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Physio-biochemical and proteome analysis of chickpea in early phases of cold stress

Leila Heidarvand, Reza Maali-Amiri*

Department of Agronomy and Plant Breeding, University College of Agriculture and Natural Resources, University of Tehran, 31587-77871 Karaj, Iran

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ABSTRACT

Intensive and short-term strategies can aid in more rapid screening with informative and reliable results for long-term investigations under cold stress (CS). The integration of cellular analysis of chickpea during 0, 2, 4, 8, and 12 h CS supplied us with novel possible responsive components and the possible interactions embedded inside, still remaining a Maze. Seedlings showed a biphasic pattern of responses over time. The transitory phase happened after 8 h, when cells are presumably experiencing a new stage of responses and setting the stage for long-term adjustments. Physio-biochemical analysis confirmed the direct effect of fatty acids composition, lipoxygenase activity and antioxidant systems in cell responses under CS. Also, proteome results using MALDI-TOF-TOF and/or LC–MS/MS were able to differentiate changes in early phases of CS. Two-dimensional gel analysis results showed the possible targets of CS as mitochondria, chloroplast, organelle–nucleus communications, storage resources, stress and defense, protein degradation and signal transduction that confirmed the cell intended to re-establish a new homeostasis, in energy and primary metabolites to adapt to long-term CS. Here we propose a time course dynamic assessing multi-dimensional approaches for CS studies as one of the first studies in short-term treatment to progressively fill in the gaps between physio-biochemical and molecular events and touch the cell architecture for a better comprehension of the nature of plant stress response.

Introduction

Chickpea (*Cicer arietinum* L.), the world's third most important food legume, is currently grown in about 52 countries around the world (Varshney et al., 2009). This crop is widely sown as a summer rain-fed system in South Asia and Australia and as an autumn or spring sown crop in Mediterranean climates (Berger, 2007; Mantri et al., 2007). Terminal water deficit is still the major abiotic stress causing more than a 40% reduction in chickpea yield globally. Many farmers sow early to minimize the crop losses due to terminal drought (Berger, 2007; Deokar et al., 2011). Sowing earlier or as autumn or spring cropping provides chickpea with a more durable growth season, efficient use of soil moisture and higher yielding (Clarke and Siddique, 2004). However, a lack of cold tolerance causes yield losses in different chickpea growth stages. Therefore, cold-tolerant chickpea cultivars could be of great value in different climate conditions so that plant development is more advanced with the arrival of the spring season.

Cold stress (CS) causes a vast range of responses in plants, including physio-biochemical responses that happen along with gene expression fluctuations (Chinnusamy et al., 2007; Heidarvand and Maali Amiri, 2010). These responses afford plants cold tolerance. However, the degree of tolerance is a genotype-based trait. Generally, responses of genes to CS are grouped in two categories, the "early" and "late" (Schade et al., 2004; Yun et al., 2010). The early response, which is a rapid-transient response in the short-term, is the determinative factor in the late response, which sustains the plant in long-term CS and helps it to survive (Hannah et al., 2005). Although responses under short-term CS can be different in the medium- or long-term, significant changes in gene expression occur in the short-term (Hannah et al., 2005). These changes can be involved in upstream signaling components and sensors or stress response activators. Thus, monitoring the dynamic of plant responses throughout the short-term CS provides the opportunity to discover and study targets in response to the stress.

The chickpea genome is relatively small (around 740 Mb) (Garg et al., 2011), but it still remains to be completely sequenced and the growing number of expressed sequence tags (ESTs) is very

Abbreviations: APX, ascorbate peroxidase; CAT, catalase; CBB, Coomassie Brilliant Blue; COX, cytochrome *c* oxidase; CS, cold stress; DBI, double bond index; DTT, dithiothreitol; ELI, electrolyte leakage index; EST, expressed sequence tag; ET, electrons transfer; FA, fatty acid; FAME, fatty acid methyl ester; FKBP, FK506-binding protein; FM, fresh mass; H_2O_2, hydrogen peroxide; JA, jasmonic acid; IEF, isoelectric-focusing; IMM, immunophilin; LOX, lipoxygenase; MDA, malondialdehyde; OEC, oxygen-evolving complex; p*I*, isoelectric point; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA, thiobarbituric acid; 2-DE, two-dimensional gel; UFAs, unsaturated fatty acids.

* Corresponding author. Tel.: +98 26 32246074; fax: +98 26 32227605.
E-mail address: rmamiri@ut.ac.ir (R. Maali-Amiri).

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small (44,157 ESTs present in the dbEST database at NCBI, release 010712). Robust and reproducible techniques have played a crucial role in providing such impressive amount of information at different cellular levels under CS. Mantri et al. (2007) have studied the response of chickpea to the three abiotic stresses, including drought, cold and salinity, using cDNA microarray technology. Other researchers have utilized comparative analysis of ESTs and SuperSAGE technology and all have focused on drought and salinity stresses (Gao et al., 2008; Molina et al., 2008; Varshney et al., 2009). However, the unavoidable gap between coding the genome and physio-biochemical responses indicates the necessity of proteome studies in different stress conditions (Amme et al., 2006; Goulas et al., 2006; Yan et al., 2006; Sarhadi et al., 2010). Chickpea sub-proteome analysis in response to drought stress is one of the few studies on chickpea proteome (Pandey et al., 2008). Thus, proteome analysis of early responses of chickpea plants to CS in the short-term still remains indeterminate.

Generally, there is an idea that specific protein patterns activated under short-term CS are responsible for the expression of a set of tolerant genes that lead to increased cold tolerance. The investigation of initial changes of total proteins using proteome analysis could be resourceful useful tool that has the ability to introduce not only many predictable proteins, but also proteins without in-depth study under CS. Unique proteins with a central role in adaptation to CS will be targets for future cold tolerance breeding programs without secondary effects on yielding. In our previous study, we assessed morpho-physiological characteristics of 10 chickpea accessions under CS regimes and the accession with better performance (Sel 96Th11439) was selected to be studied in response to CS in detail (Heidarvand et al., 2011). Investigations of physio-biochemical and proteome analysis under short-term CS in chickpea provide a close link between different cold responsive cellular–molecular levels and could develop a picture of the molecular mechanisms and their functional products in order to understand how chickpea can be cold tolerant.

Materials and methods

Plant materials and stress treatment

Chickpea accession seeds (Sel 96Th11439) provided by Dryland Agriculture Research Institute (DARI) of Iran were sterilized and germinated in Petri dishes on filter paper for 72 h at 23 °C in a thermostat. Subsequently, the seedlings were grown in potted soil (soil, sand, and farmyard manure) in a growth chamber at 23 °C, under white light (220 μmol m^{-2} s^{-1}), a photoperiod of 16 h, and 75% relative humidity. Three-week-old seedlings were exposed to a climatic chamber at 4 °C (Chilling chamber, Arvin Tajhiz Espadana, Isfahan, Iran) for 12 h. Samples from unstressed plants were collected as control condition samples (0 h). The cold stress (CS) treatment was conducted through 2, 4, 8, and 12 h after exposing the seedlings to CS. Collected samples were immediately flash frozen in liquid nitrogen and stored at −80 °C for further studies.

Cell membrane permeability

Cell membrane permeability of plantlets was assessed by the electrolyte leakage index (ELI) in tissues damaged by treatments as described previously (Heidarvand et al., 2011).

Lipid peroxidation analysis

The measurement of lipid peroxidation in leaves, which determines malondialdehyde (MDA), was assessed as described previously (Nazari et al., 2012). The amount of MDA was expressed as μmol g^{-1} fresh mass (FM).

Hydrogen peroxide (H_2O_2) assay

H_2O_2 content was determined as described previously (Nazari et al., 2012). The content of H_2O_2 was expressed in μmol g^{-1} FM.

Antioxidant enzymes activity

Samples (0.5 g) were ground in liquid nitrogen and homogenized by extraction buffer (50 mM phosphate buffer, pH 7.0) containing 1% (w/v) polyvinylpolypyrrolidone at 4 °C. Homogenates was centrifuged at 15,000 × *g*, 4 °C for 30 min. The supernatant was used for antioxidant enzymes assays. For the APX assay, 2 mM ascorbic acid was present in the extraction buffer. Total soluble protein content was determined using the Bradford method (Bradford, 1976). The activity of superoxide dismutase (SOD; EC-number: 1.15.1.1) was measured according to the method of Giannopolitis and Ries (1977). Catalase activity (CAT; EC-number: 1.11.1.6) and ascorbate peroxidase activity (APX; EC-number: 1.11.1.11) were determined as described previously (Nazari et al., 2012). Lipoxygenase activity (LOX; EC-number: 1.13.11.12) was determined as described previously (Kazemi Shahandashti et al., 2013) and the results reported based on ΔOD_{234} min^{-1} mg^{-1} protein.

Total lipid analysis

One gram of leaf samples was homogenized in glass tubes and extracted with 15 mL chloroform:methanol 2:1 (v/v) and vortexed. 5 mL distilled water was added before final centrifugation. The lower phase was collected and dried under a stream of N_2 (Bligh and Dyer, 1959). The lipid samples were processed for fatty acid (FA) analyses following the methods described by Metcalfe et al. (1966). The FAs of isolated lipids were methylated into reaction vials by refluxing with sodium methoxide (2%) for 10 min at 100 °C and then were transmethylated by refluxing with 2.175 mL boron trifluoride methanol 14% for 3 min at 100 °C. The fatty acid methyl esters (FAMEs) were extracted from the reaction vials three times with hexane, and concentrated. The FAMEs were analyzed on gas chromatography using a Unicam 4600 series (Unicam Limited, Cambridge, UK) equipped with a BPX70 capillary column (0.25 mm × 30 m, 0.2 mm film thickness), a flame ionization detector. The column oven temperature was set at 160 °C for 5 min, then increased to 180 °C at 20 °C min^{-1} and was held for 9 min, then increased to 200 °C at 20 °C min^{-1}. Components were identified by comparisons of retention times and peak curves with authentic standards. The double bond index (DBI) was calculated according to the formula of Wismer et al. (1998):
DBI = [(2 × %18:2) + (3 × %18:3)]/[(%16:0) + (%18:0) + (%18:1)],
where 16:0 = palmitic acid, 18:0 = stearic acid, 18:1 = oleic acid, 18:2 = linoleic acid, and 18:3 = linolenic acid.

Protein extraction, two-dimensional gel (2-DE) analysis and protein identification

Protein extraction and 2-DE analysis were carried out as previously described with limited modifications (Sarhadi et al., 2010). The leaf samples (0.9 g) were crushed in liquid nitrogen and suspended in 10% (w/v) trichloroacetic acid in acetone with 0.07% (w/v) dithiothreitol (DTT) at −20 °C for 1 h, followed by centrifugation at 21,000 × *g* for 20 min. The pellets were washed using ice-cold acetone containing 0.07% (w/v) DTT, incubated at −20 °C for 1 h and centrifuged at 12,000 × *g* for 15 min at 4 °C. Washing and sedimentation of the pellets was repeated three times and then pellets were freeze dried. The sample powders were then solubilized