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Validation of molecular markers associated with fruit ripening day of Japanese pear (*Pyrus pyrifolia* Nakai) using variance components

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ABSTRACT

One of the most important breeding objectives for Japanese pear (*Pyrus pyrifolia* Nakai) is to release early-ripening cultivars. To accelerate marker-assisted selection (MAS) for fruit ripening day (FRD), the molecular markers PPACS2 and BGA35, both associated with FRD, were evaluated in six populations by using variance components. The variance explained by PPACS2 ($\sigma_{ACS2}{}^2$) differed greatly among populations, ranging from 5.7 to 101.9. The percentage of total phenotypic variance accounted for by $\sigma_{ACS2}{}^2$ ranged from 8.9% to 40.4%, 22.1% on average. The variance explained by BGA35 ($\sigma_{BGA35}{}^2$) was 44.4–46.3, and the percentage of the total phenotypic variance accounted for by $\sigma_{BGA35}{}^2$ ranged from 18.3% to 24.9%, 21.6% on average. These values are high enough for these markers to be implemented in practical Japanese pear breeding. The 263-bp allele of PPACS2 and the 136-bp allele of BGA35 had early-ripening effects in six populations. No interaction was found between PPACS2 and BGA35, suggesting that the effects of both loci were additive. The 263-bp allele of PPACS2 was previously shown to increase *PPACS2* expression and ethylene production, which might have a negative effect on fruit storage and fruit drop, so further investigation of this allele is required. The information obtained in this study will accelerate MAS for FRD using PPACS2 and BGA35 in Japanese pear breeding programs.

1. Introduction

Fruit tree species have a long juvenile phase and occupy much more space than annual crops. The time and space needed to grow large seedlings pose the most serious bottleneck in fruit breeding. Marker-assisted selection (MAS), which can be used to evaluate plant genotypes at the seedling stage rather than at fruiting 3–5 years later, has been proposed as a means to speed up the selection process in practical breeding (Luby and Shaw, 2001).

Many economically important characteristics of plant species are controlled by major genes (Jiang, 2013). Such characteristics include disease resistance, male sterility, self-incompatibility, and others related to shape, color, and architecture of whole plants. Several useful DNA markers for major genes have been developed and applied to Japanese pear breeding; these include $S_4{}^{sm}$-haplotype-specific DNA markers to identify self-compatibility (Ishimizu et al., 1999; Okada et al., 2008), a molecular marker associated with the pear scab resistance gene *Vnk* (Terakami et al., 2006), and a molecular marker associated with resistance to black spot disease (Terakami et al., 2007). However, studies focused on validation of molecular markers representing quantitative trait loci (QTLs) in practical breeding populations have not been reported for fruit tree species. Both genetic variance and genetic background differ among populations, so the effects of candidate QTLs should be elucidated in multiple fruit tree breeding populations.

Japanese pear (*Pyrus pyrifolia* Nakai; Rosaceae) is a traditionally important fruit crop in Japan. The earliest records of pear cultivation in Japan date back 1300 years. At that time, pears were harvested in fall and eaten soon after harvest or preserved for eating during winter (Tamura, 2006). At present, most Japanese pear fruits are eaten fresh shortly after harvest in August and September because Japanese consumers enjoy eating the juicy fruits when the weather is hot. There are no leading cultivars that are harvested in July, even though they are in demand at that time. Thus, one of the most important objectives in Japanese pear breeding programs is to release early-ripening cultivars (Abe et al., 1995).

Fruit ripening day (FRD) was found to be controlled by several QTLs, and its narrow-sense heritability was estimated in 20

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Table 1
Genotypes of PPACS2 and BGA35 in ten parental cultivars or selections.

Cultivar/selection	Origin	Marker genotype		FRD in 2011
		PPACS2	BGA35	
290-36	Hosui × Bartlett	245/252	129/136	August 30
373-55	Chikusui × Tsukuba 43[a]	245/263	136/136	August 1
426-59	162-29[b] × 269-21[c]	245/245	136/136	August 29
Akiakari	162-29 × Hiratsuka 17[d]	245/245	136/136	September 1
Akibae	Nijisseiki × Kosui	245/245	136/136	September 16
Akizuki	162-29 × Kosui	245/245	136/136	September 24
Hatsumaru	Chikusui × Tsukuba 43	245/263	136/136	August 1
Choju	Asahi × Kimitsukawase	245/263	136/136	August 4
Shinsei	Suisei × Shinko	245/263	136/136	September 19
Taihaku	Native cultivar	245/263	129/136	September 3

FRD, fruit ripening day.
[a] Tsukuba 43: 162-29 × Hiratsuka 17.
[b] 162-29: Niitaka × Hosui.
[c] 269-21: Hosui × Osanijisseiki.
[d] Hiratsuka 17: Kumoi × Kosui.

Table 2
F_1 populations used in this study.

Cross	No. of seedlings	Year of evaluation
Akiakari × Taihaku	89	2009
Akizuki × Hatsumaru	125	2013
Akizuki × 373-55	94	2013
Akibae × Shinsei	64	2009
426-59 × Shinsei	51	2009
Choju × 290-36	61	2009

full-sib families as 0.95 (Abe et al., 1993a). In addition, its broad-sense heritability within a family was estimated in three full-sib families as 0.83 (Nishio et al., 2011). Two molecular markers associated with FRD were detected by genome-wide association study and QTL analysis using an F_1 population from a cross of Japanese pear cultivars 'Akiakari' and 'Taihaku' (Iwata et al., 2013; Yamamoto et al., 2014). One marker detects *PPACS2*, a member of the 1-aminocyclopropane-1-carboxylate (ACC) synthase gene family, and was first identified as a molecular marker associated with storage ability (Itai et al., 1999, 2003). It was later found to be associated with FRD, and is located on linkage group (LG) 15 (Yamamoto et al., 2014). The other marker is BGA35 (on LG3), for which the underlying gene(s) and physiological function(s) related to FRD are unknown. The logarithm of odds (LOD) score and the proportion of phenotypic variance explained were estimated as 3.32–3.87 and 13.7–15.1%, respectively, for PPACS2, and 5.00–5.09 and 22.0–22.5%, respectively, for BGA35. These molecular marker loci were also associated with fruit harvest time in apple (Kenis et al., 2008; Kunihisa et al., 2014; Liebhard et al., 2003).

The objective of this study was to evaluate the effects of PPACS2 and BGA35 in various populations and to evaluate the general versatility of these markers. QTL analyses of several populations performed using a dense linkage map are ideal, but genetic map construction requires considerable cost, time, and labor. Thus, we adopted single-marker regression, a simple method in which the variance explained by the molecular markers in one population is compared with the variance explained in other populations and with the environmental variance. We discuss the potential of MAS for FRD using PPACS2 and BGA35 in Japanese pear breeding programs.

2. Materials and methods

2.1. Plant materials and DNA extraction

Ten Japanese pear cultivars or selections (Table 1) were used to produce six F_1 populations (Table 2) conserved at NIFTS (NARO Institute of Fruit Tree Science). Five of the six populations were derived from intraspecific crosses of Japanese pear. The sixth was derived from a cross of a Japanese female parent ('Choju') and a Japanese–European hybrid male parent (290-36, derived from 'Hosui' [*P. pyrifolia*] × 'Bartlett' [*Pyrus communis*]). The F_1 populations of 'Akizuki' × 'Hatsumaru' and 'Akizuki' × 373-55 were evaluated in 2013. The other four populations ('Akiakari' × 'Taihaku', 'Akibae' × 'Shinsei', 426-59 × Shinsei, and 'Choju' × 290-36) were evaluated in 2009. The genotypes of PPACS2 and BGA35 in the parents of the six F_1 populations are shown in Table 1.

2.2. Evaluation of fruit ripening date

The trees were maintained with cultural techniques used in commercial production in Japan (Tamura, 2006), such as being trained on horizontal trellises, pruned in winter, and treated for pests and diseases. Fruits were thinned to one fruit per three fruit clusters in mid-May, and harvested at their respective FRD during late July to October according to a color chart that indicates the optimum ground color (underlying skin color) for picking Japanese pear (Kajiura et al., 1975). The optimum time for picking was when the fruit skin color at the calyx end had changed from green to yellow-brown (the color corresponding to Plate 4 Plate of the color chart). FRD was expressed as the number of days after July 1. The FRD of each seedling was evaluated from five fruits and was used to calculate the mean and variance of each population (n = 51–125). The frequency distribution of FRD in the six populations is shown in Supplementary Fig. 1.

2.3. Molecular marker analysis

The markers PPACS2 and BGA35, which are associated with FRD (Iwata et al., 2013; Yamamoto et al., 2014), were used to genotype the populations. The PPACS2 primers were 5′-GGTATCTTTGTCCGGCAATC-3′ (forward) and 5′-GCTCTCAAGGCTTTCTTCTCTC-3′ (reverse), and the BGA35 primers were 5′-AGAGGGAGAAAGGCGATT-3′ (forward) and 5′-GTTTCTTGCTTCATCACCGTCTGCT-3′ (reverse). Polymerase chain reaction (PCR) amplification was performed in a 20-μL reaction volume containing 10 μL of 2× Green GoTaq reaction buffer (0.4 mM of each dNTP, 3 mM $MgCl_2$, and 1 U Taq polymerase, pH 8.5; Promega, Madison, WI, USA), 20 pmol of each forward primer labeled with a fluorescent chemical (FAM or HEX) and unlabeled reverse primer, and 2.5 ng of genomic DNA. Amplification was performed using 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 7 min. PCR-amplified products were separated and detected with an ABI PRISM 3130XL DNA sequencer (Applied Biosystems, Carlsbad, CA, USA). The size of the amplified bands was determined by comparison with an internal DNA standard (400HD-ROX; Applied Biosystems) using GeneMapper 4.1 software (Applied Biosystems).

2.4. Data analysis

The variance explained by PPACS2 ($\sigma_{ACS2}{}^2$) and BGA35 ($\sigma_{BGA35}{}^2$) in each F_1 population was calculated. We use the term $m+1$ to represent the number of segregating genotypes within a population and refer to these genotypes as G_1, G_2, …, G_{m+1}. We indicate the genotypes of offspring using a column vector, **u**, of dimension m, as follows: $\mathbf{u} = (1, 0, \ldots, 0)'$ for G_1, $\mathbf{u} = (0, 1, 0, \ldots, 0)'$ for G_2, $\mathbf{u} = (0, 0, 1, 0, \ldots, 0)'$ for G_3, and so on, where " ′ " indicates the transpose of a vector. For G_{m+1}, we assume $\mathbf{u} = (0, 0, \ldots, 0)'$. The effects of marker genotype on FRD are denoted by a column vector, **a**, of dimension m, as $\mathbf{a} = (a_1, a_2, \ldots, a_m)$, where the kth component a_k indicates