RESEARCH PAPER

**PpYUC11**, a strong candidate gene for the stony hard phenotype in peach (*Prunus persica* L. Batsch), participates in IAA biosynthesis during fruit ripening

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Abstract

High concentrations of indole-3-acetic acid (IAA) are required for climacteric ethylene biosynthesis to cause fruit softening in melting flesh peaches at the late ripening stage. By contrast, the fruits of stony hard peach cultivars do not soften and produce little ethylene due to the low IAA concentrations. To investigate the regulation of IAA accumulation during peach ripening [the transition from stage S3 to stage S4 III (climacteric)], a digital gene expression (DGE) analysis was performed. The expression patterns of auxin-homeostasis-related genes were compared in fruits of the melting flesh peach 'Goldhoney 3' and the stony hard flesh peach 'Yumyeong' during the ripening stage. It is revealed here that a YUCCA flavin mono-oxygenase gene (*PpYUC11*, ppa008176m), a key gene in auxin biosynthesis, displayed an identical differential expression profile to the profiles of IAA accumulation and *PpACS1* transcription: the mRNA transcripts increased at the late ripening stage in melting flesh peaches but were below the limit of detection in mature fruits of stony hard peaches. In addition, the strong association between intron TC microsatellite genotypes of *PpYUC11* and the flesh texture (normal or stony hard) is described in 43 peach varieties, indicating that this locus may be responsible for the stony hard phenotype in peach. These findings support the hypothesis that *PpYUC11* may play an essential role in auxin biosynthesis during peach fruit ripening and is a candidate gene for the control of the stony hard phenotype in peach.

Key words: Auxin, ethylene, microsatellite, *Prunus persica* L. Batsch, ripening, stony hard, YUCCA flavin monoxygenase.

Introduction

Peach (*Prunus persica* L. Batsch) is a climacteric fruit in which the burst of ethylene production occurs at the ripening stage (Tonutti et al., 1991, 1997). Peach fruits generally soften rapidly after harvest, causing difficulties in postharvest handling and quality maintenance during storage and transportation (Yoshioka et al., 2010). Improving the fruit texture quality
of peaches is one of the most important goals of cultivar development and selection, superseded only by appearance and harvest season (Gallardo et al., 2012). Based on the characteristics of fruit firmness and textural changes during ripening, peach cultivars can be classified into three groups: melting (M), non-melting (NM), and stony hard (SH) flesh types (Bailey and French, 1932; Yoshida, 1976; Haji et al., 2005). The M phenotype shows a prominent softening at advanced stages of ripening and develops a complete melting texture, whereas NM-textured peaches soften slowly when overripe and never melt. Peaches of the SH type, the focus of the present study, have very firm and crisp flesh at ripening (both on- and off-tree), although they change colour normally and contain a high level of soluble solids (Haji et al., 2001, 2004).

Endopolygalacturonase (endo-PG), the enzyme responsible for cleaving polygalacturonic acid chains (pectins) from the cell wall, is thought to be responsible for the M/NM flesh texture (Lester et al., 1996; Callahan et al., 2004). This trait is controlled by the ‘melting’ locus, where the melting (M) is dominant over the non-melting (m) (Bailey and French, 1932). A high level of ethylene production (in the late ripening phase) characterizes the M and NM varieties. This phase, however, is absent in the SH phenotype which is thought to contribute to the inhibition of fruit softening in stony hard peaches because endogenous ethylene softens the flesh effectively; thus, the fruit softens more rapidly when a higher concentration of ethylene is applied (Haji et al., 2003; Hayama et al., 2003, 2006). Treatment of SH fruits with 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene, stimulated the fruits to synthesize ethylene and to soften (Haji et al., 2003). These results indicate that a low activity of ACC synthase is responsible for the inhibition of ethylene production and that ACC oxidase and the ethylene receptor function normally in SH peaches (Haji et al., 2003). Genetic analysis suggested that the Stony hard trait is controlled by a single recessive gene (hid) (Yoshida, 1976) and is inherited independently of the M/m flesh locus (Haji et al., 2005).

It has been assumed that transcription suppression of PpACSI in the SH phenotype is responsible for its low ethylene production (Tatsuki et al., 2006); however, mRNA transcripts of PpACSI have been detected in senescing flowers and wounded leaves as well as in immature fruits and cold-treated mature fruits of the SH phenotype (Tatsuki et al., 2006; Begheldo et al., 2008). These results indicate that stress conditions (wounding or low temperature) seem to be effective in overcoming PpACSI inhibition that has been ascribed in SH peaches to a disruption of a transcriptional factor specifically activated at ripening (Tatsuki et al., 2006). A recent study suggested that the low IAA concentrations in SH peaches may contribute to the suppression of PpACSI because the application of exogenous 1-naphthalene acetic acid (NAA), a synthetic auxin, to stony hard peaches induced a high level of PpACSI expression, the production of a large amount of ethylene, and softening (Tatsuki et al., 2013).

Multiple mechanisms regulating auxin-homeostasis have been recognized in plant organs, such as auxin metabolism (biosynthesis and conjugation) and carrier-dependent intercellular and intracellular auxin transport (Rosquete et al., 2012). In many cases, de novo IAA biosynthesis, primarily from tryptophan, is the most important source of free auxin in the plants. Genetic and biochemical studies have revealed that, in Arabidopsis and maize, auxin biosynthesis is controlled by a two-step pathway: tryptophan is first converted into indole-3-pyruvate (IPA) by the TAA family of aminotransferases, and, subsequently, IAA is produced from IPA by the YUCCA family of flavin monoxygenases (Mashiguchi et al., 2011). In addition to de novo synthesis, plants produce active IAA by hydrolysing IAA conjugates. Three major forms of auxin conjugates exist in higher plants, including IAA–amino acid conjugates, IAA–sugar conjugates, and IAA–peptide conjugates (Ludwig-Müller, 2011). Group II of the GRETCHEN HAGEN3 (GH3) family facilitates IAA to amino acids (Staswick et al., 2005) whereas the IAA-LEUCINE RESISTANT 1 (ILR1)-like family of amidohydrolases hydrolyse IAA–amino acid conjugates to free IAA (Bartel and Fink, 1995; Davies et al., 1999; LeClerc et al., 2002). Our knowledge of the metabolism of IAA–sugar conjugates and IAA–peptide conjugates is far less detailed than our knowledge of the metabolism of IAA–amino acid conjugates. UDP glucosyltransferases, such as UGT84B1 in Arabidopsis (Jackson et al., 2001) conjugate IAA to glucose. The conversion back to IAA of the presumed storage form of auxin, IBA, is catalysed by the action of peroxisomal β-oxidation enzymes, the IBRs (INDOLE-3-BUTYRIC ACID RESPONSE) (Zolman et al., 2008). In addition, the spatio-temporal distribution of auxin depends on cell-to-cell auxin transport. A few studies have demonstrated that AUXIN RESISTANT 1 (AUX1)-mediated auxin influx and PIN-FORMED 1 (PIN1)-mediated auxin efflux played a key role in the regulation of organ-level auxin transport (Bennett et al., 1996; Gälweiler et al., 1998; Müller et al., 1998; Li et al. 2011; Friml et al., 2002a, b). The low IAA concentration at the late-ripening stage of stony hard peaches would be determined by the result of one or the interaction of more mechanisms above (see Supplementary Fig. S1 at JXB online).

The above introduction indicates that auxins are involved in peach softening and that stony hard peach cultivars do not soften due to low IAA concentrations. To date, the molecular mechanism underlying this phenomenon has remained a mystery. Recently, digital gene expression profiling was conducted using deep-sequencing in melting/stony hard flesh peaches, and the results provide opportunities for investigating genes involved in IAA concentrations during peach ripening. It is reported that one gene in particular, PpYUC11, appears to be an excellent candidate gene for the stony hard phenotype in peaches, and it is proposed that the intronic TC microsatellite sequence variant controls the expression of this gene. Our study is not only helpful for understanding the molecular mechanism of IAA accumulation during peach ripening but also useful for the marker-assisted breeding of new peach cultivars with better preservability.

Materials and methods

Plant materials, sample collection, and NAA treatments

3’, ‘Hakuho’, ‘Hakoto’, ‘Hangzhou Zhao Shui Mi’, ‘Kawanakajima Hakuto’, ‘Li Chun’, ‘Matsumori’, ‘May fire’, ‘NJCl05’, ‘NJCl12’, ‘NJCl19’, ‘NJCl47’, ‘NJC77’, ‘Okitsu’, ‘Okubo’, ‘Pan Tao Huang Hou’, ‘Spring Snow’, ‘Springtime’, ‘Sunago Wase’, ‘Toobo’, ‘Yu Hua Lu’, ‘Zao Feng Wang’, ‘Zao Lu Pan Tao’, ‘Zhong Pan Tao 1’, and ‘Zhong Pan Tao 2’) and seven accessions of stony hard cultivars (‘CN 16’, ‘Hua Yu’, ‘Jing Yu’, ‘Qing Wang’, ‘Shi Jia Zhuang’, ‘Xia Cui’, and ‘Yumyeong’) were analysed in this study. All peach accessions were grown at the experimental farm of the National Fruit Tree Germplasm Repository, Zhengzhou Fruit Research Institute, Chinese Academy of Agricultural Sciences (Zhengzhou, China). Leaves, stems, and stem tips of ‘Goldhoney 3’ at the vegetative growth stage as well as flowers at the full bloom stage were collected during the spring of 2014. Fruit growth stages were defined according to Tomutti et al. (1997) and Gabotti et al. (2015). At the desired times (from the S3 to the S4 stage at eight time points), fruits of ‘Goldhoney 3’ and ‘Yumyeong’ were picked and quickly transferred to the laboratory for the determination of weight and ripening parameters (ethylene production, flesh firmness, and soluble solids content). Fruit mesocarp tissues and seeds (only for ‘Goldhoney 3’) at 106, 110, 115, 122, 127, 131, 134, and 140 days after full bloom (DAFB) were sampled. The auxin treatment was performed by dipping mature ‘Yumyeong’ whole fruits into 15 mM NAA (1-naphthalene acetic acid) mixed with 500 ppm Tween 20. All samples were immediately frozen in liquid nitrogen and then stored at −80 °C for DNA or RNA extraction.

Ethylene production, flesh firmness, and soluble solids content (SSC)

Ethylene production and flesh firmness were measured as described previously (Zeng et al., 2015). After the measurement flesh firmness, part of the mesocarp was squeezed and SSC was measured with an Atago digital refractometer (Atago, Tokyo, Japan).

Extraction and UPLC-MS/MS analysis of IAA in the peach mesocarp

Extraction and purification of IAA in mesocarp samples were performed according to a previously described method (Zourelioudou et al., 2009, Tatsuki et al., 2013), with minor modifications. To determine the IAA content in each sample, the UPLC-MS/MS analyses were carried out as previously described by Fu et al. (2012).

RNA extraction and quantitative real-time PCR

To validate the differential expression identified by DGE-seq or to detect the expression of other genes, quantitative reverse-transcription PCR (qRT-PCR) was performed as described previously (Zeng et al., 2015). Eleven auxin-homeostasis-related genes with different expression patterns during the late ripening stage between the normal and stony hard flesh phenotypes were chosen for validation and the specific primers used to quantify gene expression levels are listed in Supplementary Table S1 at JXB online. The housekeeping genes have been described by Tatsuki et al. (2013). All gene expression analyses were performed with three independent biological replicates.

Phylogenetic analysis

The amino acid sequences of the YUCCA family of flavin monooxygenases from Prunus persica and Arabidopsis thaliana were used for phylogenetic analysis. Multiple sequence alignment was carried out using ClustalX version 1.83 (Thompson et al., 1997) and adjusted manually as necessary and the phylogenetic tree was generated by the Neighbor–Joining method using MEGA version 5 (Tamura et al., 2011). Bootstrap values were calculated from 1 000-replicate analyses.

Digital gene expression profiling (DGE) library preparation, Illumina RNA-sequencing, and data processing

Total RNA was extracted using a Plant Total RNA Isolation Kit (Sangon, China) according to the manufacturer’s instructions. RNA concentrations were measured using a NanoPhotometer spectrophotometer (IMPLEN, CA, USA), and the integrity was confirmed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). DGE libraries were generated using the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer’s recommendations and index codes were added to attribute sequences to each sample. Each library’s quality was then assessed on the Agilent Bioanalyzer 2100 system. The library preparations were sequenced on an Illumina HiSeq 2000 platform. For gene expression analysis, the number of expressed tags was calculated and then normalized to RPM (Reads Per Kilobase of exon model per Million mapped reads) (Mortazavi et al., 2008).

Retrieval of auxin-homeostasis-related gene sequences

Auxin-homeostasis-related gene sequences of Arabidopsis thaliana were identified from the Arabidopsis Information Resource (TAIR, https://www.arabidopsis.org/, last accessed: 18 August 2015) and auxin-homeostasis-related gene sequences of peach were obtained through the retrieval of Prunus persica v1.0 assembly at the Genome Database for Rosaceae (Verde et al., 2013) (GDR, http://www.rosaceae.org/, last accessed: 18 August 2015) or by BLASTP search at the National Center for Biotechnology (http://www.ncbi.nlm.nih.gov/blast/, last accessed: 18 August 2015). All sequences and their accession numbers are provided in Supplementary Table S3 at JXB online.

Detection of intronic TC microsatellite variations

Genomic DNA was extracted using the modified CTAB procedure (Cheng et al., 1997). The concentration of DNA was determined with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and diluted to 25 ng μl−1 to carry out PCR amplification reactions. The DNA samples were then used as PCR templates for microsatellite region amplification. The intronic TC microsatellites were amplified using primers: 5′-CTATCTGGTATATAAGCTGAAACG-3′ and 5′-ACCTTTTT AGTTATTTTCACACAG-3′. The PCR reactions were performed using KOD-Plus-DNA polymerase (Toyobo, Osaka, Japan) according to the manufacturer’s instructions. The DNA amplification products were loaded on to 10% polyacrylamide gels. The gels were silver stained according to the protocol described by Bassam et al. (1991).

Results

Fruit phenotype during ripening: ethylene production, flesh firmness, and soluble solids content

Prunus persica fruits of ‘Goldhoney 3’ and ‘Yumyeong’ cultivars were picked at the desired ripening stages: transition from stage S3 (second exponential growth phase) to stage S4 (climacteric). The two cultivars have different fruit flesh texture: ‘Goldhoney 3’ is a melting type that softens rapidly during ripening, and ‘Yumyeong’ is a stony hard type that remains firm during ripening. Fruits from the end of stage S3 to stage S4 were measured for ethylene production, flesh firmness, and soluble solids content (°Brix, an approximate
measure of the mass ratio of dissolved solids, mostly sucrose, to water in fruit juices) (Fig. 1). Ethylene production was low in ‘Goldhoney 3’ fruit before 110 DAFB (stage S3) and it sharply increased upon reaching stage S4 whereas, in ‘Yumyeong’, ethylene production was sustained at a low level during the same period (Fig. 1A). Flesh firmness decreased substantially in ‘Goldhoney 3’ between 110 and 130 DAFB, but it decreased moderately in ‘Yumyeong’ during the same period (Fig. 1B). In ‘Goldhoney 3’ and ‘Yumyeong’, SSC was 11 °Brix at 105 DAFB; increased gradually to a peak value of 15 or 14 °Brix at 122 DAFB for the two cultivars, respectively; and then gradually decreased; the maximum SSC attained in ‘Goldhoney 3’ was higher than that of ‘Yumyeong’ (Fig. 1C). The similarity of these SSC time series is consistent with the fact that both cultivars have an identical maturity stage in Zhengzhou.

Depending on phenotypic parameters, four sampling points covering the climacteric stage at 110, 115, 122, and 127 DAFB (named S3, S4 I, S4 II, and S4 III, respectively, and marked with arrows in Fig. 1C) were selected for IAA concentration and digital gene expression (DGE) analysis.

**IAA concentration and digital gene expression (DGE) analysis of auxin-homeostasis-related genes**

Previous studies have shown that IAA levels significantly increased in a melting flesh cultivar at the climacteric stage, although the IAA concentration in the mesocarp tissue of a stony hard peach cultivar were low and did not increase (Tatsuki et al., 2013). Before the expression patterns of auxin-homeostasis-related genes in both cultivars during fruit ripening were analysed, IAA concentrations were measured with the same samples at stages S3, S4 I, S4 II, and S4 III in ‘Goldhoney 3’ and ‘Yumyeong’ (Fig. 1C). Whereas in ‘Goldhoney 3’ fruits, the IAA levels sharply increased from stage S3 to stage S4 III, the IAA levels did not increase, and the final IAA concentration was 1.049 ng g⁻¹ fresh weight in ‘Yumyeong’ fruits (Fig. 2D).

To obtain the expression profiles of auxin-homeostasis-related genes for the melting peach ‘Goldhoney 3’ and the stony hard peach ‘Yumyeong’ during the late ripening stages, total RNAs were isolated from ‘Goldhoney 3’ and ‘Yumyeong’ mesocarps at stages S3, S4 I, S4 II, and S4 III (Fig. 1C). Differential gene expression profiling was performed using high-throughput Tag-seq analysis to examine the auxin-homeostasis-related gene profiles. DGE sequencing analyses of ‘Goldhoney 3’ and ‘Yumyeong’ samples were performed on the Illumina HiSeq™ 2000 sequencing platform. ‘Goldhoney 3’ (S3, S4 I, S4 II, and S4 III) and ‘Yumyeong’ (S3, S4 I, S4 II, and S4 III) produced 7 111 569, 8 765 279, 8 115 631, 8 477 817, 8 991 034, 8 839 334, 8 479 265, and 9 126 943 clean reads, respectively. The read count data were used to analyse the differences in gene expression between the two cultivars. To estimate the gene expression levels, the read counts were transformed into RPKM (Mortazavi et al., 2008); RPKM >1 is defined as the threshold of significant gene expression. The full list of normalized expression of 28 702 peach genes is shown in Supplementary Table 2 at JXB online.

![Fig. 1.](https://academic.oup.com/jxb/article-abstract/66/22/7031/2893269)

In total, 51 auxin-homeostasis-related genes were identified from the peach genome: 12 auxin biosynthesis genes (9 YUC genes and 3 TAA genes), 27 auxin conjugation genes (9 ILL genes, 8 GH3 genes, 3 UGT genes, and 7 IBR genes), and 12 auxin transport genes (8 PIN genes and 4 AUX1 genes). Based
**PpYUC11** is the strong candidate gene for stony hard phenotype in peach on our DGE data, 34 genes out of 51 total auxin-homeostasis-related genes have significant gene expression (RPKM >1) in at least one sampling point (Fig. 2A; see Supplementary Table S3 at JXB online), constituting the subset of candidate genes involved in controlling IAA concentration in peach fruit during the ripening stage. To identify the gene or genes putatively involved in IAA accumulation during melting peach ripening, the expression profiles of these auxin-homeostasis-related genes were compared with two peach ACS and two endopolygalacturonase (endo-PG) genes by DGE (Fig. 2A).

In ‘Goldhoney 3’, the expression level of ACC synthase gene *PpACS1* (ppa004774m) increased towards harvest time along with other ripening-related genes [ppa006839m (endo-PG); ppa006857m (endo-PG)]. By contrast, the expression level of *PpACS1* in the stony hard peach stayed low and did not increase during ripening (Fig. 2A, B; see Supplementary Table S3 at JXB online). A total of 34 auxin-homeostasis-related genes were significantly expressed and their expression profiles were hierarchically clustered with that of *PpACS1* and endo-PG (Fig. 2A). Interestingly, six auxin-homeostasis-related genes were found to cluster closely with *PpACS1*: one *PIN* gene (ppa002944), one *IBR* gene (ppa107000m), one *ILL* gene (ppa005951m), two *GH3* genes (ppa003134m and ppa003126m), and one *YUC* gene (ppa008176m) (Fig. 2A).

Among these, two genes—the *YUC* gene (ppa008176m) and one of the *GH3* genes (ppa003126m)—showed an identical expression pattern to *PpACS1* for both cultivars (Fig. 2C).

To confirm the gene expression changes observed by DGE analysis, quantitative PCR analysis was performed on a selection of differentially expressed auxin-homeostasis-related genes. Overall, the quantification of 11 auxin-homeostasis-related genes by qRT-PCR exhibited close agreement with DGE-seq results (see Supplementary Fig. S2 at JXB online).
Structural features and polymorphic loci analysis of ppa008176m (PpYUC11)

Based on the observation that the expression patterns of ppa008176m (named PpYUC11, Fig. 7) and IAA concentrations of the two cultivars (Fig. 2) showed high levels of consistency, a detailed analysis was undertaken of the PpYUC11 locus variations using the hypothesis that PpYUC11 structural variation may cause differential expression of PpYUC11 between the two cultivars. PpYUC11 contains four exons, located in pseudomolecule 6 and is near the centromeric regions in the peach genome sequence (Fig. 4A, B). The PpYUC11 gene was re-sequenced from a set of homozygous cultivars. The analysed regions covered the entire transcribed region as well as the 1.7 kb sequence upstream of the start codon (the promoter region). At least 12 polymorphic loci (Fig. 4B, P1–P12), were found in the PpYUC11 gene using a combination of cloning and direct Sanger sequencing of PCR products: within the promoter region, a 25 bp insertion/deletion (indel) and two SNPs are present in PpYUC11 at positions -1068, -835, and -279 bp of the published sequence of GenBank, respectively (Fig. 4B; see Supplementary Fig. S3A at JXB online); in intron 1, a 19 bp indel, a TC dinucleotide microsatellite, and two SNPs are located at 852, 907, 910, and 1523 bp, respectively (Fig. 4B; see Supplementary Fig. S3B at JXB online); in addition, the coding region (exon 3 and exon 4) of the PpYUC11 gene contains two nonsense mutation SNPs at 2104 and 2364 bp (Fig. 4B; see Supplementary Fig. S3B at JXB online). Of these variations, the TC dinucleotide microsatellite is located just after the 19 bp indel region at positions 907–964 of the GenBank reference sequence of intron 1 of PpYUC11 gene (see Supplementary Fig. S3B at JXB online).

PpYUC11 allele distribution in peach germplasm

The sequencing of a PCR-amplified DNA fragment from a set of homozygous cultivars confirmed that the distribution of all PpYUC11 variations can be found in both flesh types, with the exception of the TC dinucleotide microsatellite (see Supplementary Fig. S3B at JXB online). To investigate the relationship between the TC microsatellite genotype of PpYUC11 and the phenotype of flesh texture (normal or stony hard), the distribution of the polymorphic loci was analysed in 36 normal and seven stony hard flesh cultivars (Table 1). To maximize genetic diversity, this set included nine American cultivars, 24 Chinese cultivars, nine Japanese cultivars, and one Korean cultivar.

![Image](https://academic.oup.com/jxb/article-abstract/66/22/7031/2893269)
PpYUC11 is the strong candidate gene for stony hard phenotype in peach.

In our results, (TC)\textsubscript{n} microsatellites were found in at least four different lengths (the repeat number ‘n’ of Hv\textsubscript{1}, Hv\textsubscript{2}, Hv\textsubscript{3}, and hd are 29, 26, 24, and 20, respectively) in these peach cultivars (Fig. 4C). The diversity of microsatellite length variants exhibited dramatic differences between the 36 normal and the seven stony hard flesh peach cultivars. All seven stony hard peaches were homozygous for hd. By contrast, all of the normal peach cultivars had at least one Hv allele (Hv\textsubscript{1}, Hv\textsubscript{2} or Hv\textsubscript{3}). These results are summarized in Table 1.

Transcriptional analysis of PpYUC11 alleles

The TC microsatellite identified to be responsible for the stony hard flesh phenotype appears to have an effect on transcriptional regulation of the gene. Direct Sanger sequencing was used to estimate the transcript levels of both alleles of PpYUC11 in the mesocarp tissue of stage S4 in several peach varieties that are heterozygous at the TC microsatellite locus. For instance, sequencing of genomic DNA showed that ‘Goldhoney 3’ carries two different alleles at its PpYUC11 locus: Hv (n=24), thymidine nucleotide (T) existing at position 2364bp, and hd with a cytidine nucleotide (C) at position 2364bp within the fourth exon of the PpYUC11 gene (Fig. 5A). By sequencing the PCR products, it was found that, whereas this SNP is heterozygous in the genomic DNA of ‘Goldhoney 3’ (both C and T), by contrast, it is homozygous in the cDNA in the mesocarp of S4 fruit (Fig. 5B), supporting the hypothesis that the TC microsatellite is responsible for the transcriptional activity of PpYUC11 and for IAA accumulation in ripening fruits and that PpYUC11 is the candidate gene controlling the stony hard phenotype in peach.

Co-ordinated variation of PpYUC11 expression, IAA concentration, PpACS1 expression, ethylene production, and fruit firmness in ripening peaches

A total of 12 peach cultivars with different genotypes in the PpYUC11 SSR loci were selected and used to investigate the relationship between PpYUC11 expression, IAA concentration, PpACS1 expression, ethylene production, and fruit firmness in the mesocarp of fruits during the ripening transition stage (S3–S4). These cultivars belong to five genotypes: ‘Xia Cui’ and ‘CN16’ is hd/hd; ‘Arctic Star’ and ‘Goldhoney 1’ is Hv\textsubscript{1}/Hv\textsubscript{3}; ‘Elberta’, ‘Kurakato Wase’, ‘Spring Honey’, and ‘Spring Snow’ is Hv\textsubscript{3}/Hv\textsubscript{3}; ‘Bao Lu’ and ‘Matsumori’ is Hv\textsubscript{2}/Hv\textsubscript{2}; and ‘CN9’ is Hv\textsubscript{1}/Hv\textsubscript{1} (Fig. 6A). For each cultivar, three samples from stage S3 to stage S4 were collected for further study. Overall, PpYUC11 and PpACS1 transcripts were highly expressed in the mesocarp of normal fleshed S4 fruits, but were either low or undetectable in the mesocarp of stony hard or S3 fruits (Fig. 6B, C). These expression profiles were accompanied by similar IAA content profiles or ethylene production in the mesocarp of these peach cultivars (Fig. 6B, C). Flesh firmness decreased towards harvest time along with ethylene emissions (Fig. 6D). These findings further confirmed that PpYUC11 may regulate the IAA content in the mesocarp during the fruit ripening stage and may lead to different ethylene emissions and firmness of the peach fruits.
Table 1. Allelic variants of PpYUC11 as related to phenotype in various melting, non-melting, and stony hard flesh peach genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
<th>ppa008176m_SSR</th>
<th>Geographical origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>May fire</td>
<td>Melting</td>
<td>Hd/Hd&lt;sup&gt;3&lt;/sup&gt;</td>
<td>USA(B)</td>
</tr>
<tr>
<td>NJC19</td>
<td>Non-melting</td>
<td>Hd/Hd&lt;sup&gt;3&lt;/sup&gt;</td>
<td>USA(B)</td>
</tr>
<tr>
<td>NJC47</td>
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<td>Hd/Hd&lt;sup&gt;3&lt;/sup&gt;</td>
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<tr>
<td>NJC77</td>
<td>Non-melting</td>
<td>Hd/Hd&lt;sup&gt;3&lt;/sup&gt;</td>
<td>USA(B)</td>
</tr>
<tr>
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<td>Non-melting</td>
<td>Hd/Hd&lt;sup&gt;3&lt;/sup&gt;</td>
<td>USA(B)</td>
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<tr>
<td>NJC112</td>
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<td>USA(B)</td>
</tr>
<tr>
<td>Springtime</td>
<td>Melting</td>
<td>Hd/Hd&lt;sup&gt;3&lt;/sup&gt;</td>
<td>USA(B)</td>
</tr>
<tr>
<td>Spring Snow</td>
<td>Melting</td>
<td>Hd/Hd&lt;sup&gt;3&lt;/sup&gt;</td>
<td>USA(B)</td>
</tr>
<tr>
<td>Elberta</td>
<td>Melting</td>
<td>Hd/Hd&lt;sup&gt;3&lt;/sup&gt;</td>
<td>USA(B)</td>
</tr>
<tr>
<td>Okitsu</td>
<td>Melting</td>
<td>Hd/Hd&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Japan(B)</td>
</tr>
<tr>
<td>Azumo</td>
<td>Melting</td>
<td>Hd/Hd&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Japan(B)</td>
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<tr>
<td>Hakuro</td>
<td>Melting</td>
<td>Hd/Hd&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Japan(B)</td>
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<td>Toobo</td>
<td>Melting</td>
<td>Hd/Hd&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Japan(B)</td>
</tr>
<tr>
<td>Okubo</td>
<td>Melting</td>
<td>Hd/Hd&lt;sup&gt;3&lt;/sup&gt;</td>
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Sequence variation in the SSR locus was analysed by means of microsatellite genotyping of PCR products. B, breeding material; L, landraces.

**YUCCA flavin monooxygenase family genes in peach**

The peach genome contains nine predicted YUC-like genes (Table 2; Fig. 7A). Based on the DGE analysis data, both *PpYUC10* (ppa007054m) and *PpYUC11* (ppa008176m) are enriched in the fruit ripening stage of ‘Goldhoney 3’, but *PpYUC11* cannot be detected in ‘Yumyeong’ fruit during the same period (Fig. 2; see Supplementary Table S3 at JXB online). The expression of the other seven YUC-like genes in this family (*PpYUC1, 2, 3, 4, 6, 8, and 9*) could not be detected in selected tissues of the two cultivars (see Supplementary Table S3 at JXB online).

To uncover the roles for each YUC gene in plant development, the varied expression profiles of the nine YUC genes were analysed during vegetative and reproductive development of ‘Goldhoney 3’ using qRT-PCR (Fig. 7C). *PpYUC1*, the gene with the highest sequence identity to *PpYUC4* in peach (Fig. 7A), shows expression patterns similar to that of *PpYUC4* in the stem, leaf, flower, and seed (Fig. 7C). Among the nine predicted YUC genes, the expression of *PpYUC1, PpYUC4, PpYUC6, PpYUC8*, and *PpYUC10* could be detected in almost all 12 tissues, of which *PpYUC1, PpYUC4, PpYUC6*, and *PpYUC8* accumulated preferentially in the vegetative tissues (stem and leaf) and seeds, and young fruit. *PpYUC10* were also enriched in the developing fruit (stages S1 through S4) (Fig. 7B). For *PpYUC3*, however, mRNA accumulation was detected only in the leaves. *PpYUC9* showed expression only in the leaves and seeds of stage S1, whereas *PpYUC2* was detected only in the leaves and flowers. Interestingly, *PpYUC11* was expressed at various stages in the seeds of ‘Goldhoney 3’ and ‘Yumyeong’ (Fig. 7B, C). However, only in the melting flesh fruit ‘Goldhoney 3’ did *PpYUC11* show obvious tissue-specific expression in S4 fruits (Fig. 7B). This result is consistent with the hypothesis that *PpYUC11* is responsible for IAA accumulation in the flesh of fruits at the ripening stage and is a candidate gene controlling the stony hard phenotype.

**Discussion**

Fruit firmness is essential for efficient harvesting, handling, marketing, and storage (Byrne et al., 2012). Due to the potential for the development of firmer peaches with tree-ripe flavour and longer storage life, the inheritance and physiological characteristics of a stony hard flesh texture have been extensively investigated for many years (Yoshida, 1976; Haji et al., 2003, 2005; Tatsuki et al., 2006, 2013; Begheldo et al., 2008). Because IAA concentration may control ethylene production (Tatsuki et al., 2013) and, in turn, the process of fruit softening in normal flesh peaches, our study investigated the expression patterns of auxin-homeostasis-related genes in the melting flesh peach ‘Goldhoney 3’ and stony hard peach ‘Yumyeong’ during the fruit ripening stage.

The distribution of auxin in plant organs is tightly controlled through synthesis, inactivation, and transport, and key regulatory steps and regulation mechanisms controlling auxin homeostasis have been extensively reviewed (Rosquete et al., 2012; Korasick et al., 2013; Sauer et al., 2013). Differential accumulation patterns of IAA in ‘Goldhoney 3’ and ‘Yumyeong’ became evident from late stage S3, and reached a maximum at stage S4 III, by which time ‘Goldhoney 3’ fruits had accumulated approximately 10-fold more IAA than ‘Yumyeong’ fruits (Fig. 2D). Accordingly, the two cultivars showed strikingly different developmental
expression patterns of auxin-homeostasis-related genes (Fig. 2A). However, the differential expression of several auxin-homeostasis-related genes appeared to be the effect, rather than the cause, of the differential IAA accumulation. For instance, in ‘Goldhoney 3’, the up-regulation of GH3 (ppa003134m), GH3 (ppa003126m), and PIN (ppa002944m) at late ripening stages appears to be caused by the high IAA levels in this phenotype because the expression levels of these genes increased in NAA-treated mature ‘Yumyeong’ fruit (Fig. 3), which is consistent with previous findings that GH3 and PIN are auxin-responsive genes (Peer et al., 2004; Terol et al., 2006). Previous genetic studies demonstrated that the flavin monooxygenases of the YUC family are key enzymes in Trp-dependent auxin biosynthesis (Cheng et al., 2007). In the present study, a YUCCA (YUC) flavin monooxygenase gene, PpYUC11, showed an identical expression pattern to both PpACS1 (Fig. 2C) and IAA accumulation (Fig. 2D) in both ‘Goldhoney 3’ and ‘Yumyeong’, suggesting that the low IAA concentrations at the late ripening stage of stony hard peaches may result from the suppressed expression of PpYUC11.

An examination of 43 peach genotypes allowed the identification of 12 polymorphisms within the PpYUC11 gene as well as in the 1.7 kb upstream flanking sequences: (i) a 25 bp indel and two SNPs upstream of the start codon; (ii) a 19 bp indel, a TC microsatellite, and two SNPs located in intron 1; (iii) an adenine nucleotide (A) indel and two SNPs located in intron 2; and (iv) two nonsense mutation SNPs in exon 3 and exon 4. Remarkably, the presence of a (TC)$_{20}$ microsatellite (an allele named hd) appears to be associated with stony hard cultivars when present in a homozygous state, whereas normal-fleshed (M or NM) cultivars possess at least one other allele (Hd$^e$, Hd$^d$ or Hd$^f$) at the PpYUC11 locus (Table 1). The transcriptional analysis of the two alleles of PpYUC11 in the heterozygous variety ‘Goldhoney 3’ and the co-ordinated variation analysis of PpYUC11 expression, IAA concentration, PpACS1 expression, ethylene emission, and fruit firmness in ripening peaches lends support to the hypothesis that PpYUC11 is a strong candidate gene for the control of the stony hard phenotype in peach.

Microsatellites, or simple sequence repeats (SSRs), represent a unique type of tandemly repeated genomic sequences (iterations of 1–6 bp nucleotide motifs), which are abundantly distributed across genomes and demonstrate high levels of allele polymorphism (Chistiakov et al., 2006). SSRs typically represent selectively neutral DNA markers. However, numerous lines of evidence have demonstrated that microsatellites are non-randomly distributed across protein coding regions, 3’-UTRs, 5’-UTRs, and introns because microsatellites can affect chromatin organization, the regulation of gene activity, recombination, DNA replication, the cell cycle, and the mismatch repair (MMR) system (see the review by Li et al., 2004). For instance, a tetranucleotide polymorphic microsatellite located in the first intron of the tyrosine hydroxylase gene regulates its gene expression (Meloni et al., 1998). In the present study, both the (TC)$_{24}$ and (TC)$_{20}$ forms of a TC dinucleotide polymorphic microsatellite localized in the first intron of PpYUC11 were found heterozygously in the...
genomic DNA of ‘Goldhoney 3’, but Sanger sequencing of the mesocarp tissue cDNA during ripening recovered only the transcript containing the \((\text{TC})_{24}\) microsatellite \((\text{Hd})\), indicating that the intronic \(\text{TC}\) microsatellite of \(\text{PpYUC11}\) may regulate the gene expression during ripening and lead to IAA accumulation. However, the causal relationship between the \(\text{TC}\) microsatellite polymorphisms and the transcriptional level remains to be formally proved. As a whole, our results strongly suggest that the \((\text{TC})_{20}\) microsatellite \((\text{hd})\) interferes with \(\text{PpYUC11}\) transcription.

Quantitative RT-PCR analysis revealed that \(\text{PpYUC11}\) is preferentially expressed in the seeds of both flesh types (‘Goldhoney 3’ and ‘Yumyeong’) during all developmental stages but is only expressed in the mesocarp of stage S4 in the melting flesh cultivar ‘Goldhoney 3’ (Fig. 7B), suggesting that there is another regulatory element, perhaps a transcription factor, that interacts with the \(\text{hd}\) locus to control the transcription of \(\text{PpYUC11}\) in both seed and flesh during different stages of fruit development. Depending upon the existence or non-existence of this transcription factor in flesh, \(\text{PpYUC11}\) will be selectively expressed in stage S4; by contrast, \(\text{PpYUC11}\) will be constitutively expressed in other tissues, such as seed. Based on this study’s results, both \(\text{PpYUC10}\) and \(\text{PpYUC11}\) are enriched in the fruit ripening stage of ‘Goldhoney 3’, but \(\text{PpYUC11}\) cannot be detected in ‘Yumyeong’ fruit in the same period (Fig. 2; see Supplementary Table S3 at JXB online), suggesting that \(\text{PpYUC10}\) is responsible for the biosynthesis of the remaining basic IAA levels in ‘Yumyeong’ and that \(\text{PpYUC11}\) is responsible for the high concentration of IAA in ‘Goldhoney 3’.

Auxin has long been considered a negative regulator for fruit ripening, as the content of auxin is low at the initiation of ripening (Nitsch, 1952). The decline of auxin content before the initiation of ripening has been suggested to be a prior

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**Fig. 6.** Co-ordinated variation of \(\text{PpYUC11}\) expression and IAA concentration, \(\text{PpACS1}\) expression and ethylene emission and fruit firmness in various ripening peaches. (A) The intron1 SSR genotype of \(\text{PpYUC11}\) in 12 peach cultivars. (B) Expression of \(\text{PpYUC11}\) and IAA contents. (C) expression of \(\text{PpACS1}\) and ethylene productions, and (D) fruit firmness in 12 peach cultivars. For each cultivar, three time points from stage S3 to S4 (from left to right) were selected. For qRT-PCR, normalization was made to the expression of the actin gene, and values are means of three technical replicates. For IAA concentrations or ethylene production, the results are the mean ± SE of measurements for at least three individual experiments; for flesh firmness, the results are the mean ± SE of measurements of at least five fruits. (This figure is available in colour at JXB online.)
PpYUC11 is the strong candidate gene for stony hard phenotype in peach | 7041

condition for fruit ripening (Given et al., 1988; Chen et al., 1999; Purgatto et al., 2002; Böttcher et al., 2010) because the application of exogenous auxins has been shown to delay the ripening of fruits (Cohen, 1996; Purgatto et al., 2002; Fabbroni et al., 2006). It has been suggested that the low auxin levels in the ripening fruit tissues may be controlled by ripening-associated GH3 genes, which are involved in auxin conjugation (Böttcher et al., 2011; Schaffer et al., 2013). It is interesting that, in contrast to most fruit species, in peach fruits, the concentration of auxin increases suddenly just before ripening and coincides with climacteric ethylene production (Tatsuki et al., 2013). Auxin induces the expression of genes encoding ACC synthase (Tatsuki et al., 2013). The observations from the current study indicate that, in peach, the flavin monoxygenase gene PpYUC11 may regulate the concentration of IAA at the late ripening stage. A higher expression level and stronger up-regulation of PpYUC11 at the transition stage from maturation to the ripening stage (S3 to S4 III) were correlated with IAA concentration and PpACS1 activation in ‘Goldhoney 3’ and another ten normal-fleshed cultivars (Figs 2, 6). By contrast, it is possible that, in ‘Yumyeong’, ‘Xia cui’, and ‘CN16’, less efficient auxin biosynthesis in the mesocarp due to the very low level of PpYUC11 gene expression may contribute to the reduced IAA level and the stony hard phenotype (Figs 2, 6). The contrasting expression profiles in melting and stony hard flesh peaches of a YUCCA flavin monoxygenase (PpYUC11) during peach fruit ripening are consistent with the hypothesis that a low IAA concentration in ripening stony hard peaches could result from the suppression of IAA biosynthesis, the acceleration of IAA inactivation and degradation, the transition from free IAA to IAA storage forms, or the inhibition of IAA transport from biosynthetic tissues (Tatsuki et al., 2013).
Table 2. Characteristics of the YUC family in Prunus persica

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<th>GDRa</th>
<th>Chromosome</th>
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<th>Length (mRNA)b</th>
<th>ESTs/cDNAs&lt;sup&gt;c&lt;/sup&gt;</th>
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<sup>a</sup> Accession numbers of the genes at the Genome Database for Rosaceae.

<sup>b</sup> Length of the predicted mRNA in nucleotides.

<sup>c</sup> Accession numbers of the ESTs/cDNAs at NCBI.

Peaches originated and diversified from China, and Chinese peach germplasm has had a great impact on breeding research in other countries (Li et al., 2013). After the introduction of ‘Chinese Cling’ (‘Shanghai Suimitsuto’) as parents in the early 20th century, Japan selected out ‘Hakuto’ (Yamamoto et al., 2003; Xu et al., 2006) and the USA released the well-known cultivar ‘Elberta’. Both ‘Hakuto’ and ‘Elberta’, selected from seedlings of ‘Chinese Cling’, were extensively used as parents for further breeding of modern cultivars (Scorza et al., 1985; Aranzana et al., 2012). ‘Hakuto’ was reported to be heterozygous (Hd/Hd) for the stony hard gene (Yoshida, 1976), accordingly, ‘Hakuto’ is heterozygous (Hd/Fd) in the intronic TC microsatellite locus in our study (Table 1). The genotype of ‘Elberta’ at the TC microsatellite locus is Hdf/Hdf, which explains the lack of any stony hard peach cultivars in modern American history (Goffreda et al., 1999). In our study, the genotype of ‘Chinese Cling’ was found to be Hdf/Hdf, a result that is in conflict with the genotype of the progeny ‘Hakuto’ (Hd/Fd). It has been suggested that ‘Chinese Cling’ was not a single but, rather, a group of cultivars (Wang and Zhuang, 2001); if so, only a subset of this group of cultivars may have carried the hd allele, and the ‘Chinese Cling’ that was tested may not have been from this subset. Similar examples are ‘Jing Yu’ (hd/hd), a well-known stony hard cultivar from China, selected from among the crosses of ‘Okubo’ (Hd/hd)x ‘Okitsu’ (Hd/hd) and ‘Qin Wang’ (hd/hd, stony hard), which is a new peach cultivar selected from the seedlings of ‘Okubo’ (Hd/hd). The pedigrees of all these cultivars are consistent with the hypothesis that the intronic TC microsatellite of PpYUC11 is the hd locus.

Stony hard peaches are characterized by the absence of both ethylene production and softening in mature fruits and are expected to be used as a genetic source for breeding new table peaches (Haji, 2014). However, stony hard fruits are often very difficult to distinguish from NM or very firm, unripe M phenotypes in the field, thereby making reliable selection difficult (Bassi and Monet, 2008; Byrne et al., 2012). Genotyping the TC microsatellite of PpYUC11 can now be used for the marker-assisted breeding of new cultivars of the stony hard flesh type.

Conclusions

In this study, DGE analysis of auxin-homeostasis-related genes in melting flesh and stony hard peaches during fruit ripening has been reported. This strategy allowed several auxin-homeostasis-related genes to be identified showing a similar expression pattern to the ACC synthetase gene PpACSI. The expression pattern, predicted function, and tissue distribution make PpYUC11 an excellent candidate gene for stony hard type peaches. It is proposed that a TC microsatellite in its first intron is responsible for regulating its gene expression in the mesocarp. In addition, the marker developed on this sequence polymorphism provides a convenient molecular tool with which to discriminate normal/stony hard flesh cultivars or individuals in breeding populations. Our data indicate that a TC microsatellite in PpYUC11 is the molecular basis of stony hard flesh peaches, and our results provide a basis for detailing breeding programmes for peach fruit texture improvement.

Supplementary data

Supplementary data can be found at JXB online.

Supplementary Fig. S1. Schematic representation of the auxin homeostasis pathway in normal or stony hard flesh peaches.

Supplementary Fig. S2. qRT-PCR validation of 11 differentially expressed genes as detected by DGE analysis.

Supplementary Fig. S3. Alignment of the PpYUC11 in melting and stony hard flesh cultivars.
Supplementary Table S1. qRT-PCR primers used in this study.

Supplementary Table S2. The full list of the normalized expression of 28 702 peach genes detected by DGE analysis in eight libraries.

Supplementary Table S3. The full list of 51 auxin-homeostasis-related genes and four ripening-related genes detected by DGE analysis in eight libraries.

Acknowledgements

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