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Transcriptome analysis of *Pyrus pyrifolia* leaf buds during transition from endodormancy to ecodormancy

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ABSTRACT

We analyzed the transcriptome of Japanese pear (*Pyrus pyrifolia* Nakai) 'Kousui' leaf buds during the dormancy transitional phases using a 10K cDNA microarray. Leaf buds were collected in October (deep endodormancy) and February (ecodormancy). Over 1000 genes were differentially expressed ($P < 0.05$, ≥2-fold change). Most of these genes are functionally related to chloroplast or plastids, electron transport or energy pathways, response to stimuli or stress, signal transduction or transcription. Of these genes detected in this study, 76 and 22 were highly induced (≥10-fold change) during endodormancy and ecodormancy phases, respectively. Comparison of selected genes between 'Kousui' and the less-dormant Taiwanese pear 'Hengshanli' (TP-85-119) identified two novel transcription factors (*NAC* and *PRR*) whose expression varied concomitantly with the dormancy phase changes. Further studies involving the genes uncovered from this microarray analysis is expected to provide new insights into the molecular events underlying their physiological role in plants including dormancy breaking for stable fruit set.

1. Introduction

Japanese pear (*Pyrus pyrifolia* Nakai) ceases growth in autumn in response to low temperatures (Heide and Prestrud, 2005) and enters into an endodormancy transitional phase which allows for survival in cold conditions. To complete the endodormancy phase, the tree needs to experience a genetically determined amount of low temperature (generally < 7.2 °C); for example, about 750 h of cumulative chilling is necessary to complete endodormancy of 'Kousui' (Sugiura and Honjo, 1997), one of the leading cultivars in Japan. After the completion of the endodormancy phase, trees may remain dormant if the environmental conditions (mainly temperature in Japanese pear) are not yet suitable for growth resumption. This latter state of growth cessation in response to adverse environmental conditions is called ecodormancy. In spring, growth resumes with bud break as temperatures rise.

Climate change (especially global warming) impacts a wide range of effects on the growth and production of fruit trees especially in Japan (Sugiura et al., 2007). Warm winters impair the breaking of endodormancy possibly owing to insufficient chilling accumulation (Yamamoto et al., 2010). Dysfunctional endodormancy breaking has become an obstacle to stable economic production of deciduous fruit trees including Japanese pear. In order to compensate for the insufficient chilling accumulation, hydrogen cyanamide has been used as a bud-break agent on grapevine (Shulman et al., 1983; Nir and Lavee, 1993), peach (Siller-Cepeda et al., 1992), and Japanese pear (Kuroda et al., 2002). To ensure greater uniformity of bud break, it is important to treat trees at the optimal stage (Kuroda et al., 2002); otherwise hydrogen cyanamide has even negative effects. In addition, hydrogen cyanamide is moderately toxic to humans (Sakamoto et al., 2010). Thus, better ways to break dormancy especially based on the regulation of internal factors (genes) are needed and efforts are made toward understanding the molecular events underlying endodormancy breaking.

Molecular techniques provide a mean of unraveling the physiological changes that occur at the gene level. Hormonal and environmental signals could influence endodormancy via the activation or repression of diverse genes (Olsen, 2003). Therefore, cataloguing the genes and comparing their expression patterns during the endodormancy and ecodormancy transitional phases

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would be helpful in efforts toward understanding the molecular events underlying the physiological processes occurring in the buds during the phases of dormancy (growth cessation) and growth resumption. Toward this end, we used a custom microarray of Japanese pear based on 9812 independently expressed sequence tags (ESTs) (Nishitani et al., 2010) to compare the gene expression patterns in the leaf buds of 'Kousui' collected on 21 October 2008 (deep endodormancy) and 12 February 2009 (ecodormancy). Furthermore, we identified two transcription factors (TFs) whose expression patterns fluctuated concomitantly with the dormancy phases and revealed the marked difference between 'Kousui', a cultivar showing distinct endodormancy and 'Hengshanli', a cultivar without distinct endodormancy (thereafter we tentatively designated 'Hengshanli' as less-dormant cultivar).

2. Materials and methods

2.1. Plant materials

Samples were collected from 34-year-old trees of Japanese pear (*P. pyrifolia*) 'Kousui' and 20-year-old trees of Taiwanese pear (*P. pyrifolia*) 'Hengshanli' grown at the orchard of the National Institute of Fruit Tree Science, Tsukuba, Japan (36°N, 140°E). Lateral leaf buds were collected from September to February approximately once a month. All samples were collected at around 0900, because dormancy in some species is controlled partly by light (Heide, 2008). The dormancy status of the trees on each date was estimated by evaluation of the cut branches from which the samples were collected (Ubi et al., 2010). As reported previously (Ubi et al., 2010), the sprouting ratios of 'Kousui' and 'Hengshanli' were as follows: the leaf buds of 'Kousui' were entering deep dormancy (sprouting rate dropped from 8 to 0%) from September to October; they were in deep endodormancy (sprouting rate was 0%) during early to mid December; they entered ecodormancy from late December, showing a sharp increase in sprouting ratio of 56% on 24 December and 93% on 12 February. In contrast, the dormancy status of 'Hengshanli' leaf buds was much less, i.e. the lowest sprouting ratio observed was over 60% (Ubi et al., 2010), confirming that 'Hengshanli' is a less-dormant cultivar and transited from endodormancy significantly earlier than 'Kousui'.

The leaf buds were immediately frozen in liquid nitrogen and stored at −80 °C until needed for RNA extraction. For microarray analysis, we used 'Kousui' leaf buds collected on 21 October 2008 (deep endodormancy) and on 12 February 2009 (ecodormancy). Lateral leaf buds were chosen for this study instead of floral buds to avoid the confounding effect of floral identity genes on dormancy-related genes.

2.2. Extraction and purification of RNA

The leaf buds were divided among three replicate samples of approximately 0.9 g each, with each of these replicate samples comprising about 30 buds. Each sample was separately ground in liquid nitrogen and used for independent extraction of total RNA by a hot borate extraction procedure (Wan and Wilkins, 1994). Approximately, 80 μg of each extracted total RNA sample was purified with an RNA clean-up kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol and then stored at −80 °C until needed for quality and purity checks in an Agilent 2100 Bioanalyzer (Palo Alto, CA, USA). RNA was precipitated by ethanol and resuspended at a concentration of 500 ng/μL for microarray analysis.

2.3. Labeling of array samples, hybridization, and signal normalization

The mRNAs in the total RNA samples were reverse-transcribed and the resultant cDNAs were labeled with a Low RNA Input Linear Amplification Kit PLUS, One-Color (Agilent, Palo Alto, CA), according to the manufacturer's instructions. The labeled samples were hybridized to custom oligoarray slides housing probes for 9812 non-redundant genes (Nishitani et al., 2010) with a Gene Expression Hybridization Kit (Agilent). The probes were designed from 11,540 representative ESTs from 24,945 sequences collected mainly from 11 'Housui' cDNA libraries (leaf bud, leaf, flower bud, flower (at pre-anthesis stage), flower (at full bloom), fruitlet at three developmental stages (immature fruit, fruit at optimum maturity for eating, and overripe fruit)) (Nishitani et al., 2009). After hybridization, arrays were washed with Gene Expression Wash Buffer (Agilent) according to the manufacturer's instructions. The dried slides were scanned on an Agilent scanner under the control of Agilent Feature Extraction software v. 9.5.1. In GeneSpring v. 7.3.1 software (Agilent), the expression of each gene was calculated as the mean of three biological replicates for each time point with the standard error of the mean (SEM), and normalized per chip and per gene.

2.4. Extraction of genes expressed differentially between endodormancy and ecodormancy stages

All expression patterns were analyzed by GeneSpring software. Genes expressed at low levels and with absent flags in at least one sample were removed. Genes that showed significant differences ($P < 0.05$) in expression (>2-fold change) between endodormant and ecodormant stages were extracted. The probes designed for the 3′ end of each cDNA sequence in the oligoarray proved reliable when compared with the results of semi-quantitative reverse-transcription PCR (Nishitani et al., 2010). Therefore, we used these probes for each gene in all of the analyses.

To annotate the genes, we selected homologous genes in the *Arabidopsis* database (TAIR9; ftp://ftp.arabidopsis.org/home/tair/Ontologies/Gene_Ontology) with high levels of sequence identity on the basis of BLASTX analysis, with the expected threshold value of 1e−10. Gene Ontology (GO) analyses were performed against the AmiGO (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi) and *Arabidopsis* GO Slim (ftp://ftp.arabidopsis.org/home/tair/Ontologies/Gene_Ontology/) databases.

To examine the changes in gene expression, we divided the genes that showed a ≥2-fold change between the endodormancy and ecodormancy stages into functional groups based on GO Slim (Table 1). GO Slim is a cut-down version of GO containing a subset of the terms. It gives a broad overview of the ontology content without the detail of the specific terms. GO Slim calculated the number of genes involved in each GO category (biological processes, molecular functions and cellular components) on the whole array and among the extracted genes and significant differences were confirmed by Fischer's exact test at $P < 0.05$.

2.5. Real-time PCR

Total RNA was isolated from the lateral leaf buds of 'Kousui' and the less dormant 'Hengshanli' collected as detailed above using a hot borate extraction method (Wan and Wilkins, 1994). The total RNA aliquot (1 μg) used in the first-strand cDNA synthesis reaction was first treated with DNase (Ambion, Austin, TX) and was reverse-transcribed using the High Capacity cDNA Transcription Kit (Life Technologies, Gaithersburg, MD, USA) based on random primers according to the manufacturer's protocol. Primers were designed based on our need to search for real-time PCR target