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Microarray analysis of gene expression patterns during fruit development in European pear (*Pyrus communis*)

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ABSTRACT

We used microarray analysis to search for novel ripening- or development-related genes in the fruits of European pear (*Pyrus communis*). Genes were classified into 20 clusters according to expression pattern. Several clusters showed stage-specific expression. Genes expressed until full bloom were dominated by photosynthesis-related genes. Genes expressed at 30 days after full bloom were dominated by those for the biosynthesis of flavonoids, lignin, and fatty acids. Since RNA from fruit skin was included in 30 days after full bloom, high expression of these genes might be due to fruit skin. Gene expressed during ripening were dominated by those for ethylene biosynthesis and cell wall modification. Among ripening-specific genes, one *cupin family protein* gene and two unannotated genes, which are not induced in Japanese pear (*Pyrus pyrifolia*), were induced in *P. communis*. These genes are likely to be involved in the ripening characteristics specific to *P. communis*. Our fruit flesh transcriptome data, spanning fruit development from before full bloom to the full ripe stage, will be helpful in studying fruit development and surveying genes important in flesh development in *P. communis*.

1. Introduction

Fruit development and ripening are complex processes. Several stages occur under the control of a large set of development- or ripening-specific genes. In climacteric fruit, an increase in ethylene production leads to high metabolic rates, a respiratory peak, and increased softening (Brady, 1987). Among *Pyrus*, there are several species with different ripening characteristics. Chinese pear (*Pyrus ussuriensis* Maxim, *Pyrus bretschneideri* Rehd.) shows increase in ethylene production, however, they do not show fruit softening (Hiwasa et al., 2004). Several Japanese pear (*Pyrus pyrifolia*) such as 'Nijisseiki' and 'Housui' produces low level of ethylene and shows low fruit softening (Itai et al., 2005). Although 'Housui' produces low level of ethylene, ethylene synthetic enzyme coding genes are induced when it ripens (Nishitani et al., 2010). On the other hand, some cultivars produce high level of ethylene and show short shelf life due to high level of fruit softening. European pear (*Pyrus communis* L.) shows high level of ethylene production, and then shows dramatic fruit softening. Although *P. communis* shows typical climacteric characteristics, it has particular ripening characteristics. Unlike other climacteric fruit, *P. communis* fruits on the tree generally do not develop the characteristic buttery, juicy texture expected by consumers (Murayama et al., 1998). They possess varying degrees of resistance to ripening at harvest even when harvested at the appropriate stage, and require a period of chilling or ethylene exposure to ripen properly.

During the ripening of *P. communis*, chilling treatment induces the expression of *1-aminocyclopropane-1-carboxylic acid oxidase* (*ACO*) and *1-aminocyclopropane-1-carboxylic acid synthase* (*ACS*) (El-Sharkawy et al., 2004), followed by cell-wall-modifying genes, such as *β-galactosidase* (Mwaniki et al., 2005), *α-arabinofuranosidase* (Mwaniki et al., 2007), *β-xylosidase*, *expansin*

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(Fonseca et al., 2004a), and *polygalacturonase* (Hiwasa et al., 2004). This enzyme activity softens the fruit flesh. Although genes related to ethylene biosynthesis and cell wall breakdown in *P. communis* have been well studied, other ripening characteristics and flesh development related genes have not.

Genes with stage-specific expression patterns are likely to control important developmental or ripening events. Microarray analysis can reveal such genes. Microarrays have been used to examine gene expression during development in strawberry (Aharoni et al., 2002), peach (Trainotti et al., 2006), apple (Lee et al., 2007; Schaffer et al., 2007; Janssen et al., 2008), citrus (Shimada et al., 2005; Fujii et al., 2008), grape (Deluc et al., 2007; Pilati et al., 2007; Grimplet et al., 2007), and kiwifruit (Crowhurst et al., 2008). In *P. communis*, however, the limited transcriptome profile has supported only a cDNA spot array with 1300 *P. communis* expressed sequence tags (ESTs) (Fonseca et al., 2004b). This array revealed several genes important in development and ripening, but had far fewer ESTs than microarrays of apple, grape, or citrus (~15 000–21 000; Fonseca et al., 2004b). Instead, a *P. pyrifolia* oligoarray with 11 540 ESTs (Nishitani et al., 2010) has been used for *P. communis* (Nishitani et al., 2012) because of the close genetic relationship.

Here, we used the same oligoarray to analyze the transcriptome of *P. communis* fruits. To search for genes specific to developmental stage, we analyzed fruit from 7 days before full bloom to harvest (162 days after full bloom: DAFB) and the ripe stage (after chilling treatment).

2. Materials and methods

2.1. Plant materials

Fruits (30, 68, 97, 132, and 162 DAFB) or flower bud (−7 and 0 DAFB) of *P. communis* 'La France' were collected from an orchard in Yamagata Prefecture, Japan. Fruits harvested at 162 DAFB were incubated at 2 °C for 12 days and then at room temperature (15–20 °C) for 20 days to ripen. At least three fruits were harvested on each date. Weights of corrected fruits or flower buds were measured. The petiole, skin, and seeds were removed, and the samples were frozen in liquid nitrogen and stored at −80 °C (except those collected at −7 and 0 DAFB). The skin could not be removed from fruit at 30 DAFB. Samples collected at −7 and 0 DAFB were dissected by laser microdissection to avoid contamination (Nashima et al., 2013).

2.2. RNA extraction

Total RNA was extracted from the receptacle of samples collected at −7 and 0 DAFB with a PicoPure RNA isolation kit (Molecular Devices, Sunnyvale, CA, USA), and quantified with a Quant-iT RiboGreen RNA reagent kit (Invitrogen, San Diego, CA, USA). Total RNA was extracted from later samples by the hot borate method as described by Wan and Wilkins (1994). All isolated RNA was purified with an RNeasy Mini Kit (Qiagen, Hilden, Germany).

The quality of the total RNA was assessed with an RNA 6000 Pico Kit (−7 and 0 DAFB) or an RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA) on an Agilent 2100 Bioanalyzer. Total RNA with an RNA integrity number of >5.0 (calculated with 2100 Expert software v. B.02.02; eukaryote total RNA pico mode; Agilent) was used for microarray analysis.

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

From total RNA (20 ng for −7 and 0 DAFB, 500 ng for others), mRNA was reverse-transcribed and labeled cRNA was synthesized with a Low Input Quick Amp Labeling Kit, one color (Agilent), according to the manufacturer's instructions. The labeled cRNA was hybridized to the *P. pyrifolia* custom 44K oligo microarray (GPL13124) with the Gene Expression Hybridization kit (Agilent) hybridization buffer at 65 °C for 17 h in a hybridization chamber. The oligoarray was designed from 11 540 ESTs collected mainly from 11 *P. pyrifolia* 'Housui' (syn. 'Hosui') cDNA libraries (leaf bud, leaf, flower bud, unopened flower, flower at full bloom, fruitlets at three developmental stages, immature fruit, fruit at optimum maturity for eating, and over-ripened fruit) (Nishitani et al., 2009).

After hybridization, the array slides were washed with Gene Expression Wash Buffer (Agilent), and the dried slides were scanned with an Agilent microarray scanner (G2505C). Raw scan data were captured by Agilent Feature Extraction software (v. 10.7.1.1). Three or four biological replicates were used at each stage.

2.4. Microarray analysis

The datasets were normalized in the Subio Platform software (Subio Inc., Tokyo, Japan), using low signal cutoff (mean raw signal < 100 at all stages), log-transformation, global normalization, and centering. Probes whose normalized signals were between −2 and 2 among all samples were removed. Cluster analysis by the *k*-means cluster analysis method was performed in the R software (http://www.r-project.org/).

Gene ontology (GO) categorization was performed according to the Arabidopsis annotation of each probe (Nishitani et al., 2010). Arabidopsis GO terms were obtained from The Arabidopsis Information Resource (TAIR, http://www.arabidopsis.org/) (ATH_GO_GOSLIM, updated 2013 May 15).

2.5. Real-time RT-PCR

Real-time RT-PCR was performed on an ABI 7300 system (Life Technologies, Gaithersburg, MD, USA) in accordance with the manufacturer's instructions. Random primers were used for first-strand cDNA synthesis.

Primer pairs for real-time RT-PCR were designed with the Primer3 Web interface (Rozen and Skaletsky, 2000; http://frodo.wi.mit.edu/primer3/input.htm). Primer-picking conditions used a primer size of 20–22 bp, a primer melting temperature of 60–63 °C (optimum 62 °C), and a product size range of 100–200 bp. After confirmation that each cDNA was amplified with the same efficiency by each primer pair, *EF1α* (NCBI accession FS999622, Nishitani et al., 2012) was used as the reference gene.

3. Results

3.1. Development of fruit

European pear fruit showed active enlargement from 30 to 68 DAFB, and it continued until harvest stage (Fig. 1). Generally, active cell division occurs before active cell enlargement starts. Ripening started after the chilling treatment.

3.2. Genes expressed during fruit development

The *k*-means cluster analysis of 11,854 genes categorized 3416 into 20 sets (Fig. 2). Sets 2, 3, 4, 5, and 9 showed gradual decreases in expression, and Sets 13, 18, and 19 showed gradual increases. Several sets showed stage-specific expression patterns: Set 1, high expression until full bloom; Set 2, high expression until 30 DAFB; Set 8, low expression at the ripe stage; Set 15, high expression at 30 and 68 DAFB; Sets 16 and 17, high expression at 30 DAFB; Set 20, high expression at the ripe stage.