg) in a 1.5 ml siliconized microcentrifuge tube. 20 μl of 0.1 M spermidine was added with constant vortexing followed by 50 μl of 2.5 M calcium chloride solution was added drop by drop to the mixture with constant vortexing. The cocktail was set aside for 10 min and centrifuged at 10,000 RPM for 10 s. The pellet containing DNA-coated gold particles was washed with 200 μl of absolute alcohol, and the supernatant was decanted after centrifugation. Finally, the pellet was resuspended with 60 μl of absolute alcohol. A 10 μl of DNA-coated gold suspension was coated onto the sterile macrocarrier for particle bombardment. Bio-Rad PDS 1000/He Biolistic System at a pressure of 1100 Psi of helium was used for bombardment. The explants were bombarded at a distance of 4 and 8 cm from stopping screen. The bombarded explants were incubated in the dark at 25 °C for 24 h. The slides were prepared by staining the bombarded calli with 0.1% propidium iodide (nuclear stain) for 1 h in the dark (Palaniswamy et al. 2016) and examined in both RFP (530 nm -593 nm) and GFP (470 nm – 525 nm) channels of an EVOS FL color fluorescence microscope (Life Technologies, USA).

Quantitative Real-Time PCR (qRT-PCR) Analysis

For qRT-PCR experiments, *SsCor413* primers (Forward Primer-5'-AGCTTCCTGGTTCCATCATC-3′, Reverse Primer-5'-CATCCAATCGCAAGGCATATC-3′) were designed using a FastPCR tool (Kalendar et al. 2017). Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene (Forward Primer-5'-AAGGGTGGTGCCAAGAAGG-3′, Reverse Primer-5'-CAAGGGGAGCAAGGCAGTT-3′) was used as an endogenous control. To perform qRT-PCR experiments, the cDNA converted using total RNA samples isolated from samples of low temperature (leaf and root), water deficit (leaf), salinity (leaf), and control plants were used as a template. qRT-PCR was performed on a StepOnePlus Real-Time PCR system (Applied Biosystems, Canada) using the SYBR-green dye method. Each reaction was carried out in triplicates. In brief, each qRT-PCR reaction consists of 50 ng cDNA, 2.5 pmol primers, 12.5 μl of 2X MESAGREEN Master Mix (Eurogentec Belgium) and the final volume was made up to 25 μl with sterile water (Dharshini et al. 2018). qRT-PCR reaction conditions used were as follows: denaturation for 10 min at 95 °C followed by annealing and extension at 1 min for 60 °C (40 cycles). The fold change of the target genes was determined using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Results

Isolation and Sequence Analysis of *SsCor413* Gene

A low-temperature responsive uncharacterized gene *SsCor413* was identified from our previous low-temperature

stress transcriptome profiling of *Saccharum spontaneum* (Dharshini et al. 2016). A full-length coding region (CDS) of *Cor413* was isolated from *S. spontaneuem* collected from a low-temperature region. Sequence analysis of *SsCor413* revealed that the length of the ORF is 642 nucleotides which encode a putative protein of 213 amino acids (Fig. 1). The molecular weight of the protein was 25.6 kDa and an isoelectric point (Pi) of 9.69. The instability index (II) is computed to be 23.89 for protein and hence it classifies as stable protein. The basic local alignment search tool for nucleotide (BLASTN) and protein (BLASTP) sequence analysis showed that *SsCor413* has 95% and 96% similarity to the *ZmCor413* (EU965484.1), respectively.

The secondary structure analysis of putative *SsCor413* showed the dominance of α helices (69.04–76.99%) followed by random coils (21.59–31.60%) and strands (0–5.16%). The prediction of secondary structure using different server is given in supplementary Table S1, and Fig. S1 represents the secondary prediction. The *SsCor413* protein was predicted with one *WCOR413* domain and its position ranged from 19 to 200 amino acids (supplementary Fig. S2). Further to confirm, ten monocot *Cor413* proteins were subjected to Batch CD server, and results indicated that all queries assigned to *WCOR413* domain and Pfam 05562 as accession number.

The Kyte and Doolittle hydrophobicity plot of *SsCor413* protein showed a pattern of high hydrophobicity with the Grand Average of Hydropathy (GRAVY) value of 0.808 and 35 hydrophobic leu residues (Fig. 2a). TMHMM analysis based on (Krogh et al. 2001) Hidden Markov Model predicted that *SsCor413* protein possesses five transmembrane helices at positions "53–75," "88–105," "131–150," "157–175," and "190–212" which is complemented with high hydrophobic nature of *SsCor413* protein (Fig. 2b).

Conserved region analysis revealed the comparison of *Cor413* proteins of the *Poaceae* family showed ten conserved blocks at amino acid position "40'AARKLANHA," "48' VLGGGLGF," "67' AAVYLL," "81'NWKTNMLT," "90', LLVPYIFFTLP," "122'LRLFFPRHFPDWLELPGS," "146'VAP," "170'LGCYLL," "177'EHI," and "207'YPVW" suggesting the highly conserved nature of *Cor413* among grass family (Fig. 3). *SsCor413* protein showed the presence of serine at positions "7, 59, 103, 147, 178, 192, and 194", threonine at "13, 58, 82, 86, 96, 142, and 152," and tyrosine residues at "8, 71, 92, 169, and 203" revealed the possible regulatory mechanism of protein function through post-translation modification. ngLOC protein subcellular localization prediction server reveals that putative *SsCor413* protein is likely to be localized in the nucleus. But low prediction score (19.8) in this analysis has given low chance of localization in the nuclear lumen. Hence, *SsCor413* protein sequence was further analyzed for NLS using cNLS Mapper which has predicted presence of importin α-dependent few bipartite NLS sequences in *SsCor413* amino acid sequence position ranging from 34 to 67 with a minimum score of 2.9 and 60–88 with a score of 2.4. Importin alpha is known to bind to NLS sequence of nucleus-targeted protein which recognize and transports the NLS-containing protein across the nuclear membrane. SignalP server does not predict with any signals and cleavage sites; LocSigDB server displays

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1   ATGGGGAAGGGGTTCGCGTCGTACCTGGCGATGAAGACGGGGCCGGAGGGCGGCGGCGCTGCGGCGGCGGCGCAGCAGGCGCTGATCGAC
    M  G  K  G  F  A  S  Y  L  A  M  K  T  G  P  E  G  G  G  A  A  A  A  Q  Q  A  L  I  D  30

91  GCGGACCTGCGGGAGCTCGGCGTCGCGGCACGGAAGCTCGCCAACCACGCCTTCGTCCTCGGCGGCGGGCTGGGGTTCGGCACTTCGTTC
    A  D  L  R  E  L  G  V  A  A  R  K  L  A  N  H  A  F  V  L  G  G  G  L  G  F  G  T  S  F  60

181 CTCAAGTGGCTGGCCTTCCTCGCCGCAGTGTATCTCTTGATATTGGACCGCGCGAATTGGAAGACCAACATGCTGACAGCTCTCTTGGTT
    L  K  W  L  A  F  L  A  A  V  Y  L  L  I  L  D  R  A  N  W  K  T  N  M  L  T  A  L  L  V  90

271 CCTTACATTTTCTTCACTCTGCCTAATGTGCTGTTTTCTCTGATCAGAGGCGAGGTGGGGAAATGGATTGCGATTATTGCTGTTATTCTG
    P  Y  I  F  F  T  L  P  N  V  L  F  S  L  I  R  G  E  V  G  K  W  I  A  I  I  A  V  I  L  120

361 CGTCTATTCTTTCCACGCCACTTCCCAGATTGGTTAGAGCTTCCTGGTTCCATCATCCTGCTCACAGTGGTTGCTCCCAGCCTGTTCGCA
    R  L  F  F  P  R  H  F  P  D  W  L  E  L  P  G  S  I  I  L  L  T  V  V  A  P  S  L  F  A  150

451 GACACCTTCAGGGGTGACCTCGTTGGTGTCTTGATATGCCTTGCGATTGGATGCTACCTGCTCCAAGAGCACATCAAGGCGTCAGGTGGA
    D  T  F  R  G  D  L  V  G  V  L  I  C  L  A  I  G  C  Y  L  L  Q  E  H  I  K  A  S  G  G  180

541 TTCAGGAACGCCTTCAGGAAGGGCAATGGCGTGTCGAACTCCATTGGCATCCTCCTGCTCTTCGTCTACCCTGTCTGGGCCGCGGTGCTG
    F  R  N  A  F  R  K  G  N  G  V  S  N  S  I  G  I  L  L  L  F  V  Y  P  V  W  A  A  V  L  210

631 CGAGTCCTGTAG
    R  V  L  *                                                                              213
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Fig. 1 The full-length cDNA sequence and deduced amino acid sequence of *SsCor413*. The primers used for amplification were underlined and * represents stop codon. The deduced amino acid sequence was shown beneath the nucleotide sequence and the amino acids were numbered on the right-hand side of the sequence. The cDNA sequence has been deposited in GenBank under accession No. MF680545

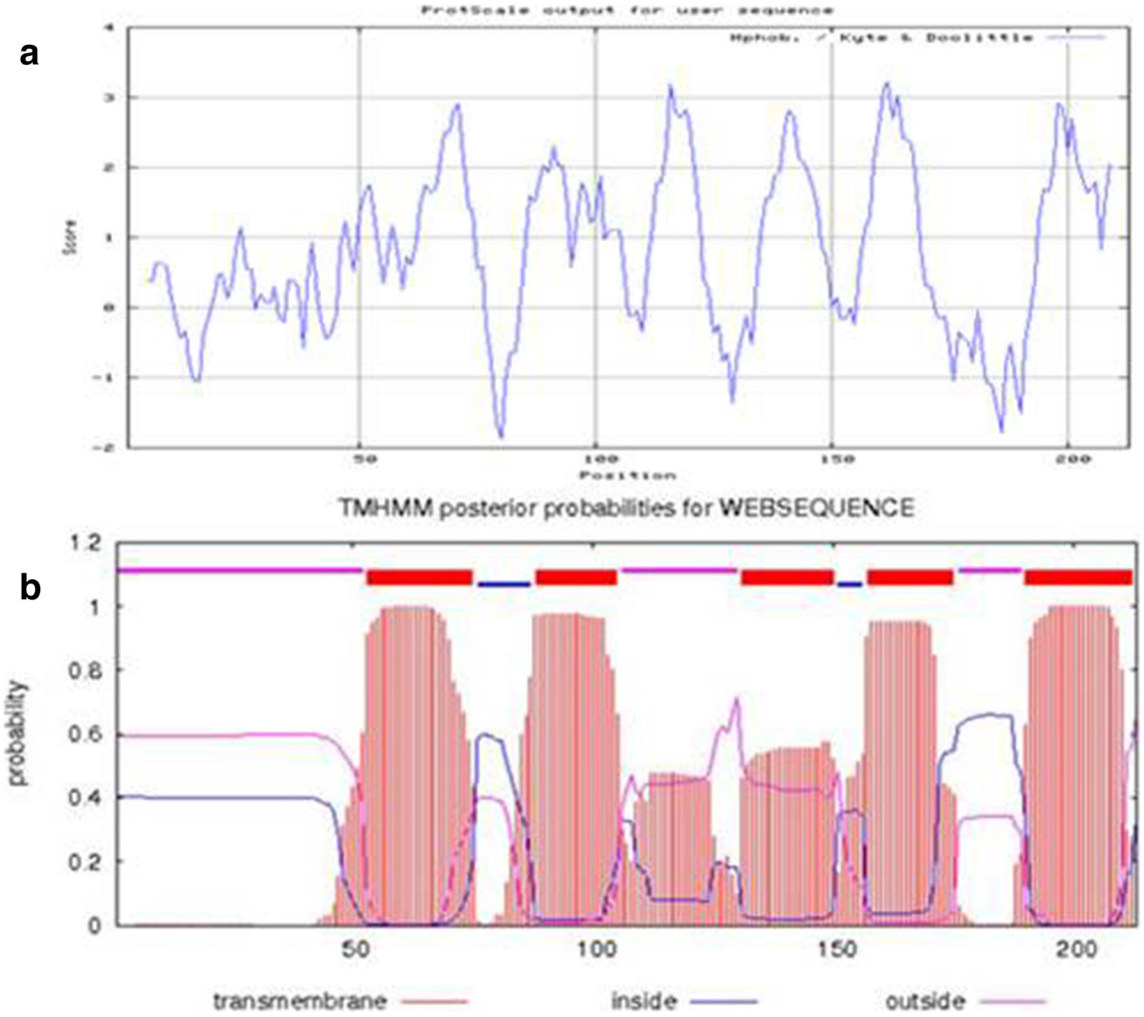

Fig. 2 Hydropathy plot (**a**) and prediction of transmembrane domain (**b**) of *SsCor413*

protein sorting signals of *SsCor413* to endoplasmic reticulum at the position 173–176 and lysosomes at positions 7–11, 70–74, 91–95, 202–206.

STRING Network and Gene Ontology of *Cor413*

Clustered analysis using STRING database indicated that *SsCor413* protein grouped with proteins like *Cor413*-like, Rab28, late embryogenic abundant proteins (LEA)-Lea4 and Lea14-A, oleosin18, and xyloglucan endotransglucosylase, which are reported to be involved in low-temperature tolerance (Fig. 4). *SsCor413* protein annotated with gene ontology terms such as integral components of membrane (AT2G15970), plasma membrane (AT2G15970), cold acclimation (AT1G29395), cellular response to cold (AT1G29395), cellular response to water deprivation (AT2G15970), and response to abscisic acid (AT1G29395). These results suggest that *SsCor413* protein might respond to cold, drought, and abscisic acid stress. A pictorial representation of *SsCor413* protein GO terms are given in supplementary Fig. S3.

Chromosomal Location of *Cor* Gene

Chromosomal location of *Cor* gene and its isoforms were identified in this study. The location of *Cor* gene includes in chromosome 1 at position "7829564–7830587,"; chromosome 3 at positions "30565359–30566967," "30585588–30586911," "30594461–30594810," and "50326281–50326556,"; and chromosome 9 at position "33099810–33103744." These results indicate that *Cor* gene located at four positions in chromosome 3 and located at a single position in chromosome 1 and chromosome 9.

Development of *SsCor413* Phylogeny

Amino acid sequences of a *Cor413* family of monocot species were retrieved from the NCBI database. The phylogenetic analysis of *SsCor413* proteins showed its close evolutionary relationship with *Sorghum bicolor* and *Zea mays* with high similarity (Fig. 5). The ten *Cor413* protein sequences from

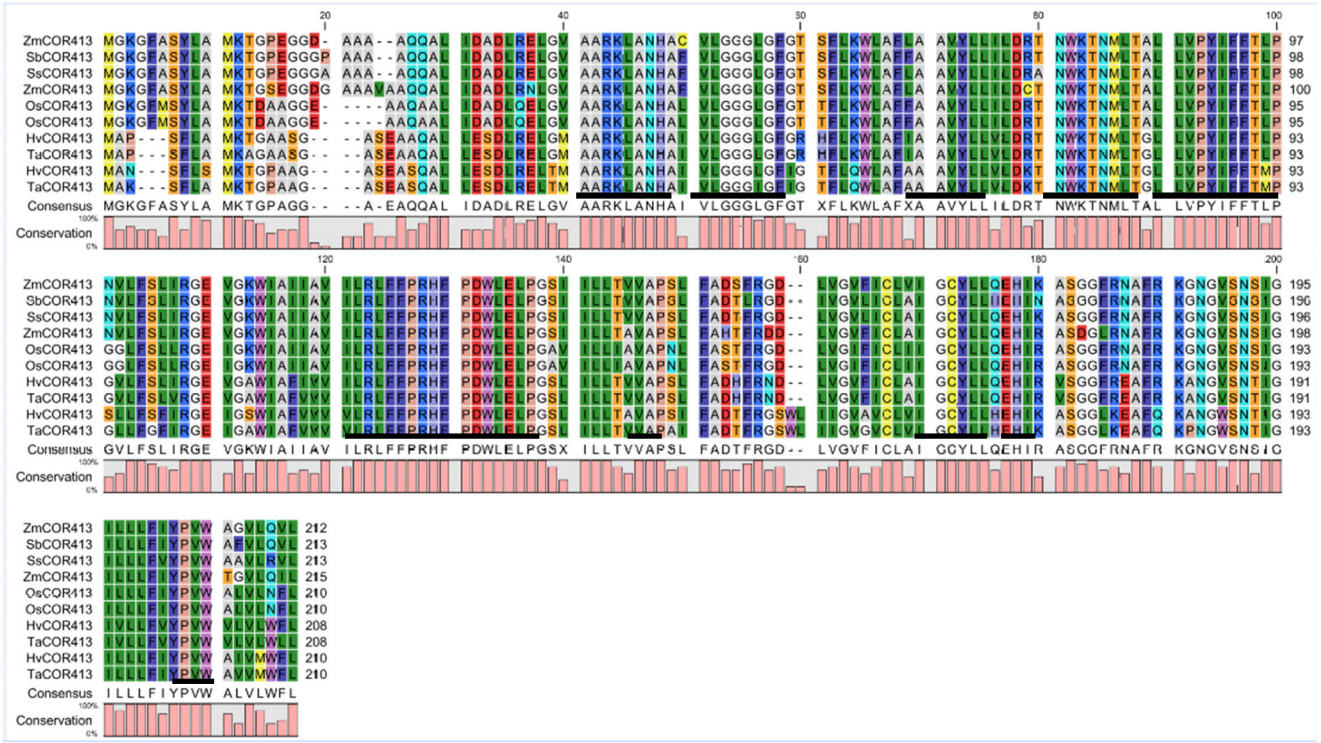

Fig. 3 Protein sequence homology of *SsCor413* with *Cor413* from *Saccharum* and other plant species. Ss, *Saccharum spontaneum*; *Zm, Zea mays*; Sb, *Sorghum bicolor*; Os, *Oryza sativa*; Hv, *Hordeum vulgare*; Ta, *Triticum aestivum*. The conserved blocks are underlined

monocots showed a good similarity level. Also, 154 protein sequences which contain the *WCOR413* domain from different species were retrieved and phylogenetic tree analysis also revealed that the *WCOR413* domain was highly conserved among monocots.

Sub-Cellular Localization of *SsCor413*

Localization of *SsCor413* using C-terminal GFP (*SsCor413-GFP*) and N-terminal GFP (*GFP-SsCor413*) was studied using sugarcane calli and onion epidermal cells. The diagrammatic representation of *GFP-COR* constructs is given in Fig. 6. Control and GFP fused plasmids were bombarded on the sugarcane calli and onion epidermal cells. The microscopic slides were prepared using bombarded explants stained with propidium iodine (Nuclear stain) and observed under a fluorescent microscope. The results revealed that control GFP constructs exhibit fluorescence strongly throughout the cell in sugarcane calli and onion cells. The fused plasmids such as *GFP-SsCor413* and *SsCor413-GFP* showed strong GFP expression in the nucleus compared with plasma membrane of both sugarcane calli (Fig. 7) and onion epidermal cells (Fig. 8).

Expression Pattern of *SsCor413* upon LT, Water Deficit, and Salinity Stresses

To understand the regulation of the *SsCor413* protein under abiotic stresses such as low temperature, drought, and salinity in sugarcane, the gene expression pattern was studied through quantitative real-time PCR at different points of time in leaf and root samples. The qRT-PCR results indicated that *SsCor413* transcripts are strongly up-regulated in leaf and root tissues at 24 h upon LT stress. A gradual up-regulation of *SsCor413* was observed in root tissue at 3 h (0.70 fold), 6 h (3.5 fold), 12 h (4.21 fold), and reached a maximum at 24 h (7.26 fold) and then declined at 48 h (0.32 fold) under LT stress. Similarly, *SsCor413* transcripts in leaf tissue showed up-regulation at 3 h (1.02 fold), 6 h (3.78 fold), 12 h (3.98 fold), and reached a maximum (5.3 fold) at 24 h and declined at 48 h (4.0 fold) under LT stress (Fig. 9). During water deficit conditions, *SsCor413* expression was up-regulated by 0.95-, 1.20-, 3.22-, 3.41-, 3.22-, and 1.47-fold at 1st, 2nd, 3rd, 4th, 5th, and 7th days, respectively (Fig. 10a). Upon 200 mM salinity treatment, *SsCor413* showed 3.94-, 2.77-, 1.84-, and 1.48-fold change at 1 h, 3 h, 12 h and 24 h, respectively (Fig. 10b).

Discussion

In this study, a full-length *SsCor413* gene, which encodes a polypeptide of 213 amino acids, was cloned from *S. spontaneum*, a low-temperature tolerant clone (Dharshini et al. 2016). The amino acid sequence of *SsCor413* showed 96% homology to *ZmCor413* (EU965484.1). The molecular

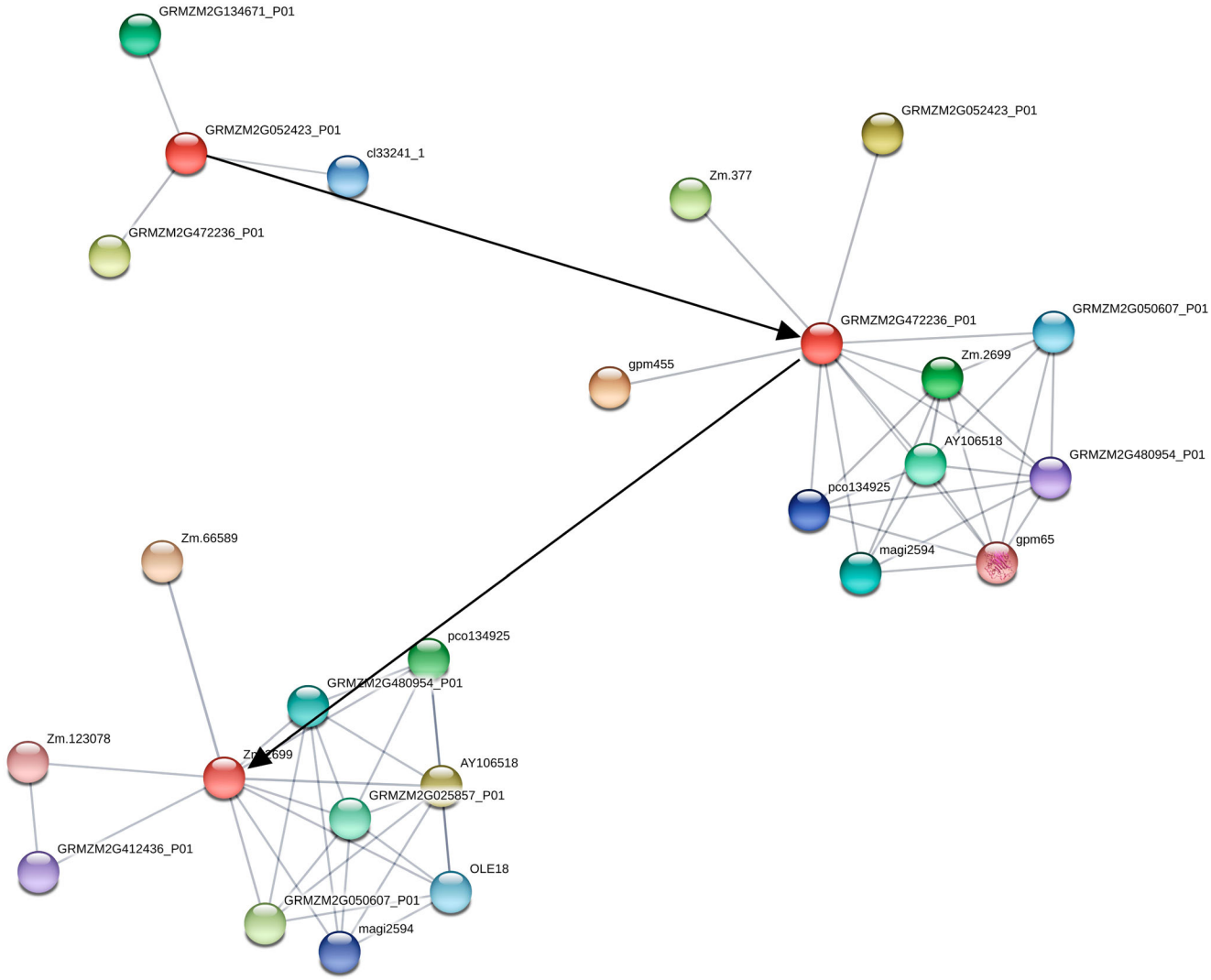

Fig. 4 Interactome analysis of *Cor413* protein

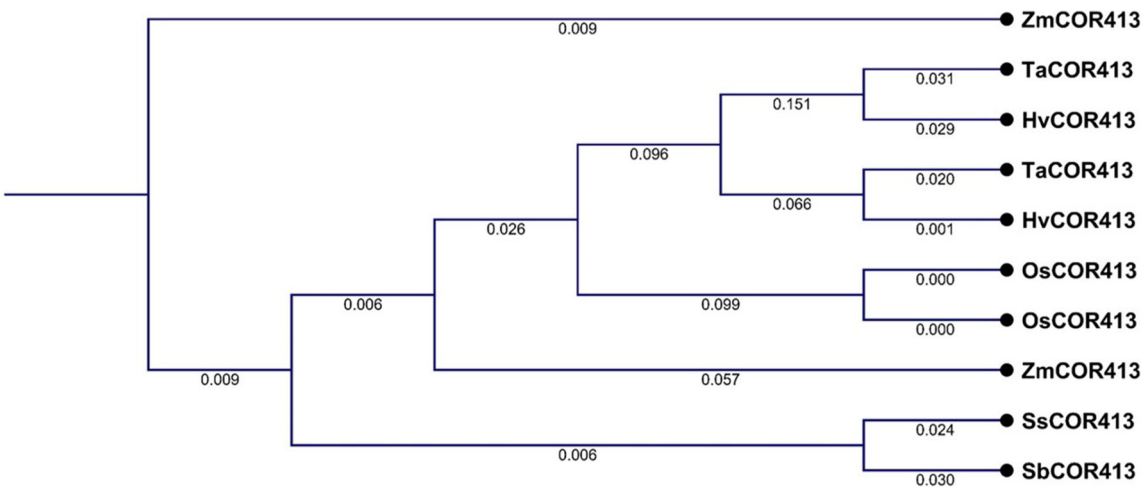

Fig. 5 Phylogentic relationships of *SsCor413* protein among monocots

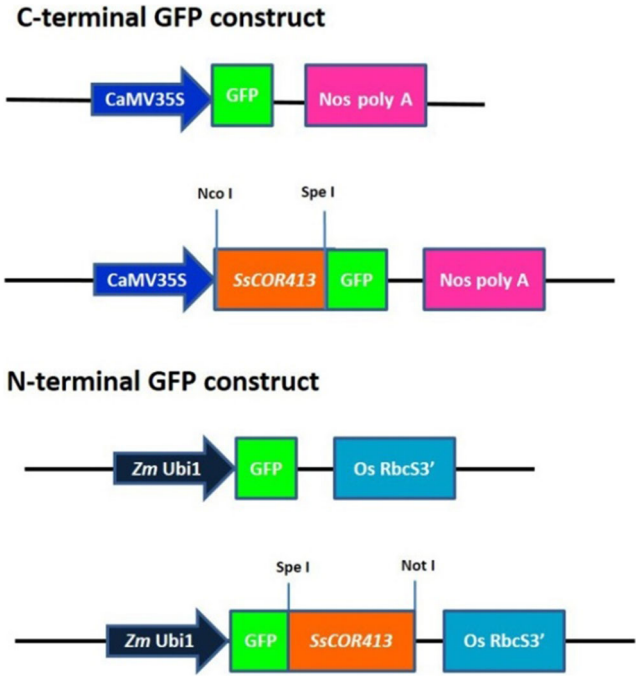

Fig. 6 Diagrammatic representation of *CorGFP* constructs

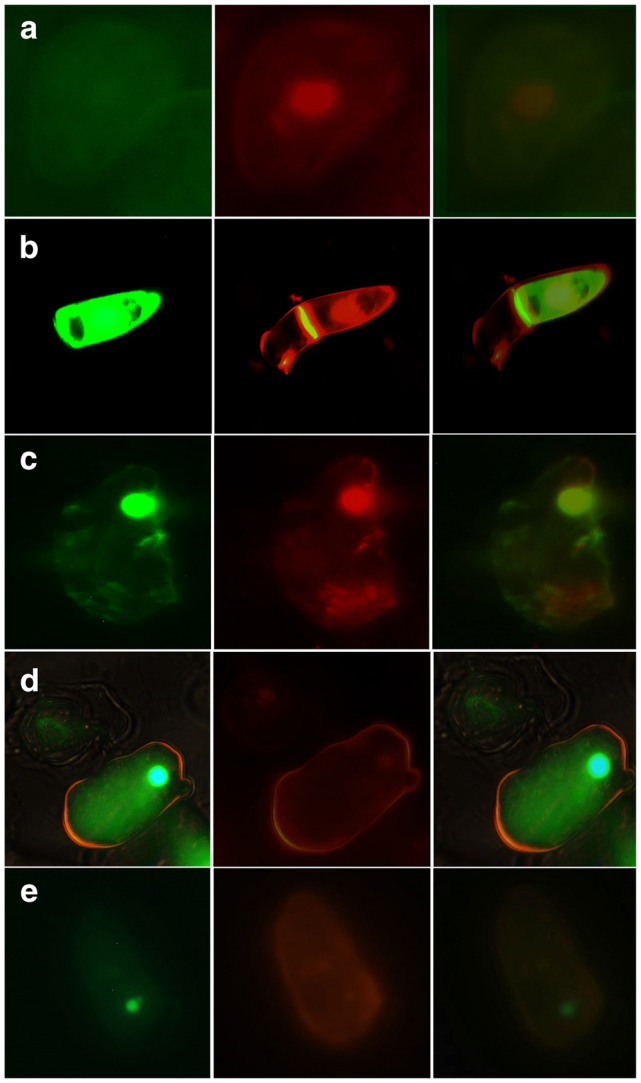

Fig. 7 Subcellular localization of *SsCor413-GFP* in sugarcane. **a** Fluorescent microscopic images of untransformed sugarcane calli. **b** N-terminal control plasmid shows GFP expression throughout the callus. **c** Fluorescent microscopic images of sugarcane calli showing the transient expression of *SsCor413-GFP* in nucleus and plasma membrane. **d** C-terminal control plasmid shows GFP expression throughout the callus. **e** Fluorescent microscopic images of sugarcane calli showing the transient expression of *GFP-SsCor413* in nucleus. Middle vertical lane from A to E represents nuclear staining with the propidium iodide

weight of the *SsCor413* is 25.6 kDa, which almost equal to *ZmCor413* (25.4 kDa) (Breton et al. 2003). *Cor413* protein with molecular weight of 22.74 kDa was also cloned from *Gossypium barbadense* (Wang et al. 2007). Isoelectric point (pI) value indicates the pH at which protein is stable and has no net charge. The computed pI value of *SsCor413* is 9.69. Hence, it is classified that the protein is alkali in nature. The *Cor413* protein is seen to be stable with instability index (II) of 23.89. Instability index lower than 40 is considered to be stable proteins (Guruprasad et al. 1990). The amino acids of *SsCor413* protein are hydrophobic, suggesting that this protein could be a membrane protein (Wang et al. 2007). The amino acid residue ratio is one of the essential criteria that decide the role and the activity of its protein. Multiple sequence alignment was performed to find out the conserved amino acids residue, and this revealed that the leucine (leu) residue is abundant among all cold-regulated proteins. For example, *GbCor413* contains 25 leu residues and aids stabilization of the transmembrane structure during cold stress (Wang et al. 2007). In this study, *SsCor413* contains 35 leu residues and perhaps plays a vital role in stabilization. The percentage of leu in *SsCor413* protein is very close to that of *ZmCor413* (Breton et al. 2003) and *GbCor413* (Wang et al. 2007) characterized from *Z. mays* and *G. barbadense*, respectively. The residues such as serine (ser), tyrosine (tyr), and threonine (thr) are associated with phosphorylation sites of a protein. The *Cor413* protein reported to contain phosphorylation sites (Wang et al. 2007). The *SsCor413* protein contains 8 ser, 7 thr, and 5 tyr residues. *SsCor413* contains eight pro residues. Other amino acids such as pro and cys were also conserved among different *Cor413* proteins (Breton et al. 2003), and *Cor413* from *Arabidopsis* and cereals contain five pro residues and is conserved among other *Poaceae* families. These residues are involved in the possible regulatory mechanism of protein function through post-translational modification. *SsCor413* protein possesses five transmembrane helices as that of cold-regulated *Cor413* proteins of cereals and *Arabidopsis* family which are identified as a novel stress-regulated multi-spanning transmembrane protein family (Breton et al. 2003), whereas only four transmembrane helices

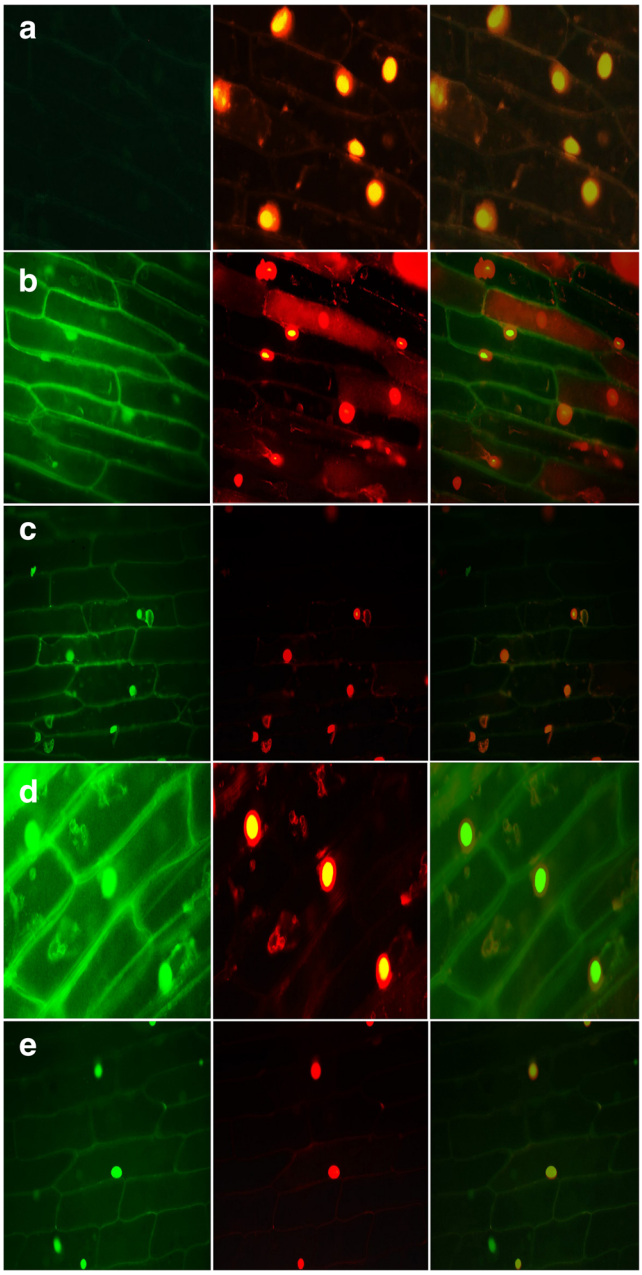

Fig. 8 Subcellular localization study in onion epidermal cells. **a** Fluorescent microscopic images of untransformed onion epidermal cells. **b** N-terminal control plasmid shows *GFP* expression throughout the onion epidermal cells. **c** Fluorescent microscopic images of onion epidermal cells showing the transient expression of *SsCor413-GFP* in nucleus and plasma membrane. **d** C-terminal Control plasmid shows GFP expression throughout the onion epidermal cells. **e** Fluorescent microscopic images of epidermal cells showing the transient expression of *GFP-SsCor413* in nucleus and plasma membrane. Middle vertical lane from A to E represents nuclear staining with the propidium iodide

were found in *GbCor413* protein (Wang et al. 2007). SignalP result showed the absence of signal peptide in *SsCor413* sequence as reported in Wang et al. (2007). But interestingly, LocSigDB server predicted with protein sorting signals to ER and lysosome. Based on earlier reports and present study, *Cor413* protein exhibits different number of transmembranes, different protein sorting signals suggesting us that *Cor413* protein could be diverse.

Domains are distinct functional and structural units of a protein containing conserved sequence patterns. They are recognized as building blocks and may rearrange to modulate its protein function. *SsCor413* protein was detected with one *WCOR413* domain. Domain-based evolutionary studies indicated that *WCOR413* domain found in many species such as *Lolium temulentum*, *Brassica rapa*, *Cucumis sativus*, and *Gossypium barbadense* was grouped within its family and plays a significant role in freezing tolerance mechanism. Early studies reported that there were two conserved blocks in *ZmCor413* (Wang et al. 2007). In this study, we were able to identify a few more conserved blocks in *Cor413* sequence among monocot families. The phylogenetic analysis revealed that *SsCor413* was highly conserved among *Poaceae* family. The gene ontology (GO) for *SsCor413* protein was annotated with cold responsive and cold acclimation terms indicating the *SsCor413* might play a significant role upon cold stress. The STRING network analysis revealed that *SsCor413* protein is connected to LEA, Rab28, and oleosin18 which was reported to enhance cold tolerance in plants. Rab, an LEA protein and Oleosin help in freezing tolerance in *A. thaliana* (Puhakainen et al. 2004; Shimada et al. 2008). This level of mining suggests that *SsCor413* protein is involved in low-temperature tolerance mechanism, as it connects to LEA proteins. The secondary structure of *SsCor413* predicted with alpha-helix structures.

Cellular localization of *SsCor413* protein using C′-terminal GFP (*SsCor413*-GFP) and N′-terminal GFP (GFP-*SsCor413*) was performed in sugarcane callus and onion epidermal cells. The result indicated that *SsCor413* is likely to be localized in nucleus. This localization result correlated with in silico ngLOC prediction and further supported by cNLS Mapper prediction. Prediction of the importin α-dependent nuclear localization signals (NLS) in *SsCor413* protein sequence indicated its localization in nucleus. In general, the NLSs are short stretches of amino acids recognized by nucleo-cytoplasmic transporters (karyopherins) that promote active transport of proteins into the nucleus (Xu et al. 2010). Also, our localization study showed low abundance of *SsCor413-GFP* signals in plasma membrane which might be because of presence of sorting signals to ER and lysosomes. Usually, after ER synthesis, lysosomal transmembrane proteins are transported as glycosylated proteins to the trans Golgi Network (TGN) where they follow the secretory route to the plasma membrane. Thus, the result of localization of *SsCor413* was differed from other reports of *Cor413* protein which were reported to be localized in thylakoid

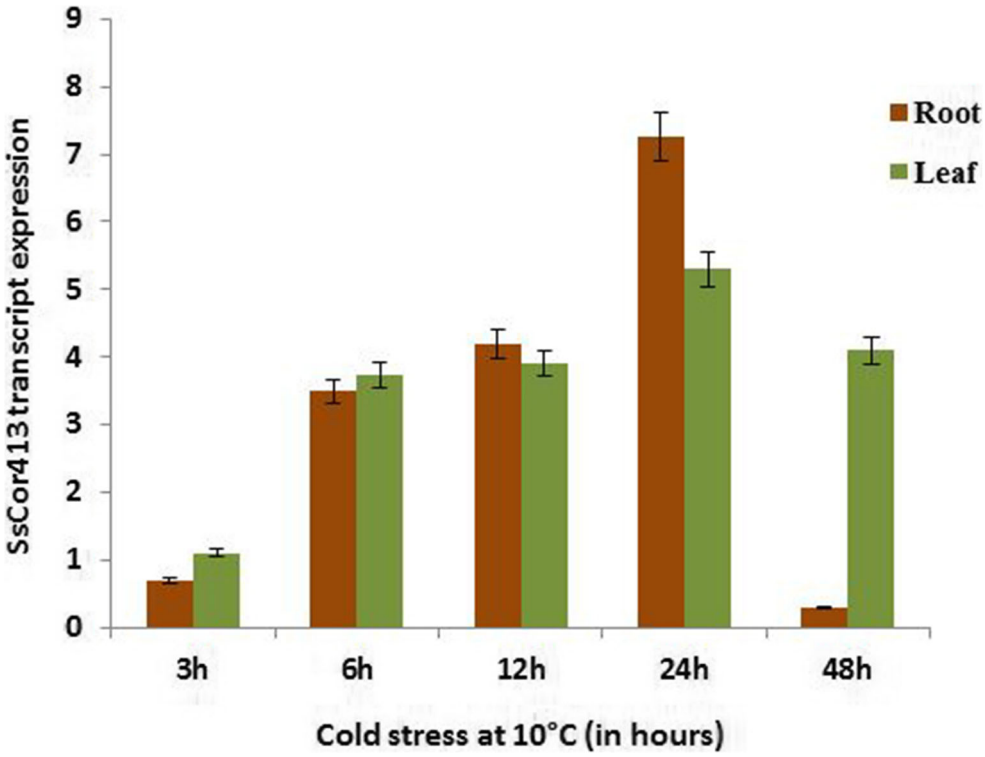

Fig. 9 qRT-PCR analysis of *SsCor413* transcript upon low temperature condition. Error bars show SD of the values from three replicates

membrane and plasma membrane. Zhou et al. (2018) reported that GFP construct containing transmembrane regions of *Cor413* protein (*PsCor413*-TM1-TM4-GFP) was localized to the nucleus and the cytoplasm.

SsCor413 transcript up-regulated under low-temperature stress and showed a maximum accumulation in leaf and root tissues at 24 h of stress. *TaCor413-pm1* is expressed abundantly in the leaves and root under freezing conditions (Breton et al. 2003). During water deficit stress conditions, *SsCor413* expression showed a gradual increase until the 4th day of stress and declines at 5th and 7th day. Zhao et al. (2016) reported that *Cor413* was up-regulated during water deficit and low-temperature stress conditions and thus playing a double role and widespread cross talk between the cold and water deficit stress response pathway in sheepgrass. *SsCor413* showed an early response to salt stress in sugarcane. Other stresses as well triggered *Cor* genes such as *Cor15A*, *Cor6.6* in response to cold treatment, ABA, and water deficit stresses (Thomashow 1998). All cereals contain homologous low-temperature responsive genes in their genome, but the gene expression is found only in cold-tolerant cereal variety under low-temperature stress conditions (Sarhan and Danyluk 1998). Accumulation of *Cor* proteins has a close relationship between freezing tolerance and this protein can be used as a molecular marker to select for freezing tolerance (Houde et al. 1992). In line with previous reports, *SsCor413* can be used as molecular markers and would serve as a potential candidate gene for developing abiotic stress tolerance variety in sugarcane.

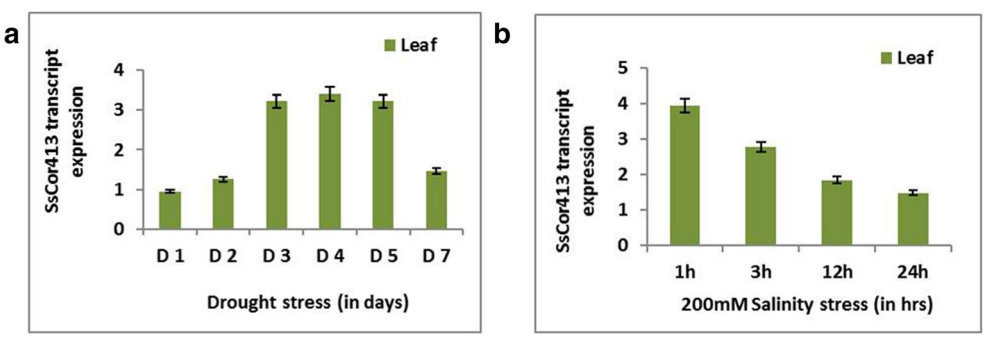

Fig. 10 qRT-PCR analysis of *SsCor413* transcript upon water deficit (**a**) and salinity (**b**) conditions. Error bars show SD of the values from three replicates

Conclusion

SsCor413 gene was isolated from *S. spontaneum* (low-temperature tolerant) and for the first time to gain insight into features of protein sequence, localization, and expression analysis of different stress conditions such as low temperature, water deficit, and salinity stresses were studied. Results of physicochemical characterization showed that *SsCor413* protein was stable and hydrophobic in nature. The evolutionary and conserved region analysis suggested ten conserved blocks which showed high conservation among monocots. *SsCor413* contains five potential transmembrane domains which are stabilized by the presence of 35 leu amino acids upon adverse conditions. *SsCor413* was significantly up-regulated and might actively participate in the cold acclimation process. Also, *SsCor413* was up-regulated upon water deficit and salinity stress conditions, suggesting that this protein responds to multiple stresses. Localization study revealed *SsCor413* protein presumably to be localized in the nucleus and plasma membrane. This is the first report which studied the transcriptional regulation of *SsCor413* under low temperature, salinity, and water deficit conditions in *S. spontaneum*. *SsCor413* has shown potential candidate for developing multiple abiotic stress-tolerant sugarcane varieties either through marker-assisted breeding or transgenic approaches.

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Author's Contributions DS and AC designed the experiments. DS performed the experiments and wrote the manuscript. SGS provided localization control vectors, assisted in microscopic studies, and manuscript revision. MVM and ANJ supported in cloning. SPTS did the artwork for figs. RK and MRM helped in bioinformatics analysis. MM, BR, and AC revised the manuscript. All authors read and approved the final manuscript.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Abbreviations *Cor*, Cold regulated; *ORF*, Open reading frame; *GFP*, Green Fluorescent Protein; *LEA*, Late Embryogenic Abundant Protein; *GO*, Gene Ontology; *LT*, Low temperature; *MS*, Murashige and Skoog

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