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Research article

Transcription factors and anthocyanin genes related to low-temperature tolerance in *rd29A*:*RdreB1BI* transgenic strawberry

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ABSTRACT

Dehydration-responsive element-binding (DREB) transcription factors play critical roles in plant stress responses and signal transduction. To further understand how DREB regulates genes expression to promote cold-hardiness, Illumina/Solexa sequencing technology was used to compare the transcriptomes of non-transgenic and *rd29A*:*RdreB1BI* transgenic strawberry plants exposed to low temperatures. Approximately 3.5 million sequence tags were obtained from non-transgenic (NT) and transgenic (T) strawberry untreated (C) or low-temperature treated (LT) leaf samples. Over 1000 genes were differentially expressed between the NT-C and T-C plants, and also the NT-C and NT-LT, as well as the T-C and T-LT plants. Analysis of the genes up-regulated following low-temperature treatment revealed that the majority are linked to metabolism, biosynthesis, transcription and signal transduction. Uniquely up-regulated transcription factors as well as anthocyanin biosynthetic pathway genes are discussed. Accumulation of anthocyanin in the stolon and the base of the petiole differed between non-treated NT and T plants, and this correlated with gene expression patterns. The differentially expressed genes that encode transcription factors and anthocyanin enzymes may contribute to the cold hardiness of *RdreB1BI* transgenic strawberry. The transcriptome data provide a valuable resource for further studies of strawberry growth and development and DREB-mediated gene regulation under low-temperature stress.

1. Introduction

Environmental factors such as drought, high salt levels, heat, and low temperature (chilling and freezing temperature) are major abiotic stresses during the sessile lifetime of a plant (Kasuga et al., 1999). In particular, low temperature is a key environmental factor that adversely affects plant growth and development, thereby limiting crop productivity and geographical distribution. Surviving such stress led to plants acquiring protection mechanisms involving membrane stabilisation, the synthesis and accumulation of specific solutes and changes in enzyme activity (Thomashow, 1999). Various functional genes, and regulator genes with their target proteins, play important roles in regulating low-temperature tolerance. Among low-temperature induced genes, the dehydration responsive element binding protein (DREB)/C-repeat binding factor (CBF) genes encode key transcription factors in the major transcriptional cascade that responds to low temperature (Shinozaki and Yamaguchi-Shinozaki, 2000). These genes belong to the ERF (ethylene responsive element binding factor) family of transcription factors. Transcription factors of the DREB class exclusively recognise and bind the CRT/DRE (C-repeat/dehydration responsive element) *cis*-acting element (core motif: G/ACCGAC) in the promoter regions of cold-regulated genes (Stockinger et al., 1997). They also regulate the expression of genes associated with transcription, metabolism, osmolyte biosynthesis, membrane transport, hormone metabolism, ROS detoxification and signalling pathways and some others with known or putative cellular protective functions (Thomashow, 2010). Since the critical role of DREB transcription factors was discovered in the abiotic stress response, many DREB homologues have been isolated from many commercial key plant

Abbreviations: CBF, C-repeat binding factor; CRT/DRE, C-repeat/dehydration responsive element; DAA, day after anthesis; DEG, differentially expressed gene; DGE, digital gene expression; DREB, dehydration responsive element binding protein; ERF, ethylene responsive element binding factor; FDR, false discovery rate; GO, gene ontology; KEGG, kyoto encyclopedia of genes and genomes; TPM, number of transcripts per million clean tags.

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species such as rice (*OsDREB*), soybean (*GmDREB*) and maize (*ZmDREB*).

Enhanced cold tolerance was acquired in different plant species where DREB genes were constitutively ectopically expressed. Constitutive overexpression of wheat *TaDREB2* and *TaDREB3* in barley resulted in marked improvement in cold tolerance, and genes expressing LEA/COR/DHN proteins, which are known to protect cells from damage and death under cold stress (Morran et al., 2011) were clearly up-regulated. In apple, overexpression of a peach DREB, *PpCBF1* resulted in a 4–6 °C increase in cold hardiness in both the acclimated and non-acclimated states compared with those of untransformed trees (Wisniewski et al., 2011). Although great progress has been made in recent years in understanding cold signal transduction in plants, especially DREB transcription factor networks, further study is required to identify DREB target genes in order to better understand the molecular mechanisms of DREB transcription factor function in abiotic stress tolerance.

OsDREB1B from rice, which is highly regulated by cold and other stresses, was shown to play a critical role in plant response to abiotic stress (Dubouzet et al., 2003). Transgenic rice and Arabidopsis overexpressing *OsDREB1B* showed the induction of stress-related genes and heightened tolerance to cold, drought and salt stress (Datta et al., 2012). Furthermore, *OsDREB1B* appears to play an important role in crosstalk between various stress signalling pathways (Figueiredo et al., 2012). Qin et al. (2007) isolated an AP2/EREBP transcription factor, *RdreB1B* (AY319971), from a rice cDNA library, that was subsequently found to be identical to *OsDREB1B* (AF300972). Because its high GC content and secondary structure makes it hard to perform transcriptional expression analyses, *RdreB1B* was reconstructed using codon optimisation and named *RdreB1BI*. Ectopic expression of this rice homologous gene led a higher tolerance to cold stress in Arabidopsis.

In some areas where strawberry (*Fragaria* x *ananassa)* is grown in a perennial growing system, low temperature becomes a major limiting factor in the strawberry industry. Functional analyses performed *in vivo* by transforming DREB transcription factors into plants are important to understand the molecular mechanisms of stress tolerance in plants and are also effective ways to provide new varieties of strawberry with cold-stress tolerance. *RdreB1BI*, under the control of the *rd29A* promoter (*rd29A*::*RdreBIB1*), which harbours cold, ABA, and salt stress response element (Msanne et al., 2011) was transformed into strawberry as described (Wang et al., 2014). Subsequent proteomic analyses showed that the gene was expressed at both the transcriptional and translational levels (Gu et al., 2013).

In the present study, we investigated gene expression in cold treated non-transgenic and *rd29A*:*RdreB1BI* transgenic strawberry plants using next-generation Illumina/Solexa sequencing. The results lay the foundation for further dissection of the DREB transcription factor network by providing an overall view of gene expression patterns, as well as by revealing metabolic networks and the correlated transcription factors.

2. Methods

2.1. Plant materials and RNA extraction

The single copy *rd29A*::*RdreBIB1* transgenic strawberry (*F. ananassa* Duch. cv. Benihoppe) line that exhibited the highest level of cold tolerance in previous studies (Wang et al., 2014) was chosen for the present study. Non-transgenic and transgenic plants, obtained from the laboratory of Fruit Crops Biotechnology of Nanjing Agricultural University in Nanjing, P. R. China, were transplanted into plastic pots with a mixture of autoclaved peat:vermiculite:perlite (9:3:1, v/v), and were grown in a growth chamber at 25/20 °C (16/8 h) day/night temperature, relative humidity ranging from 70 to 80%, and a photon flux density of 500 μmol m^{-2} s^{-1}. One group of six-week-old plants without stolons was treated at 0 °C in the light, at the same relative humidity and illumination intensity for 6 h. Plants without treatment were used as a control. Young expanding leaves were harvested from three separate plants after cold treatment for DGE analysis. Leaves, petioles and stolons were collected from a different group of 12-week-old plants maintained under control conditions in a growth chamber. Fruit was also collected at 30 d after anthesis (DAA) from these plants for measurement of anthocyanin content and related gene expression. All samples were frozen immediately in liquid nitrogen and stored at −70 °C. Total RNA was extracted from collected samples using TRIzol® reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. An Agilent 2100 Bioanalyser was used to assess RNA quality. First-strand cDNA was synthesised with 1 μg of total RNA using the PrimeScript™ RT reagent Kit with gDNA Eraser and oligo dT as primer (TaKaRa, Japan), according to the instruction manual.

2.2. Measurement of anthocyanin content

Anthocyanins from leaf, petiole, stolon and fruit were extracted using a pH-differential method (Wrolstad et al., 1982). Briefly, 1.0 g (5.0 g for fruit) of frozen powder from each sample was extracted with 25 mL 0.1 M HCl. An aliquot of the extract (6 mL) was diluted with 9 mL KCl–HCl buffer pH 1.0 and a separate 6 mL aliquot was diluted with 9 mL Na_2HPO_4-Citric acid buffer pH 5.0. The absorbance of the diluted solutions was measured at 510 nm (UV-2450, Shimadzu, Kyoto, Japan). The content of anthocyanin was calculated as cyanidin-3-glucoside equivalents.

2.3. DGE library preparation and Illumina sequencing

Illumina's DGE tag sequencing was carried out by BGI (Shenzhen, China) using the Illumina Cluster station and the Illumina HiSeq™ 2000 System. Six μg of total RNA from each sample was purified using oligo (dT) magnetic bead adsorption, and oligo (dT) was used as a primer to synthesize cDNA. Two types of endonuclease: *Nla*III or *Dpn*II, can be used to generate the 5′ ends of tags. Usually, *Nla*III, which recognises and cuts at the CATGA sites, is used to subsequently digest the bead-bound cDNA. The fragments apart from the 3′ cDNA fragments attached to oligo (dT) beads were washed away and the Illumina adaptor 1 was ligated to the sticky 5′ end of the digested bead-bound cDNA fragments. The endonuclease *Mme*I, has separate recognition and digestion sites; the recognition site, CATG, which is the junction of the Illumina adaptor 1 and the digestion site 17 bp downstream of the CATG site, where *Mme*I cuts to produce tags with adaptor 1. After removal of 3′ fragments with magnetic bead precipitation, Illumina adaptor 2 was ligated to the 3′ ends of tags, acquiring tags with different adaptors at both ends to form a tag library. After 15 cycles of linear PCR amplification, 105 bp fragments were purified by 6% TBE PAGE gel electrophoresis. The single-chain molecules were fixed onto the Illumina Sequencing Chip (flowcell) for sequencing. Over 3 million 49 bp raw reads were obtained per sample.

2.4. Bioinformatic analysis and mapping of DGE tags

Raw sequence reads were filtered through the Illumina pipeline according to BGI Shenzhen, China. Briefly, filtering included: removal of low-quality sequences such as the 3′ adaptor sequence; removal of empty reads with only 3′ adaptor sequences but no tags; removal of low-quality tags that contain unknown sequences 'N';