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A nuclear-localized protein, *KOLD SENSITIV-1*, affects the expression of cold-responsive genes during prolonged chilling in Arabidopsis

Sarah J. Purdy[a,b], John D. Bussell[a], David C. Nelson[a,b], Dorthe Villadsen[c], Steven M. Smith[a,b,*]

[a] *Australian Research Council Centre of Excellence in Plant Energy Biology, The University of Western Australia, Stirling Highway, Crawley, Perth, Western Australia, 6009, Australia*
[b] *Centre of Excellence for Plant Metabolomics, The University of Western Australia, Stirling Highway, Crawley, Perth, Western Australia, 6009, Australia*
[c] *Institute of Molecular Plant Sciences, University of Edinburgh, Edinburgh, UK*

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ABSTRACT

Plants respond to cold by transcriptional and metabolic responses which underlie tolerance and acclimation mechanisms, but details at the molecular level are incomplete. Here we describe *KOLD SENSITIV-1* (*KOS1*), a new gene required for responses to cold. KOS1 protein is predicted to have coiled-coil, Structural Maintenance of Chromosomes and nuclear-targeting domains. GFP-labeled KOS1 localizes to the nucleus. Null mutants could not be isolated but two independent knockdown T-DNA mutants were obtained. Growth and development of *kos1* knockdown mutant plants was comparable to wild type when grown at 21 °C. However, when grown at 4 °C these mutants exhibited accelerated leaf yellowing and smaller rosette size than wild type. Quantitative RT-PCR revealed that in the cold *kos1* mutants had reduced expression of cold-responsive transcripts *COR15A*, *COR15B*, *BAM3* and *AMY3*. Metabolite profiling revealed that ascorbate levels were lower in the mutants in the cold relative to wild type. *KOS1* therefore represents a new gene that influences the regulation of transcript and metabolite levels in response to prolonged chilling temperatures.

Introduction

In order to survive and acclimatise to chilling temperatures plants must coordinate the timing of transcriptional and metabolic responses that ensure cellular protection and adaptation. Some responses to cold occur within minutes and return to uninduced levels after only a short period of time even if the cold temperatures remain (Fowler and Thomashow, 2002). Other responses remain in place throughout the cold period but revert back after a return to higher (optimal) temperatures. Another category of cold-responsive changes, such as histone modifications during vernalization remain in place indefinitely, through mitosis (Gendall et al., 2001; Sung and Amasino, 2004). The phenomenon by which plants adapt to survive freezing conditions by exposure to chilling is called acclimation (Chinnusamy et al., 2003).

The best characterized cold-responsive transcriptional pathway in *Arabidopsis thaliana* is the C-repeat binding factor/dehydration responsive element binding (CBF/DREB1) pathway (Yamaguchi-shinozaki and Shinozaki, 1994; Thomashow, 2001; Fowler et al., 2005). Comprising three principle transcription activating proteins, CBF1, CBF2, and CBF3 (or DREB1b, 1c, 1a, respectively), these cold-inducible transcription factors bind to the C-repeat/dehydration responsive element (CRE/DREB) found in the promoters of many cold-responsive genes (Baker et al., 1994; Yamaguchi-shinozaki and Shinozaki, 1994; Fowler and Thomashow, 2002). Upon exposure to low temperatures the *CBF*/*DREB1* transcripts are rapidly induced, followed by expression of their downstream targets (Stockinger et al., 1997; Gilmour et al., 1998; Fowler et al., 2005). Examples of downstream targets of the CBF/DREB1 family include *COR15A* and *COR15B*. The mature form of COR15A, COR15Am acts as a cryoprotectant (Lin and Thomashow, 1992) within the chloroplast stroma ensuring enzyme function at low temperature (Nakayama et al., 2007).

Specific metabolite changes have also been observed in plants during cold acclimation. Upon exposure to cold stress, starch is broken down to provide an immediate source of soluble sugars (Kaplan and Guy, 2004, 2005). Maltose and maltotriose are the first carbohydrates to accumulate upon cold shock, followed by the hexose phosphates glucose-6-phosphate, fructose-6-phosphate, galactose-6-phosphate and mannose-6-phosphate (Kaplan et al., 2004, 2007). An essential enzyme for starch breakdown is β-amylase BAM3 which is responsible for the production of maltose

Abbreviations: AMY, alpha-amylase; BAM, beta-amylase; CBF, C-repeat binding factor; CIP, COP1-intercative protein; COP, constitutive photomorphogenesis; COR, cold-responsive; Cp, crossing point; DAPI, 4′,6-diamidino-2-phenylindole; DREB, dehydration responsive element binding protein; FLX, FLC expressor; GC–MS, gas chromatography–mass spectrometry; GFP, green fluorescent protein; KOS, *KOLD SENSITIV*; PHS, starch phosphorylase; qRT-PCR, quantitative real-time polymerase chain reaction; SMC, Structural Maintenance of Chromosomes.

* Corresponding author at: Molecular and Chemical Sciences Building M316, The University of Western Australia, 35 Stirling Highway, Crawley, Western Australia, 6009, Australia. Tel.: +61 8 6488 4403; fax: +61 8 6488 4401.
E-mail address: ssmith@cyllene.uwa.edu.au (S.M. Smith).

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through the hydrolysis of α-1-4 linkages from the non-reducing ends of glucan chains (Kaplan and Guy, 2005; Edner et al., 2007). Experiments with *bam3* RNA interference plants demonstrated a freezing-sensitive phenotype that correlated with the inhibition of maltose accumulation upon cold shock (Kaplan and Guy, 2004, 2005). Another enzyme of starch breakdown is the alpha-amylase AMY3, which although not essential for starch breakdown at 20 °C (Yu et al., 2005) may have a role in the cold (Kaplan and Guy, 2005). Other metabolites with potential cryoprotective properties such as proline and trehalose have also been shown to accumulate and antioxidants such as ascorbate increase to scavenge reactive oxygen species (Cook et al., 2004).

In a search for proteins of unknown function potentially targeted to plastids we studied At3g23980 but found that this protein is targeted to the nucleus rather than plastids. During further studies of the function of At3g23980 we subjected mutants to several abiotic stresses and observed that they were cold sensitive and assigned the gene a Danish name *KOLD SENSITIV-1* (*KOS1*) to distinguish it from numerous other cold sensitive mutants (http://www.arabidopsis.org/portals/nomenclature/symbol_main.jsp). We discovered that *KOS1* has a role in acclimation to chilling including regulation of transcript levels of genes potentially required for cryprotection, and levels of ascorbate and malate.

Materials and methods

Plant growth

Arabidopsis thaliana T-DNA mutant seeds were obtained from the Arabidopsis Biological Resource Centre (ABRC), stock numbers SALK_005655, SAIL_107_D04 and SAIL_107_D04. Oligonucleotide primers for genotyping are listed in Supplementary Table 1. Seeds were surface sterilized in 70% (v/v) ethanol and 0.05% (v/v) Triton X-100 for 5 min and then washed successively in 70% (v/v) and 100% (v/v) ethanol and then left to dry in a sterile laminar flow cabinet. Sterilized seeds were germinated on 0.5 strength MS basal salt medium with 0.8% (w/v) agar. Seeds were stratified for 3 days at 4 °C and transferred to growth chambers (150 $_{\mu}$E cm^{-2}, 21 °C) under continuous light, long-day (16 h light:8 h dark), medium-day (12 h light:12 h dark) or short-day (8 h light:16 h dark) photoperiods. Ten-day-old seedlings were transferred to soil and grown in the same growth chamber as for germination. For cold treatment, plants were germinated and grown on soil as above for 2 weeks before transfer to 12 h days or continuous light at 4 °C and 150 $_{\mu}$E cm^{-2}. For plants grown on agar medium at 4 °C, seeds were germinated as above at 21 °C and then grown at 4 °C.

Transcript analysis

Four mid-stage rosette leaves (excluding the four oldest leaves and expanding leaves) were harvested from each plant 1 h before lights out and frozen in liquid nitrogen. RNA was extracted using the Plant RNeasy mini kit (Qiagen, Doncaster, Australia). Contaminating genomic DNA was removed by DNAse treatment with Turbo DNA Free (Ambion, Austin, Texas, USA). cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Gladesville, Australia). qRT-PCR was performed on a Roche LC480 using LightCycler 480 SYBR Green I Master (Roche, Dee Why, Australia). Cycle conditions were 95 °C for 10 min; 45 cycles of 95 °C for 20 s, 60 °C for 20 s and 72 °C for 20 s followed by melt curve analysis. Where possible primer pairs were designed across introns using the AtRT-Primer website (Han and Kim, 2006). Primer sequences are listed in Supplementary Table 1. Crossing point (Cp) values were calculated with Roche LightCycler 480 software using the high confidence algorithm. The Cp value of two technical replicates was used to calculate expression relative to an internal reference gene. Several potential reference genes identified by Czechowski et al. (2005) were investigated, and At1g67350 and At5g08290 (yellow-leaf-specific protein 8; YLS8) selected. At least three biological replicates for each genotype were used for every analysis. The mean relative expression and standard deviation for the biological replicates were determined.

Metabolite extraction and GC–MS analysis

Plant material was harvested in exactly the same way as for transcript analysis. Approximately 15 mg of tissue was rapidly weighed and frozen. Frozen samples were extracted in a solution containing: 20:2:1 ratio of methanol:water:ribitol solution (0.2 mg ml^{-1} in water). Derivatisation was automated using an autosampler (Gerstel MPS2 XL-Twister). GC–MS retention time was locked to the internal standard, ribitol (retention time = 25 min). GC (Agilent 7890) comprised a Varian Factor 4 column, inlet temperature of 300 °C, 2 μl splitless injection with a constant flow rate of 1.55 ml min^{-1}. MS (Agilent 5975) parameters: solvent delay of 10 min, mass range 50–600 amu with 4.4 scans per second. Temperatures of the quadrupole and ion source were 150 °C and 230 °C, respectively. Data were analysed using Chemstation software (Agilent) and peaks were manually integrated using a target ion for each peak.

GFP localization studies

GFP fusion proteins were created according to Tian et al. (2004) with modifications as described in Eubel et al. (2008). In summary: GFP5 was inserted 11 amino acids upstream from the C-terminus and driven by the 35S promoter for transient expression by biolistic bombardment in onion (*Allium cepa*) epidermal cells (see Eubel et al., 2008). Stable transformations of Arabidopsis with this construct were made by the floral dip method (Clough and Bent, 1998). Full-length templates for the amplification of *KOS1* were cloned from total cDNA. The region corresponding to the N-terminus was amplified using KOS-GFP P1 and P2 (for complete list of primers see Supplementary Table 1). The P1–P2 fragment, GFP5 and oligos P3 and P4, encoding the C-terminal 11 amino acids and the stop codon had overlapping adapters and were used directly in a second round of PCR. This triple-template PCR-product was gateway cloned into pDONR207 and subsequently into gateway-modified pGREEN vector as described in Eubel et al. (2008). Fluorescence images were obtained using an Olympus BX61 epifluorescence microscope with HQ GFP (U-MGFPHQ) filters and CellR software. Confocal images were obtained with a Leica TCS 2P2 AOBS multiphoton confocal microscope with laser excitation of GFP at 488 nm and DAPI (4′,6-diamidino-2-phenylindole) at 360 nm. Emission collection was in the ranges of 500–530 nm and 394–450 nm, respectively. Confocal images were captured using Leica confocal software.

Results

KOS1 has a predicted coiled-coil and Structural Maintenance of Chromosomes domain

KOS1 encodes a 714 amino acid protein predicted to have a coiled-coil structure (http://www.coiled-coil.org) similar to that of Structural Maintenance of Chromosomes (SMC) proteins. SMC proteins are involved in chromosome maintenance and condensation and their coiled-coil domains mediate protein-protein interactions (Hirano, 2002). An amino acid sequence comparison of KOS1, COP1-interactive protein1 (CIP1), FLC expressor (FLX), a floral promoting protein also with an SMC domain (Andersson et al., 2008) and an Arabidopsis condensin protein orthologous to yeast SMC2