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Upregulation of *CBF/DREB1* and cold tolerance in artificial seeds of cauliflower (*Brassica oleracea* var. *botrytis*)

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ABSTRACT

An effective protocol for cauliflower micropropagation and artificial seed production was optimized and applied in this study. However, in order to be a viable alternative to traditional seeds, cauliflower artificial seeds need to show a high capacity to withstand abiotic stresses such as cold and desiccation. Therefore, in order to increase cauliflower abiotic stress tolerance, the effect of cold acclimation and drought on the cold tolerance of both cauliflower microshoots and mature plants were investigated. Moreover, the effect of cold and drought treatments on the induction of *CBF/DREB1* gene regulation was tested. Both cold acclimation and drought improved the cold tolerance in both cauliflower microshoots and mature plants. However, whilst cold acclimation up-regulated *CBF/DREB1* in cauliflower mature plants and microshoots, drought had the capacity only to up-regulate this gene in mature plants. Therefore, the high effect of cauliflower developmental stage on the *CBF/DREB1* regulation was confirmed. Moreover, a small reduction in soil moisture had the capacity to unregulated this gene in mature cauliflower plants. The results presented in this study have an important role in the improvement of cauliflower micropropagation and the effectiveness of the artificial seed production protocol. Furthermore, the results contribute to an understanding of the cold tolerance mechanism in *Brassica oleraceae* var botrytis.

1. Introduction

It is widely known that the exposure of most temperate plants to non-freezing low temperature (0–5 °C) for a period of time (7–14 days) increases their freezing tolerance and this process is known as cold acclimation (Thomashow, 1999). Because of its importance to agriculture, great efforts have been made and many experiments have been conducted to improve the understanding of this important phenomenon (Thomashow, 2001). Multiple polygenic traits appear and various physiological and biochemical changes occur during the progress of acclimation and these changes often involve modifications in membrane lipid structure (Lynch and Steponkus, 1987; Uemura and Steponkus, 1994). Acclimation also causes an increase in the production of antioxidants, abscisic acid and compatible osmolytes such as soluble sugars and proline (Chen et al., 1993; Dörffling et al., 1997; Kishitani et al., 1994; Koster and Lynch, 1992; Lynch and Steponkus, 1987; Murelli et al., 1995; Nomura et al., 1995; Tao et al., 1998; Uemura and Steponkus 1994). The improvement of cold tolerance by acclimation involves abroad reprogramming of gene expression and metabolism. Recent studies describing full genome transcripts and mutational and transgenic plant analysis have provided a great deal of information about the complex transcriptional systems that function under cold acclimation (Jan et al., 2009).

It has been reported that there is a set of genes which are highly up-regulated during the process of acclimation and these genes encode a specific family of proteins called cold- regulated (COR) proteins (Gilmour et al., 2004). Several types of *COR* genes have been recognized in both monocotyledonous and dicotyledonous plants (Sharma et al., 2005; Sun et al., 2009). It has been demonstrated that abscisic acid (ABA) can have an important role in acclimation and it has been shown that ABA-dependent and ABA-independent pathways are the main two pathways intermediating the induction of *COR* genes expression. In the ABA-dependent pathway, the accumulation of endogenous ABA observed under the effect of cold triggers the basic leucine zipper (bZIP) transcription factor, which then induces ABA-dependent *COR* genes through ABA-regulated elements (Uno et al., 2000; Xiong et al., 2002). Also it has been demonstrated that ABA accumulates under the effects of other environmental stresses such as drought (Leung and Giraudat, 1998). The accumulation of ABA causes several physiological adaptations including stomatal closure and growth inhibition. Moreover, ABA induces the expression of several genes other than the *COR* genes (Kurkela and Franck, 1990; Lång and

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Palva, 1992).

In the ABA independent pathway, cold induces the expression of C-repeat binding factor (*CBF*) transcription factors. This family of genes has an essential role in activating downstream *COR* genes which in turn improve the freezing tolerance in plants (Sun et al., 2009). The *CBF* transcription factor has been identified and characterized in many plant species including rape (*Brassica napus*), broccoli (*Brassica oleracea*), alfalfa (*Medicago sativa*), tomato (*Lycopersicon esculentum*), corn (*Zea mays*), rice (*Oryza sativa*), strawberry (*Fragaria ananassa*), soybeans (Glycine max) wheat (*Triticum aestivum*), barley (*Hordeum vulgare*),woody plants, apple and peach (Al-Issawi et al., 2015a; Al-Issawi et al., 2015b; Artlip et al., 2014; Choi et al., 2002; Dubouzet et al., 2003; Francia, 2004; Gao et al., 2002; Owens et al., 2002; Rihan et al., 2014; Vágújfalvi et al., 2003; Wisniewski et al., 2014, 2015).

Under drought stress a similar mechanism exists and dehydration-responsive element binding factor (DREB) are up-regulated. Both *CBF*s and *DREB*s are transcription factors which induce the expression of cold and dehydration stress regulated gene in plants (Gilmour et al., 1998; Liu et al., 1998a; Shinwari et al., 1998). These transcription factors bind to specific regulatory sequences in the promoters of cold and dehydration responsive genes. These sequences are C-repeat (CRT: TGGCCGAC) and dehydration-responsive elements (DRE: TACCGACAT). Both of these sequences contain the highly conserved core 5-bp sequence of CCGAC, which has the capacity to regulate transcription under drought and low temperature and also under salinity (Baker et al., 1994; Gao et al., 2007; Yamaguchi-Shinozaki and Shinozaki, 1994). Furthermore, a crosstalk between the response of different abiotic stresses and some of the genes up-regulated by drought or salt stress have been reported in different plant species (Seki, 2002; Zhao and Zhu, 2016).Thus *CBF* induces the expression of *COR* genes (the genes which contain the *COR* sequence) and these genes play an essential role in the improvement of plant abiotic stress resistance.

Cauliflower is species which demonstrates cold tolerance through a *CBF* mediated pathway (Hadi et al., 2011, Rihan 2013) and also a remarkable capability for plant tissue culture. Cauliflower curd can be homogenised, sieved, grown into microshoots and converted to artificial seed (Kieffer et al., 2006; Rihan et al., 2012a, b, c). Rihan et al. (2011) reported high growth capacity of cauliflower artificial seeds in commercial substrates which is considered a promising step for their direct use *in vivo*. However, cauliflower artificial seeds should ideally show high cold and drought tolerance in order to survive the vagaries of establishment in the field and be a competitive alternative to traditional seeds. This study aimed to investigate the effect of cold acclimation and drought on the cold tolerance of cauliflower artificial seeds. Moreover, it aimed to investigate the effect of these parameters on the induction of *CBF/DREB1* gene expression at different developmental stages (microshoots and mature cauliflower plants) and to determine the partial sequence of *CBF/DREB1* gene in cauliflower.

2. Material and methods

2.1. Cauliflower microshoot production

Large pieces of cauliflower curds (cv.Dionis) (1–5 cm) were sterilized by immersion in diluted un-thickened domestic bleach (10% v:v, 0.06% sodium hypochlorite) for 15 min, followed by a double wash with sterile distilled water. Explants were produced mechanically by eliminating the mass of non-responsive tissue (stem branches) and shaving off the upper meristematic layer using a sterilized scalpel whilst working in a laminar flow cabinet. The meristimatic clusters were then homogenised using a commercial blender (Waring model 800) at approximately 1700 rev min^{-1} in liquid maintenance S23 medium (4.4 g L^{-1} MS salts (Murashige and Skoog 1962)) supplied by Sigma™ and 3% w/v sucrose) for 30 s to produce a homogenate of micro-explants. The micro-explants were size graded by passing the homogenate through a series of sieves with aperture sizes of 212, 300 and 600 μm (Endacotts Ltd). A small volume (74 μL) of the 212–300 μm homogenate fraction was cultured in 30 mL S23 medium, supplemented with 2 mg L^{-1} Kinetin and 1 mg L^{-1} IBA in 125 mL plastic pots. In order to preserve culture sterility, the culture media was supplemented with 1 mL L^{-1} PPM™ (Plant Preservative Mixture) which was used with all treatments. The 26 day old cultures were divided into two groups:

The first group was transferred to the cold room at 4 °C for acclimation. Samples of microshoots, each consisting of 2 culture pots, were sampled at 0 (control), 1, 6, 12, 18, 24 h after the transfer to the new temperature. These samples were stored at −80 °C until the RNA was extracted. The samples (100 ± 10 mg) were then ground to a powder in liquid nitrogen with a mortar and pestle and the total RNA was isolated using the Spectrum plant total RNA kit (Sigma Aldrich: spectrum plant total RNA kit, Cat # STRN50) according to the manufacturer's instructions. The total extracted RNA was quantified using the Nano-drop 1000 spectophotometer method to estimate its concentration. The purity of the RNA was assessed spectrophotometrically by examining the absorbance ratio at 260 and 280 nm. The reverse transcription was carried out using M-MLV Reverse Transcriptase (Sigma: M1302) in 20 μL volume. Sequence specific primers for *CBF/DREB1* (Forward primer 5-ACTTTCCTAACCGCCGAC, Reverse primer 5-TCTCAGCCTGAAAAGCCA-3) and for the Actin 1 mRNAs (endogenous control) (Forward primer 5-CCCAAAGGCCAACAGAGAGAAG-3-3) (Reverse primer 5-CACCAGAGTCCAGCACAATACC-3) were designed using Primer-BLAST (Ye et al., 2012) and synthesized by Eurofin MWG/Operon (Germany).

The cDNA for the samples was used as a template for gel electrophoresis PCR (Applied Biosystems, Veriti) (Sigma kit). A Master mix was prepared consisting of (for each sample) 1 μL Red tag polymerase + 2.5 μL Red tag polymerase buffer + 0.5 μL forward primer + 0.5 μL reverse primer + 0.5 μL dNTPs + 18 μL sterile nuclease free water. The master mix was prepared for all samples together and 23 μL from the mixture was added to 2 μL of each sample in nuclease free 1.5 mL microcentrifuge tubes. The PCR thermal cycle was optimized to be as follows, initial denaturation at 94 °C for 2 min once followed by 40 cycles of denaturation at 94 for 30 s, annealing 57 °C for 30 s, extension at 72 °C for 30 s and then final extension at 72 °C for 5 min and then 4 °C ∞.

The PCR products were analysed using 1.4% high melting agarose gel (Fisher, EP1356-100) melted in TAE (Tris-acetate + EDTA) and with 0.005% of SYBR™ safe. The PCR products were compared with a PCR 100 bp low scale DNA ladder (Fisher BioReagents, BP2581-200) consisting of 10 DNA fragments with sizes of 50, 100, 200, 300, 40, 500, 700, 1000, 1400, 1500, 2000 bp. Band intensities were semi-quantitatively measured using Image j software. The same procedures were followed in all PCR experiments reported in this study.

The second group of microshoots was used for the production of artificial seeds. Microshoots were mixed with sterilized (by tyndallisation) sodium alginate 2% (w/v) and dropped into a sterilized (autoclaved) solution of calcium chloride 15 g L^{-1} using a sterilized pipette to form gel beads. Microshoots were left in the calcium chloride for 30 min for full complexion of their encapsulating beads. The artificial seeds were then transferred to S23 liquid media (without plant growth regulators (PGRs)) for 30 min followed by a quick wash with sterile distilled water.

The artificial seeds produced were divided into two groups. The first group was incubated at 4 °C for 15 days for acclimation and the second group was used as a control (kept at room temperature). Cultures were exposed to 16 h photoperiod. Frost tolerance analysis of both acclimated and non-acclimated artificial seeds was carried out to test the effect of acclimation process. Artificial seeds were exposed to different temperatures as follows, 20, 0, −2, −4, −6, −8, and −10 °C. The artificial seeds were placed in sterile petri dishes together with a small piece of ice (prepared from sterilized water) to ensure ice nucleation. The petri dishes were placed in a Sanyo programmable chamber to the various freezing temperatures in sequence with a hold of two hours at