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Identification of Chinese cabbage genes up-regulated by prolonged cold by using microarray analysis

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Abstract

Vernalization is induced by prolonged cold and accelerates flowering. To monitor the genome-wide transcriptome change that identifies the genes that are differently up-regulated depending on the duration of cold exposure, we fabricated a Chinese cabbage cDNA microarray and used it to identify genes that are up-regulated by short-term (1-week) and long-term (4-week) cold. The overall expression patterns between 1- and 4-week cold treated seedlings were very similar but some genes in the cell growth/division/DNA synthesis functional category were regulated differently. Prolonged cold specifically up-regulated several *heat shock proteins* (*HSPs*), and *small HSPs* were more significant. Among the up-regulated *sHSPs*, we chose the *small HSP 17.6-II* and confirmed its up-regulation in response to long- but not short-term cold exposure by Northern analysis. And its constitutive overexpression moderately accelerated flowering.

Keywords: cDNA microarray; Chinese cabbage; Expression sequence tag; Heat shock proteins; Cold; Vernalization

1. Introduction

Low temperature is a primary environmental stress that influences plant growth, development and productivity and thus plants have evolved a variety of temperature-regulated adaptive mechanisms, including cold acclimation and the vernalization response [1]. Both mechanisms have a common feature triggered by non-freezing cold temperatures but differ in the duration of cold exposure. Cold acclimation is achieved relatively quickly and mainly serves to protect the plant from cold injury without influencing the flowering time [2]. In contrast to the cold acclimation, vernalization requires several weeks of prolonged cold (e.g. winter) and induces or accelerates the ability to flower (reviewed in [3]). Thus, the molecular mechanism behind vernalization differs from that of acclimation in that it is related to the ability of the plant to flower. Several evidences supporting these facts are the down-regulates the floral repressor '*Flowering Locus C* (*FLC*)' [4] and up-regulates *AGAMOUS-LIKE 24* (*AGL24*) by prolonged cold condition [5]. Moreover, recent discoveries concerning the molecular mechanisms that relate to how plants remember they have been vernalized have provided a framework for understanding how genes involved in vernalization behave (reviewed in [3]). However, to date, no genome-wide transcriptome analysis has been performed which distinguishes the period-dependently up-regulated genes under the prolonged cold. In this context, to better understand vernalization mechanisms, we have used microarray technology to identify the genes that are differently up-regulated depending on the duration of cold exposure.

Together with the availability of complete genome sequences of model plants and comprehensive sets of EST sequences (http://www.ncbi.nlm.nih.gov), the microarray technique has led to major advances in our understanding of many aspects of plant biology [6–8], including the discovery of important agricultural traits of several important crop plants [9–11]. One such plant is Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*), a widely

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used vegetable and its functional and structural genomics have been investigated. The Multinational *Brassica* Genome Project (MBGP) has also been initiated to establish communal genomic resources (http://www.brassica.info/). Here, in coordination with international efforts for *Brassica* Genomes, we describe the fabrication of the first Chinese cabbage cDNA microarray as a genomics tool and its application to identify genes that are up-regulated by prolonged cold in order to better understand of vernalization.

2. Materials and methods

2.1. cDNA library construction and sequencing

Total RNAs were isolated as described [12] from 4-day-old green seedlings, etiolated seedlings, NaCl-treated seedlings and root tips of a Chinese cabbage cultivar (*Brassica campestris* L. ssp. *pekinensis* cv. Jangwon) grown in MSO-agar medium containing Murashige and Skoog salts (Duchefa, Haarlem, The Netherlands) and 3% sucrose. Poly(A)$^+$ RNA was isolated by using a commercially available poly(A)$^+$ purification kit (Amersham Pharmacia Biotech, Uppsala, Sweden). cDNA was synthesized with a SUPERSCRIPT plasmid cDNA library kit (Invitrogen, San Diego, CA, USA) and cloned into pSPORT I (Invitrogen) according to the manufacturer's instructions. A flower bud library was constructed as previously described [13].

Plasmid DNA was isolated with a plasmid miniprep kit (Omega, Doraville, GA, USA) or a 96-well Turbo Plasmid prep system (Qiagen, Hilden, Germany). Its quality was checked by 1% agarose gel electrophoresis. The plasmids were then sequenced. Clones were partially sequenced from the 5′-end with ABI377XL or ABI310 sequencers using BigDye-terminator chemistry (ABI Perkin-Elmer, Wellesley, MA, USA).

2.2. Microarray fabrication

Unique ESTs (6233) were selected from 9646 ESTs by the EST analysis program 'GeneMaster' (Ensolteck, Daejon, Korea). The selected clones and 167 controls were amplified and purified according to the published protocol [14]. The purified amplicons were mixed with Micro Spotting Plus (Arryit, Sunnyvale, CA, USA), and each cDNA was spotted in duplicates onto CMT-GAPS 2 slides (Corning, Corning, NY, USA) using a MicroGrid II Arrayer (Genomic Solutions, Ann Arbor, MI, USA). Apart from the positive and negative controls needed to ensure microarray reliability, various Chinese cabbage housekeeping genes in the EST collection, including tubulins, actins, translation initiation factors, translation elongation factors, elongation factors, glyceraldehyde-3-phosphate dehydrogenases, ubiquitins, and ribonuclease, were placed in 17–19 columns of the last row in every sub-array as internal controls to permit scanning normalization and to eliminate single-control normalization error [15]. After printing, post-processing was performed as described by Corning (http://www.corning.com/). Whole array information is available at the ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) in European Bioinformatics Institute with array accession number A-MEXP-115.

2.3. Fluorescent probe preparation, hybridization and scanning

Chinese cabbage seeds were sown on MSO medium (Duchefa) containing 0.25% phyta-gel (Sigma, St. Louis, MO, USA) and incubated at 4 °C for 3 days under short-day conditions (8 h light) to induce synchronous germination. They were then transferred to a growth chamber and grown at 22 °C for 4 days under long-day conditions (16 h light). Thereafter, they were incubated in a cold chamber (4 °C) for 1, 2, 3 or 4 weeks, respectively, under long-day conditions. Total RNA was isolated from seedlings treated with cold for 0, 1, and 4 weeks as previously described [16]. A 3 DNA Array 50 kit (Genisphere, Hatfield, PA, USA) was used for labeling and hybridization. Microarrays were scanned in both the Cy3 and Cy5 channels with a ScanArray Lite (GSI Lumonics, Billerica, MA, USA) at 5 μm resolution. To compensate for variable backgrounds, the Cy5/Cy3 signal intensities were adjusted on the basis of the exogenously added control (Lambda DNA polyA$^+$ RNA-A: Takara, Shiga, Japan) and internal control genes placed in different sections of the microarray slides. To minimize inherent microarray errors [15], each comparison was performed with dye-reversal hybridization at least twice using independently isolated RNA samples as the starting material. At least four replicates were analyzed.

2.4. Data analysis

The fluorescence intensity of the raw scan data was quantified by using QuantArray software (Packard BioScience, Billerica, MA, USA). By calculating the correlation coefficient of replicates, reproducible arrays were selected for further analysis. Normalization between the Cy3 and Cy5 fluorescent dye intensities was achieved by locally weighted linear regression (lowess) sub-grid normalization (GeneTraffic software; Iobion, La Jolla, CA, USA). The genes were then filtered for cluster analysis on the basis of the following criteria: (1) the fluorescent signal intensity was at least 1000; (2) genes whose percentage of values were lower than 80% were removed; and (3) of all probe pairs for any given gene, at least one pair had an examined probe versus reference ratio that was greater than two-fold. With reference to published studies [17] and our own self–self hybridization experiments, we selected a cutoff of two-fold up- or down-regulation to define differential expression. Complete experiment information is available at the ArrayExpress with experiment accession number E-MEXP-190.