Genetic Mapping of *PcDw* Determining Pear Dwarf Trait

Caihong Wang and Yike Tian¹

Department of Horticulture, Qingdao Agricultural University, Qingdao, China 266109

Emily J. Buck and Susan E. Gardiner

The New Zealand Institute for Plant & Food Research Limited, Palmerston North Research Centre, Palmerston North 4474, Private Bag 11 600, Palmerston North 4442, New Zealand

Hongyi Dai and Yanli Jia

Department of Horticulture, Qingdao Agricultural University, Qingdao, China 266109

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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.

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¹Corresponding author. E-mail: yktian123@yahoo.com.cn.

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 between ‘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.

RANDOM AMPLIFIED POLYMORPHIC DNA MARKER ANALYSIS. Polymerase chain reaction (PCR) amplification was performed in a 25-μL volume containing 50 ng of genomic DNA, 2.5 μL 10× PCR buffer, 1.8 mM magnesium chloride (MgCl₂), 1.0 U TaqE polymerase (Takara, Tokyo, Japan), 0.2 mM of each dNTP (Sangon, Shanghai, China), and 0.45 μM RAPD primer. Reactions were 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.

CONVERSION OF RANDOM AMPLIFIED POLYMORPHIC DNA 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 *Eco*RI 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 of TaqE polymerase (Takara), 0.2 mM of each dNTP (Sangon), 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 25 cycles of 94 °C for 30 s, 62 °C for 35 s, and 72 °C for 1 min followed by a final extension step at 72 °C for 5 min. The products were visualized on a 1.3% agarose gel as previously described.

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., 2002; 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 (Kryazma, 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

Fig. 1. The dwarf (A) and standard (B) phenotypes in the progeny derived from the cross ‘Aihuali’ (*Pyrus communis*) × ‘Chili’ (*Pyrus bretschneideri*). The seedling trees are 4 years old.

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	— ^z	Pear	NH007b	16 ^{y, x}	Pear
NB103a	5 ^y	(Yamamoto et al., 2002c)			(Yamamoto et al., 2002b)
NB105a	11 ^y				
NB106a	9 ^y		CH04f10	16 ^w	Apple
NB109a	3 ^y		CH02a03	16 ^w	(Liebhard et al., 2002)
NB110a	9 ^y , 17 ^y		CH02d10a	16 ^w	
NB111a	11 ^y		CH05a04	16 ^{w, x}	
NB113a	3 ^{y, x}		CH05a09	16 ^{w, x}	
NH019b	— ^z		CH05c06	16 ^{w, x}	
NH020a	5 ^y		CH01f03a	16 ^x	
NH021a	13 ^y				
NH022a	— ^z		AU301431	16 ^w	Apple
NH023a	3 ^y		Hi01a08	16 ^w	(Silfverberg-Dilworth et al., 2006)
NH024b	— ^z		Hi02b10	16 ^w	
NH025a	15 ^y		Hi02h08	16 ^w	
NH026a	16 ^{y, x}		Hi04e04	16 ^w	
NH027a	15 ^y		Hi08d09	16 ^w	
NH029a	9 ^y		Hi08f12	16 ^w	
NH030a	— ^z		Hi12a02	16 ^w	
			Hi15a13	16 ^w	
KA4b	1 ^y	Pear	Hi15g11	16 ^w	
KA5	— ^z	(Yamamoto et al., 2002a)	Hi22f06	16 ^w	
KA14	16 ^{y, x}				
KA16	12 ^y		TsuENH022	16 ^x	Pear
KB16	6 ^y		TsuENH042	16 ^x	(Celton et al., 2009a)
KU10	2 ^y		TsuENH079	16 ^x	
BGA35	3 ^y		TsuENH052	16 ^x	
BGT23b	2 ^y				
HGA8b	3 ^y				

^zNot mapped to any genetic linkage groups of apple or pear to date.

^yFrom Yamamoto et al. (2007).

^xFrom Celton et al. (2009a).

^wFrom Silfverberg-Dilworth et al. (2006).

phenotype by visual inspection. Among the progeny, 169 individuals were determined as dwarf and the others as standard (non-dwarf) phenotype. Based on a chi-square test, the segregation of 169 dwarf:183 standard fitted a 1:1 ratio ($P > 0.05$), indicating a monogenic inheritance. This means the dwarf character from ‘Aihuali’ is highly likely to be controlled by a single dominant gene, which was designated as *PcDw*. ‘Aihuali’ is heterozygous for the *PcDw* gene inherited from its ancestor ‘Nain Vert’.

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-SCAR₉₃₀ (the forward primer is 5’GTCTTACCCCTTCC CATCTTC3’ and the reverse primer is CACCGCCCCATACAAAAA CT) and S1212-SCAR₃₁₈ (the forward primer is GGATCGTCGGAT CAAATGAATTG and the reverse primer is GGATCGTCGGCATGT 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 (S1127-SCAR₉₃₀ and S1212-SCAR₃₁₈), 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

was positioned on the same side of *PcDw* and the whole linkage map covered ≈ 10 cM. The marker TsuENH022

Fig. 2. The random amplified polymorphic DNA (RAPD) products from the S1172 (A) and S1212 (B) primers screened over the parents [P1 = dwarf 'Aihuali' (*Pyrus communis*), P2 = standard 'Chili' (*Pyrus bretschneideri*)], the two bulks (mixed by 12 progenies with the corresponding phenotype, respectively; B1 = dwarf; B2 = standard), and 19 progenies from the 'Aihuali' × 'Chili' population for identification of RAPD markers linked to the pear dwarf gene *PcDw* run alongside M, a DL 2000 DNA marker (Takara, Tokyo, Japan). Progeny 1 to 10 are dwarf phenotype and 11 to 19 standard phenotype trees. The specific fragments of interest are indicated by the arrows.

Fig. 3. Amplification patterns of the sequence-characterized amplified regions (SCARs), S1172-SCAR₉₃₀ (A) and S1212-SCAR₃₁₈ (B) linked to the pear dwarf gene *PcDw* screened over the two bulks (B1 = dwarf, B2 = standard), the parents [P1 = 'Aihuali' (*Pyrus communis*), P2 = 'Chili' (*Pyrus bretschneideri*)], and 19 progenies from the 'Aihuali' × 'Chili' population run alongside M, a DL 2000 DNA marker (Takara, Tokyo, Japan). Progeny 1 to 10 are dwarf phenotype and progeny 11 to 19 are standard phenotype trees.

mapped 0.9 cM from the *PcDw* locus and appears to be the closest marker to *PcDw* followed by S1212-SCAR₃₁₈, KA14, and S1172-SCAR₉₃₀ (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 *Co* for columnar habit (Lapins, 1976) and *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 *PcDw*, which is in accordance with Decourtye's report in 1967 (Fideghelli et al., 2003). As a dominant dwarf mutant that affects internode length, *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 offspring of 'Nain Vert', is characterized by a dwarf growth habit. The height of the original 10-year-old mother tree is ≈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 *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-SCAR₉₃₀ and S1212-SCAR₃₁₈ are linked to the *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 *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 *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 *PcDw*. However, further markers need to be identified for *PcDw* so that the position of the *PcDw* gene can be identified more

Fig. 4. Amplification products from KA14 (A) and TsuENH022 (B) pear simple sequence repeat (SSR) markers screened over the two bulks (B1 = dwarf, B2 = standard), the parents [P1 = 'Aihuali' (*Pyrus communis*), P2 = 'Chili' (*Pyrus bretschneideri*)], and 13 progenies from the 'Aihuali' × 'Chili' population run alongside M, a DL 2000 DNA marker (Takara, Tokyo, Japan). Progeny 1 to 7 are dwarf phenotype and 8 to 13 are standard phenotype. The arrows indicate the polymorphic fragments related to the dwarf/standard trait.

Fig. 5. The partial genetic linkage map around the *PcDw* locus in 'Aihuali' (*Pyrus communis*) constructed by JoinMap 4.0 software (LOD = 10.0) using the F₁ population with 111 individuals derived from the cross 'Aihuali' × 'Chili' (*Pyrus bretschneideri*) aligned to LG 16 of 'Bartlett' (*P. communis*) (Celton et al., 2009a) by two simple sequence repeat markers.

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 Pomoi-deae 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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