Genetic Mapping of PcDw Determining Pear Dwarf Trait

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ABSTRACT. European pear (Pyrus communis) ‘Aihuali’ carrying the dwarf character originating from ‘Nain Vert’ was crossed with ‘Chili’ (Pyrus bretschneideri). A total of 352 F1 progenies was produced to investigate the inheritance of the dwarf trait, and 111 of these were used to develop molecular markers. Chi-square analysis showed that the character fitted a 1:1 ratio indicative of a single dominant gene, which we have named PcDw. Using a bulked segregant analysis approach with 500 random amplified polymorphic DNA (RAPD) and 51 simple sequence repeat (SSR) markers from pear (Pyrus pyrifolia and P. communis) and apple (Malus ×domestica), four markers were identified as cosegregating with the dwarf character. Two of these were fragments produced by the S1212 and S1172 RAPD primers, and the other two were the pear SSR markers KA14 and TsuENH022. The RAPD markers were converted into sequence-characterized amplified regions (SCARs) and designated S1212-SCAR318 and S1172-SCAR930 and, with the SSR markers KA14 and TsuENH022, were positioned 5.9, 9.5, 8.2, and 0.9 cM from the PcDw gene, respectively. Mapping of the KA14 and TsuENH022 markers enabled the location of the PcDw gene on LG 16 of the pear genetic linkage map.

Pears are cultivated commercially in more than 50 temperate countries across the world. Their current mode of cultivation is one of intense fruit production, similar to that of apple. In the last century, many dwarfing apple rootstocks and dwarf cultivars have been developed, which have become crucial for current intense orchard management and production systems in apple. However, for pear, there is a significant lack of suitable germplasm to breed dwarf scion cultivars and rootstocks. In the 1930s, a French Pyrus communis cultivar named Nain Vert, originating from a chance seedling (Fideghelli et al., 2003; Rivalta et al., 2002) and exhibiting the dwarf characteristic, was released. This cultivar forms a bush between 0.9 and 1.2 m high with very compact internode branches, and in 1991, we obtained open-pollinated seeds from ‘Nain Vert’ from East Malling Research Station, UK. Several seedlings with the dwarf character were raised, one of which was named ‘Aihuali’, and these trees now serve as an important genetic resource for breeding pear cultivars with the dwarf tree form. It remains to be determined whether ‘Nain Vert’ and ‘Aihuali’ can be used as dwarfing rootstocks.

Most fruit crops are perennial plants with a long juvenile period, which acts as one of the main obstacles to fast progress in conventional breeding. To help accelerate breeding, breeders are now applying marker-assisted selection (MAS), which allows selection of seedlings for marker-linked traits before maturity. For apple, molecular markers for many different genes determining important traits such as pest and disease resistances (Bus et al., 2008; Gardiner et al., 2007; Gessler et al., 2006), columnar growth habit (Tian et al., 2005), and the apple rootstock dwarfing gene Dw1 (Rusholme Pilcher et al., 2008) have been developed, some of which are being used effectively in apple breeding programs (Bus et al., 2009). However, information on molecular markers associated with pear horticultural traits is presently limited to genes for fireblight (Erwinia amylovora) or scab (Venturia nashicola, Venturia pyrina) resistance (Cho et al., 2009; Dondini et al., 2004; Pierantoni et al., 2007; Terakami et al., 2006) as well as fruit red skin (Dondini et al., 2008), self-incompatibility (Yamamoto et al., 2007), and fruit internal ethylene concentration (Oraguzie et al., 2009). Several different types of molecular markers have been used for marker-assisted breeding and mapping in apple and pear. Many microsatellite or SSR markers have been developed from apple (Liebhard et al., 2002; Silfverberg-Dilworth et al., 2006) and pear (Celton et al., 2009a; Fernández-Fernández et al., 2006; Yamamoto et al., 2002a, 2002b, 2002c), and a number of SSRs from apple have been located on pear genetic maps (Pierantoni et al., 2004; Yamamoto et al., 2002c, 2004, 2007). The recently published apple rootstock maps contain 49 SSRs developed from Pyrus genome sequences (Celton et al., 2009b). The maps of Japanese pear ‘Hosui’ (Pyrus pyrifolia) and European pear ‘Bartlett’ were successfully aligned to the apple consensus map using SSR markers, and all pear linkage groups corresponding to its basic chromosome number (x = 17) could be anchored to homologous apple groups (Celton et al., 2009a; Yamamoto et al., 2004, 2007). These studies are significant for speeding up pear genetic map construction and will also have importance for comparative mapping of traits from apple to the more poorly resourced pear.
This study used SSR markers derived from both pear and apple as well as RAPD markers for bulked segregant analysis (BSA) (Michelmore et al., 1991) across an F1 population derived from a cross of ‘Aihuali’ and the Chinese pear ‘Chili’ (Pyrus bretschneideri) to dissect the genetics behind the dwarf trait and identify markers linked to the character.

**Materials and Methods**

**PLANT MATERIAL.** ‘Aihuali’, an open-pollinated offspring of ‘Nain Vert’, was crossed with ‘Chili’ in 2002. A total of 352 seedlings was produced and visually assessed for the dwarf character after 2 years in the nursery (the dwarf plant height was less than 1.0 m with compact internodes and usually fleshy shoots). Of these, 111 randomly selected individuals, 55 with dwarf phenotype and 56 with standard phenotype, were transplanted with a planting distance of 2.5 × 1.0 m and 2.5 × 0.5 m for standard and dwarf non-grafted trees, respectively, and used for molecular marker identification and development.

**ISOLATION OF GENOMIC DNA.** Genomic DNA was isolated from dormant buds collected in early spring using a variation of the Doyle and Doyle (1987) extraction method. DNA concentration was estimated against DNA samples of a known concentration using agarose gel electrophoresis.

**BULKED SEGREGANT ANALYSIS.** The progenies were classified into two groups by phenotype: dwarf and standard tree architecture (Fig. 1). Equal amounts of DNA extracted from 12 individuals in each group were mixed to form two contrasting bulks: dwarf versus standard. RAPD and SSR primers were then screened over the two bulks as well as DNA from the parents (Michelmore et al., 1991). Markers identified as associated with the dwarf phenotype were then screened over the whole population (111 individuals) to determine their extent of linkage to the dwarf or standard phenotype.

**MARKERS INTO SEQUENCE-CHARACTERIZED AMPLIFIED REGION MARKERS.** Polymorphic RAPD marker fragments that were linked to the dwarf phenotype were excised from the agarose gel and extracted using the Silver Beads DNA gel-extraction Kit (Sangon) according to the manufacturer’s recommendations. The marker fragments were cloned into the pGEM-T Easy Vector (Promega, Madison, WI) and transformed into Escherichia coli cells (DH5α) according to the manufacturer’s instructions. Plasmid DNA was extracted and digested with EcoRI to verify whether the cloned fragment was of the expected size. Cloned fragments were sequenced and specific primers were designed within the fragment sequence. PCR amplification was performed in a 25-μL volume containing 25 ng of genomic DNA, 2.25 mM MgCl2, 2.5 μL of 10× PCR buffer, and 1.0 U Taq polymerase (Takara), 0.2 mM of each dNTP (Sangon, Shanghai, China), and 0.2 μM of each forward and reverse primer. Reactions were then subjected to an initial denaturation at 94 °C for 4 min followed by 40 cycles of 94 °C for 1 min, 38 °C for 1 min, and 72 °C for 1.5 min followed by a final extension step at 72 °C for 5 min in a PCR machine (PTC-200; Bio-Rad Laboratories, Hercules, CA). A total of 500 arbitrary decamer RAPD primers (Sangon) was used. The amplified products were separated on 1.3% agarose gels, stained with ethidium bromide (0.5 μg·mL⁻¹), and photographed under ultraviolet light.

**SIMPLE SEQUENCE REPEAT MARKER SCREENING.** A total of 51 SSR primers (Table 1) was used in the BSA approach to identify SSR markers linked to the dwarf character. Of these, 33 SSR primer pairs originated from pear (Celton et al., 2009a; Yamamoto et al., 2002a, 2002b, 2002c) and 18 SSR primer pairs were developed from apple (Liebhard et al., 2006; Silfverberg-Dilworth et al., 2006). Most of these markers had been previously positioned on either the pear or apple genetic linkage map (Celton et al., 2009a, 2009b; Silfverberg-Dilworth et al., 2006; Yamamoto et al., 2002c, 2007). The SSR PCR reactions and conditions were carried out as described in the original publications with amplification products detected after electrophoresis on a 4% agarose gel.

**LINKAGE ANALYSIS.** Linkage analysis between the DNA molecular markers and PcDw was performed using JoinMap 4.0 software (Kyazma, Wageningen, The Netherlands). Map distances in centiMorgans were calculated from recombination frequencies using the Kosambi mapping function (Kosambi, 1944) and a LOD score of 10.

**Results**

**INHERITANCE OF THE DWARF CHARACTER.** A total of 352 F1 seedlings obtained from the cross between ‘Aihuali’ and ‘Chili’ was classified into the two groups of dwarf and standard
Table 1. The 51 simple sequence repeat (SSR) markers screened by bulked segregant analysis for identifying DNA molecular markers related to the pear dwarf trait.

| SSR locus | Linkage group no. | Origin of primer sequence | SSR locus | Linkage group no. | Origin of primer sequence |
|-----------|-------------------|---------------------------|-----------|-------------------|---------------------------|
| NB102a    | 11                | Pear                      | NH007b    | 16               | Pear                      |
| NB103a    | 5′                | (Yamamoto et al., 2002c)  |           |                   |                           |
| NB105a    | 11′               |                           |           |                   |                           |
| NB106a    | 9′                | CH04f10                   | 16        | Apple             |                           |
| NB109a    | 3′                | CH02a03                   | 16        | (Liebhard et al., 2002) |
| NB110a    | 9′, 17′           | CH02d10a                  | 16        |                   |                           |
| NB111a    | 11′               | CH05a04                   | 16        |                   |                           |
| NB113a    | 3′, x              | CH05a09                   | 16        |                   |                           |
| NH101b    | — x               | CH05c06                   | 16        |                   |                           |
| NH102a    | 5′                | CH01f03a                  | 16        |                   |                           |
| NH103a    | 13′               |                            |           |                   |                           |
| NH1022a   | — x               | AU301431                  | 16        | Apple             | (Silfverberg-Dilworth et al., 2006) |
| NH1023a   | 3′                | Hi01a08                   | 16        |                   |                           |
| NH1024b   | — x               | Hi02b10                   | 16        |                   |                           |
| NH1025a   | 15′               | Hi02h08                   | 16        |                   |                           |
| NH1026a   | 16′, x            | Hi04e04                   | 16        |                   |                           |
| NH1027a   | 15′               | Hi08d09                   | 16        |                   |                           |
| NH1029a   | 9′                | Hi08f12                   | 16        |                   |                           |
| NH1030a   | — x               | Hi12a02                   | 16        |                   |                           |
| KA4b      | 1′                | Pear                      | Hi15g11   | 16                |                           |
| KA5       | — x               | (Yamamoto et al., 2002a)  | Hi22f06   | 16                |                           |
| KA14      | 16′, x            |                            |           |                   |                           |
| KA16      | 12′               | TsuENH022                 | 16        | Pear              | (Celton et al., 2009a)   |
| KB16      | 6′                | TsuENH042                 | 16        |                   |                           |
| KU10      | 2′                | TsuENH079                 | 16        |                   |                           |
| BGA35     | 3′                | TsuENH052                 | 16        |                   |                           |
| BGT23b    | 2′                |                            |           |                   |                           |
| HGA8b     | 3′                |                            |           |                   |                           |

*Not mapped to any genetic linkage groups of apple or pear to date.
†From Yamamoto et al. (2007).
‡From Celton et al. (2009a).
§From Silfverberg-Dilworth et al. (2006).

Identification of random amplified polymorphic DNA markers linked with PcDw. A total of 500 RAPD primers were screened over the two DNA bulks and the two parents from the population. From these, 68 polymorphic fragments were identified that could be associated with the dwarf phenotype, which were then screened over 111 individuals (55 dwarf and 56 standard phenotypes). Two RAPD markers derived from the S1172 and S1212 primers showed significant linkage to the PcDw gene with a recombination frequency of 8.1% and 6.3%, respectively. The amplified pattern exhibited can be seen in Figure 2.

Conversion of random amplified polymorphic DNA markers to sequence-characterized amplified region markers. The sequencing result showed that the RAPD marker fragments amplified from the S1172 and S1212 primers were 940 and 318 bp in length, respectively (GenBank accession numbers EU251429 and EU251430, respectively). Specific SCAR primers were designed from these sequences and screened over the whole population. These markers, designated as S1172-SCAR 930 (the forward primer is 5′ GTCTTACCTTTC CATCTTC3′ and the reverse primer is CACCGCCCATACAAAAA CT) and S1212-SCAR 318 (the forward primer is GGATCGTCGGAT CAAATGAGT and the reverse primer is GGATCGTGGCATGT GAAGT), gave amplification products of 930 and 318 bp, respectively (Fig. 3), with segregation profiles identical to those from the original RAPD fragments.

Simple sequence repeat markers linked to the PcDw gene. Of the 28 pear SSR primer pairs (Yamamoto et al., 2002a, 2002c) (Table 1) tested by BSA, only one of them, KA14, was found to be linked to PcDw (Fig. 4). Of the 111 individuals, 10 showed recombination events between this marker and PcDw, i.e., the recombination frequency is 9.0%. In the pear genetic map published by Yamamoto et al. (2002c), KA14 was previously located on LG 10 of ‘Bartlett’ [now designated LG 16 (Yamamoto et al., 2004)]. Therefore, PcDw is positioned on the same linkage group as KA14, LG 16. Although SSRs, NH007b developed from pear and CH05c06 developed from apple, have been located on LG 16 of ‘Bartlett’ (Yamamoto et al., 2004), they were monomorphic in the mapping population. According to the aligned pear and apple reference map, LG 16 of pear (Celton et al., 2009a; Yamamoto et al., 2004) corresponds to LG 16 of apple. Seventeen other SSRs distributed over LG 16 of apple (Silfverberg-Dilworth, et al., 2006) and five SSRs (one of them is CH01f03a derived from apple) mapped to LG 16 of pear (Celton et al., 2009a) were also tested, and another SSR marker, TsuENH022, showed tight linkage to PcDw locus with the polymorphic band appearing on the standard phenotype; only one of the 111 individuals exhibited recombination events. The amplified profiles are presented in Figure 4.

Linkage map. A partial genetic linkage map consisting of the PcDw locus, the two SCAR markers (S1172-SCAR 930 and S1212-SCAR 318), and the two SSR markers (KA14 and TsuENH022) was constructed using JoinMap 4.0. All four markers were positioned on the same side of PcDw and the whole linkage map covered ≈10 cM. The marker TsuENH022

Figure 2. The amplified pattern exhibited can be seen in the figure.
mapped 0.9 cM from the \( \text{PcDw} \) locus and appears to be the closest marker to \( \text{PcDw} \) followed by S1212-SCAR 318, KA14, and S1172-SCAR 930 (Fig. 5).

**Discussion**

Fruit tree breeders are greatly interested in developing cultivars with dwarf or dwarfing characters because of the potential for savings on tree management and production costs and for early fruiting. However, only a few tree architecture characters in apple are known to be controlled by major loci such as \( \text{Co} \) for columnar habit (Lapins, 1976) and \( \text{Dw1} \) for dwarfing tendency (Rusholme Pilcher et al., 2008). We determined that the dwarf character in pear originating from ‘Nain Vert’ was controlled by a single dominant locus, first designated here as \( \text{PcDw} \), which is in accordance with Decourt’s report in 1967 (Fideghelli et al., 2003). As a dominant dwarf mutant that affects internode length, \( \text{PcDw} \) probably is a regulator element and not a structure gene, which needs to be confirmed by other research. The fruit quality of the several selections made from ‘Nain Vert’ and the subsequent backcross progeny with reduced growth habit were too poor to allow the release of these genotypes, but some of them could be used as ornamental plants as a result of their compact, globe-shaped canopy (Fideghelli et al., 2003; Rivalta et al., 2002). ‘Aihuali’, an offshoot of ‘Nain Vert’, is characterized by a dwarf growth habit. The height of the original 10-year-old mother tree is \( \approx 1.8 \) m, and the fruit are pear-shaped, large-sized (180 g) with greenish skin and an irregular surface; however, the overall quality is still undesirable for fruit production. Therefore, further crosses need to be performed to develop selections with a combination of dwarf trait and excellent fruit quality. From the interspecies cross ‘Aihuali’ × ‘Chili’, we aim to introduce this dwarf trait to the Chinese pear and create new selections that can be used for fruit production. The mapping of four genetic markers within 10 cM of \( \text{PcDw} \) will be a very useful resource after validation for future breeding of new dwarf pear scion cultivars using MAS or by using a biotechnology strategy involving gene cloning and transformation.

S1172-SCAR 930 and S1212-SCAR 318 are linked to the \( \text{PcDw} \) gene at distances of 9.5 and 5.9 cM, respectively, and were the first DNA molecular markers identified for this locus. However, further work was required to identify the linkage group to which it mapped. SSR markers are generally regarded as being valuable for gene location on maps, because they tend to be more transferable across genetic background than other markers such as RAPDs, amplified fragment length polymorphisms, or SCARs. We located the \( \text{PcDw} \) locus to LG 16 of the genetic map of European pear cultivar Bartlett published by Yamamoto et al. (2004) using SSR marker KA14, which mapped at a distance of 8.2 cM from the locus. We then attempted to identify more SSR markers linked to the \( \text{PcDw} \) gene at distances of 9.5 and 5.9 cM, respectively, and were the first DNA molecular markers identified for this locus. However, further work was required to identify the linkage group to which it mapped. SSR markers are generally regarded as being valuable for gene location on maps, because they tend to be more transferable across genetic background than other markers such as RAPDs, amplified fragment length polymorphisms, or SCARs. We located the \( \text{PcDw} \) locus to LG 16 of the genetic map of European pear cultivar Bartlett published by Yamamoto et al. (2004) using SSR marker KA14, which mapped at a distance of 8.2 cM from the locus. We then attempted to identify more SSR markers linked to the \( \text{PcDw} \) gene, screening several SSRs already mapped on LG 16 of ‘Bartlett’ (Celton et al., 2009a; Yamamoto et al., 2004). In this way we obtained the tightly linked SSR marker TsuENH022, which was located only 0.9 cM from \( \text{PcDw} \). However, further markers need to be identified for \( \text{PcDw} \) so that the position of the \( \text{PcDw} \) gene can be identified more
precisely and closer or flanking markers can be found for MAS within the preferred 5-cM distance ( Tanksley, 1983).

There are many examples of the extent to which SSRs are conserved among related species. To date, the apple consensus genetic map is the most advanced in pome fruits. It contains a large number of codominant SSR markers (Celton et al., 2009b; Silfverberg-Dilworth et al., 2006); however, progress in pear genetic map construction is much slower than that of apple. One of the distinctive disadvantages is the lack of sufficient SSR markers evenly distributed over the pear map compared with apple to date (Celton et al., 2009b; Silfverberg-Dilworth et al., 2006; Yamamoto et al., 2007). Because the genome structure is highly conserved between pear and apple [both belong to the subfamily Pomoideae in Rosaceae with identical chromosome number (2x=34) and similar genome size [pear 1.11 pg/2C, apple 1.57 pg/2C (Celton et al., 2009a)], comparative mapping could serve as an important way to improve pear map construction. Genetic linkage maps of the Japanese pear cultivar Housui and European pear cultivar Bartlett were successfully aligned to the apple consensus map by using apple SSRs as anchor loci, which suggested genetic synteny between pear and apple (Yamamoto et al., 2004, 2007) and pear SSRs were used in construction of apple rootstock maps (Celton et al., 2009a, 2009b). Based on the genetic synteny and the SSRs’ conserved character between pear and apple genomes, 17 SSRs evenly distributed on LG 16 of apple (Silfverberg-Dilworth et al., 2006) were tested in this population; however, none cosegregated with PcDw. However, the release of the apple genome sequence (Velasco et al., 2010) as well as the smaller scale sequencing of the pear genome taking place mean that many more SSRs as well as single nucleotide polymorphism will be soon identified, which will provide an abundant resource for further development of markers linked to this locus. The four DNA molecular markers reported here will be useful for MAS, particularly the closest TsuENH022, as well as for initiating fine mapping and subsequent cloning of the PcDw gene.

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