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The investigation of pellicle peelability on Japanese chestnut cultivar of 'Yakko' (*Castanea crenata* Sieb. et Zucc.)

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ABSTRACT

Japanese chestnuts (*Castanea crenata* Sieb. et Zucc.) generally have difficult-peeling pellicles even after heating, making easy-peeling pellicle (EPP) an important breeding target. Recently, EPP cultivars 'Porotan' and 'Porosuke' were released by a government-funded breeding program. However, very few genotypes carry the major recessive gene responsible for the EPP trait, resulting in inbreeding within a narrow gene pool. To discover other genetic materials having the potential for EPP breeding, we evaluated the pellicle peelability of 59 accessions (51 Japanese local cultivars and 8 wild individuals) by using the high-temperature oil peeling method. We discovered that 'Yakko' had an exceptionally high pellicle peelability score (87%), close to that of 'Porotan' (94%). The results of segregation ratio analysis of pellicle peelability and genotype prediction by simple sequence repeat (SSR) markers among F_1 seedlings suggested that the EPP alleles of 'Porotan' and 'Yakko' are at the same locus. However, a haplotype structure analysis of the EPP genome region with SSR markers revealed that both haplotypes of 'Yakko' differed from those of 'Porotan', suggesting that the EPP gene of 'Yakko' had a different origin from that of 'Porotan' or was inherited from a common ancestor many generations ago.

1. Introduction

There are four major chestnut species: Japanese chestnut (*Castanea crenata* Sieb. et Zucc.), Chinese chestnut (*C. mollissima* Bl.), European chestnut (*C. sativa* Mill.), and American chestnut (*C. dentata* Borkh.). Japanese chestnut is naturally distributed and is grown in Japan and the Korean Peninsula, and many local cultivars have been developed in Japan (Pereira-Lorenzo et al., 2012). Chinese chestnut is grown mainly in China. European chestnut is commercially grown in Europe, Asia Minor, and North Africa. American chestnut was a common species in eastern North America until the early 20th century, when it was decimated by the accidental introduction of chestnut blight (Woodroof, 1979). Japanese chestnut cultivars are believed to have been selected from wild chestnuts of Japanese origin (Kotobuki, 1994). This hypothesis is supported by the considerable genetic distance between local Japanese chestnut cultivars and Chinese chestnut accessions, as determined using amplified fragment length polymorphism markers (Yamamoto et al., 1998).

Many cultivars of Chinese chestnut and European chestnut have a pellicle that is easy to peel (hereafter, an easy-peeling pellicle: EPP). In contrast, Japanese chestnut cultivars generally have a pellicle that is difficult to peel (hereafter, a difficult-peeling pellicle: DPP), even after heating (Kikuchi, 1948; Miller et al., 1996; Pereira-Lorenzo et al., 2012; Tanaka et al., 1981). The pellicle of Japanese chestnut can be scraped away by hand using a knife, but this is laborious and costly. Thus, releasing new Japanese chestnut cultivars with EPP has been an important target for Japanese chestnut breeding, in addition to large nut size, high eating quality, and high productivity. This program started in 1947 at a national level and is currently managed by the Institute of Fruit Tree and Tea Science, National Agriculture and Food Research Organization (NIFTS). Recently, the breeding program released two Japanese chestnut cultivars with the EPP trait: 'Porotan' in 2006 (Saito et al., 2009) and 'Porosuke' in 2016 (Saito et al., 2017). The area planted to 'Porotan' has been increasing rapidly, reaching 212 ha in 2014. The EPP trait of 'Porotan' is controlled by a single major recessive gene: the pellicle peelability locus has been designated *P/p* (Takada et al., 2012), and a molecular marker linked to this locus was developed (Nishio et al., 2013). Today, marker-assisted selection (MAS) is

Abbreviations: APR, average peeling rate; DPP, difficult-peeling pellicle; EPP, easy-peeling pellicle; HOP, high-temperature oil peeling; MAS, marker-assisted selection; NARO, National Agriculture and Food Research Organization; NIFTS, Institute of Fruit Tree and Tea Science, NARO; QTLs, quantitative trait loci; SSR, simple sequence repeats

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available for the EPP trait in cross-derived populations, allowing selection using large seedling populations and eliminating the need to raise the plants until they are old enough to produce nuts, which is laborious and time-consuming.

So far, very few genotypes (offspring, selections, or cultivars) have been found to carry the EPP gene. This is a concern because repeated crossing among specific genetic resources within a narrow gene pool results in inbreeding depression, such as decreased tree vigor and productivity, in woody fruit crops, including Japanese pear (Sato et al., 2008) and persimmon (Yamada et al., 1994). This depression has not yet been observed in Japanese chestnut, but based on the results for other tree species, seems likely to develop as breeding progresses. Outcrossing can mitigate or eliminate inbreeding depression by incorporating genes from accessions that are genetically distant from the current cross parents in breeding, thus increasing genetic diversity.

Both 'Porotan' and 'Porosuke' are early-maturing cultivars, which results in early cessation of EPP nut production in areas of cultivation and a concentration of harvest dates within a brief period. Therefore, the development of a mid- or late-maturing cultivar with EPP, which would extend the season when fresh nuts are available and give farmers more time to harvest their crops, is a current chestnut breeding target at NIFTS. Kotobuki et al. (1984) suggested that nut harvest time is controlled by quantitative trait loci (QTLs), and Nishio et al. (2017) detected QTLs for nut harvesting date. Thus, we wish to identify later-ripening Japanese chestnut accessions with some level of EPP as cross parents for the breeding of mid- or late-maturing cultivars.

In books published about a century ago, Nakaoka (1913), Yagioka (1915), and Tanaka (1933) described local Japanese chestnut cultivars having EPP on the basis of their observations, but they did not report any test results. This suggests that some unidentified EPP genotypes might exist among Japanese chestnut genetic resources, including the local cultivars mentioned in those books. Our previous study suggested the possibility of breeding novel EPP cultivars by crossing among DPP accessions with relatively easily peeled pellicles (Takada et al., 2017). Thus, it is necessary to identify accessions with relatively high pellicle peelability for breeding novel EPP cultivars. The objective of this study was to discover Japanese chestnut accessions with the EPP trait or with relatively high pellicle peelability by surveying 59 Japanese chestnut accessions that were not included our previous study (Takada et al., 2017).

2. Materials and methods

2.1. Pellicle peelability of 51 local cultivars and 8 wild individuals

We tested a total of 59 Japanese chestnut accessions, consisting of 51 local cultivars and 8 wild individuals, and used 'Porotan' as the standard for the EPP trait (Table 1). We grew one tree per accession at NIFTS, in Tsukuba, Ibaraki (36°02′56″N, 140°05′56″E), Japan. The pellicle peelability of each accession was evaluated in either 2004 or 2007 (Table 1). All trees were grown following standard cultural techniques used in commercial production in Japan.

The harvest day for each accession was the first day that ≥10 nuts could be harvested. In 2004, it ranged from 25 August for 'Yamaguchiwase' to 6 October for 'Daihachi', 'Katayama', and 'Kinshiu'. In 2007, it ranged from 22 August for 'Hassaku', 'Tanabata', and 'Toyotamawase' to 17 October for 'Choubei' and 'Shimokatsugi'. Among the 33 accessions harvested in 2004, 24 were harvested again in 2007. The average harvest day of these 24 accessions was 18 September in 2004 and 27 September in 2007. Although there was a difference of about 10 days in mean harvest day between the two years, the relative maturities of the accessions were similar in each of the two years. Nuts were harvested after the bur opened and were then stored at 5 °C for 1 month.

Ten nuts per accession were randomly used to evaluate pellicle peelability. For accessions harvested in both 2004 and 2007, peelability was assessed only in 2004. After the shells were removed, the nuts were fried in canola oil at 190 °C for 2 min (the high-temperature oil peeling [HOP] method; Shoda et al., 2006). The pellicle peelability of each nut was then determined by means of hand-peeling with a paring knife and was scored by visual estimation of the percentage of the surface area that peeled away without scraping ("peeling rate"), on a scale graded in 10% increments, where "0%" represents 0%, "5%" represents 0% < and ≤10%, "15%" represents 10% < and ≤20%, … "85%" represents 80% < and ≤90%, and "95%" represents 90% < and ≤100% (Takada et al., 2017). Pellicle peelability was quantified as the average peeling rate of 10 nuts per genotype evaluated (APR; %). The accessions with APR values ≥75% were classified as EPP; those with APR < 75% were considered DPP.

2.2. Inheritance of pellicle peelability of 'Yakko'

As described in Results, 'Yakko' had an exceptionally high APR value relative to the other accessions, suggesting that it has a major EPP gene. To test whether the mode of inheritance of pellicle peelability of 'Yakko' was the same as that of 'Porotan', we examined the segregation ratio of pellicle peelability among F_1 seedlings of crosses made using 'Yakko' as a parent. We crossed 'Porotan' (*p/p*) × 'Yakko' in 2006 and 2010, and 'Tanzawa' (*P/p*) × 'Yakko' in 2005 and 2006. 'Tanzawa' was previously shown to be heterozygous for the *p* allele found in 'Porotan' (Takada et al., 2012; Nishio et al., 2013). Two-year-old offspring were planted in a space of 2 m × 5 m in the NIFTS orchard. Nuts were harvested from each seedling of 'Tanzawa' × 'Yakko' in 2011 and of 'Porotan' × 'Yakko' in 2013 after the bur opened and stored at 5 °C for 1 month. Ten nuts from each seedling were randomly evaluated for pellicle peelability by the HOP method as described in section *2.1*. As above, seedlings having average APR values of ≥75% were regarded as EPP. The segregation ratio of pellicle peelability for the seedlings of 'Tanzawa' × 'Yakko' was tested by the chi-square goodness-of-fit test for the hypotheses of a 1:1 segregation ratio.

2.3. Association between pellicle peelability and genotype estimated by simple sequence repeat markers

Because 'Yakko' had an exceptionally high APR value, similar to that of 'Porotan', we hypothesized that both cultivars had the same *p/p* genotype. Thus, we estimated the pellicle peelability genotypes of F_1 seedlings derived from 'Tanzawa' (*P/p*) × 'Yakko' (described in section 2.2) by determining which allele from 'Tanzawa' was present in each seedling. Two simple sequence repeat (SSR) markers closely linked to the *P/p* locus of 'Tanzawa' (PRB28 and PEB62; Nishio et al., 2013) were used to genotype each seedling.

Genomic DNA was extracted from young leaves or young buds using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Polymerase chain reaction products were separated and detected with a 3130xl Genetic Analyzer (Life Technologies, Carlsbad, CA, USA). The size of each amplified band was determined by comparison with a set of internal standard DNA fragments (400HD-ROX, Life Technologies) in GeneMapper v. 5.0 software (Life Technologies).

2.4. Haplotype structure around the P/p locus of 'Yakko' and 'Porotan'

To determine the haplotype structure around the *P/p* locus of 'Yakko' and 'Porotan', we investigated an F_1 population derived from 'Porotan' × 'Yakko' (described in section *2.2*). Genomic DNA was extracted as in section *2.3*. The seedlings were genotyped using 10 SSR markers associated with the *P* gene locus (PEA18, PEA41, PEB62, PEB102, PRA51, PRB25, PRB28, PRD2, PRD52, PRD58; Nishio et al., 2013). The size of each amplified band was determined as described in section *2.3*. The order and spacing of the markers were obtained from Nishio et al. (2013).