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ORIGINAL PAPER

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Development of a genomic library of near isogenic lines (NILs) in melon (*Cucumis melo* L.) from the exotic accession PI161375

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Abstract A doubled haploid line (DHL) population of melon derived from a cross between the Korean cultivar "Songwhan Charmi" accession PI161375 (SC), included in the horticultural group *conomon*, and the Spanish cultivar "Piel de Sapo" (PS), included in the horticultural group *inodorus*, was used to develop a collection of near isogenic lines (NILs). These parental lines represent very different melon cultivar groups, with important differences at fruit, plant, disease response and molecular level. This cross is one of the most polymorphic ones within melon germplasm. Selected DHLs were backcrossed to PS and further backcrossing and selfing was performed, monitoring introgressions from SC using molecular markers covering the melon genetic map. A final collection of 57 NILs was obtained, containing a unique independent introgression from SC in the PS genetic background. The introgressions within the collection cover at least 85% of the SC genome with an average introgression size of 41 cM, corresponding to 3.4% of the SC genome. The average resolution for mapping genes or quantitative trait loci is 18.90 cM. This set of NILs is a potentially powerful tool for the study of quantitative trait locus involved in melon fruit quality and other important complex traits, and the introduction of new genetic variability in modern cultivars from exotic sources. The NILs can also be used as precompetitive breeding lines in melon breeding projects.

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Introduction

Melon (*Cucumis melo* L.) is an economically important species of the Cucurbitaceae family grown in temperate and tropical regions worldwide. Melon fruits have wide morphological, physiological and biochemical diversity (Whitaker and Davis 1962; Kirkbride 1993; Liu et al. 2004). This high level of phenotypic variability corresponds to a high-genetic variability as has been confirmed using molecular markers. African, Indian and Oriental melon germplasm, which can be considered exotic melon germplasm for European/North American cultivars (Stepansky et al. 1999; Mliki et al. 2001; Akashi et al. 2002; Monforte et al. 2003), includes most of this genetic variability. Exotic germplasm has been used to search for resistance genes, but the potential of this germplasm as a source of new major genes and quantitative trait locus (QTL) alleles with favourable effects on fruit quality had not been thoroughly investigated. Furthermore, the genetic control of the phenotypic variation is largely unknown. Much of the variation could be due to allelic variability for a large number of genes, increasing the difficulty of studying them. Some QTLs involved in fruit quality traits have been detected using segregating populations such as F_2, double haploid lines (DHLs) and recombinant inbred lines (RILs) (Périn et al. 2002a; Monforte et al 2004a). The precision of the estimates of QTL effects and position in the genome using these populations is quite limited (Hyne et al. 1995; Melchinger et al. 1998; Lebreton et al. 1998). QTLs need to be located more precisely for a better understanding of the origin, nature, number, allele multiplicity and phenotypic value of genes involved in quantitative variation (Morgante and Salamini 2003).

Wherhahn and Allard (1965) demonstrated that individual QTL effects could be efficiently estimated using backcross inbred lines (BILs) with a low proportion of donor genome. Eshed and Zamir (1994; 1995) further developed the idea, constructing an introgression line (IL) population consisting of a set of lines where

each one contained a single homozygous chromosome segment from a donor parent, in the genetic background of an elite cultivar, which together provided complete coverage of the donor parent genome. These lines have, intentionally, a high percentage (mostly higher than 95%) of the recurrent parent genome, so they are also defined as near isogenic lines (NILs). A NIL population can be defined as a genomic library where inserts are chromosome fragments of the donor parent genome and the vector is the recurrent parent genome.

QTL analysis is very efficient in using NIL collections. Eshed and Zamir (1995) detected a minimum of 23 QTLs for some traits, whereas only five or six QTL are detected when using standard mapping populations (Kearsey and Farquhar 1998). NILs have been used to verify QTL effects (Tanksley et al. 1996), study QTL × environment, QTL × genetic background and QTL × QTL interactions (Monforte et al. 2001). They have also been used to introduce new genetic variability from wild species into the elite germplasm (Tanksley and McCouch 1997; Zamir 2001), determine metabolic profiles (Overy et al. 2004), correlate metabolic and expression profiles (http://tomet.bti.cornell.edu/) and for QTL cloning (Frary et al. 2000; Fridman et al. 2000). The use of NILs has transformed the task of QTL cloning: it is now similar to that performed for simple Mendelian traits, with the exception that phenotyping requires more detailed replicated measurements (Paran and Zamir 2003).

Several sets of NILs have been developed in crop plant species including tomato (Eshed and Zamir 1994; Bernacchi et al 1998; Monforte and Tanksley 2000a; Chetelat and Meglic 2000), cabbage (Ramsay et al. 1996), rice (Lin et al. 1998; Wan et al. 2004), wheat (Pestova et al. 2001), barley (von Korff et al. 2004), lettuce (Jeunken and Linhout 2004) and *Arabidopsis thaliana* (Koumprouglou et al. 2002). Similar strategies have been followed in mice (Singer et al. 2004). Thus, there is increasing interest in the scientific community to develop these populations as the basis for understanding the genetics of complex traits.

Interspecific crosses between *C. melo* and wild *Cucumis* species are not viable (Chen and Adelberg 2000). Thus, the construction of a NIL collection in melon needs to start from an intraspecific cross. In the current report, a Spanish "Piel de Sapo" cultivar (PS), belonging to horticultural group *inodorus* was chosen as recipient genotype. PS fruits are very sweet, oval, white-flesh-coloured and non-climateric (Monforte et al. 2004). The Korean cultivar "Songwhan Charmi" accession PI161375 (SC), included in the horticultural group *conomon*, was chosen as donor genotype based on phenotypic and molecular data. The genetic distance between PS and SC is one of the highest distances described between two cultivars within melon germplasm. (Garcia-Mas et al. 2000; Monforte et al. 2003). For example, 49% of single sequence repeat (SSR) markers were found polymorphic between SC and PS (Gonzalo et al. 2005). The average frequency of single nucleotide polymorphisms (SNPs) was one every 441 and one indel was also found every 1,666 bp (Morales et al. 2004). SC presents some resistance to some diseases and plagues as Cucumber Mosaic Virus (Lecoq et al. 1998), Melon Necrotic Spot Virus (Coudriet et al. 1981), *Fusarium oxysporum* (Risser 1973) and virus transmission by *Aphis gossypii* (Pitrat y Lecoq 1980). SC fruits are pear-shaped, green-flesh-coloured, with low-sugar content (Monforte et al. 2004). The objective of the current study is to obtain a NIL collection from these two melon genotypes, covering the complete genome of this exotic accession in the PS genome background. The newly developed set of melon NILs will constitute a genomic resource for the study of complex traits in melon and the evaluation of the potential of exotic germplasm as a source of new genetic variability for fruit quality melon breeding programs.

Materials and methods

Plant materials and NIL development

The base population for NIL development was a set of 80 DHLs previously developed from a wide cross between an inbred line of Spanish cultivar "Piel de Sapo" (PS) and the exotic Korean cultivar "Songwhan Charmi" (SC) accession PI161375 (Gonzalo 2003). The PS genome was chosen as the recipient genome and SC genome as the donor genome. The breeding scheme used to develop the NILs population is depicted in Fig. 1. The first selection was performed in the DHL population based on percentage of donor genome. By the time the DHL selection was performed, the molecular marker map in this population was not completed. To prevent the loss of genomic regions, a conservative strategy was followed. Twenty-five DHLs were selected with the following criteria: (1) the entire known genome was represented at least twice with overlapping known donor segments, and (2) the selected plants contained the lowest possible proportion of the donor genome. Additionally, five DHLs carrying a high percentage of the donor genome were also selected. All 30 selected DHLs were backcrossed to PS and given that the genetic structure of the DHL × PS lines is similar to a backcross, the selected 30 DHL × PS were considered as our initial BC_1 population (first generation of backcrossing). All 30 BC_1 genotypes were backcrossed to PS. By then, the molecular marker map was finished (Gonzalo et al. 2005). Twelve BC_1 progenies were chosen for the next round of selection, ten of them having a low percentage of donor genome and two a high percentage. Sixteen plants of each of the 12 BC_2 families (192 plants in total) were grown in the greenhouse for backcrossing to PS and genotyped with 65 molecular markers, covering the whole genome (see below). BC_3 families with candidate introgressions and a lower proportion of donor genome were selected according to BC_2 genotypes.

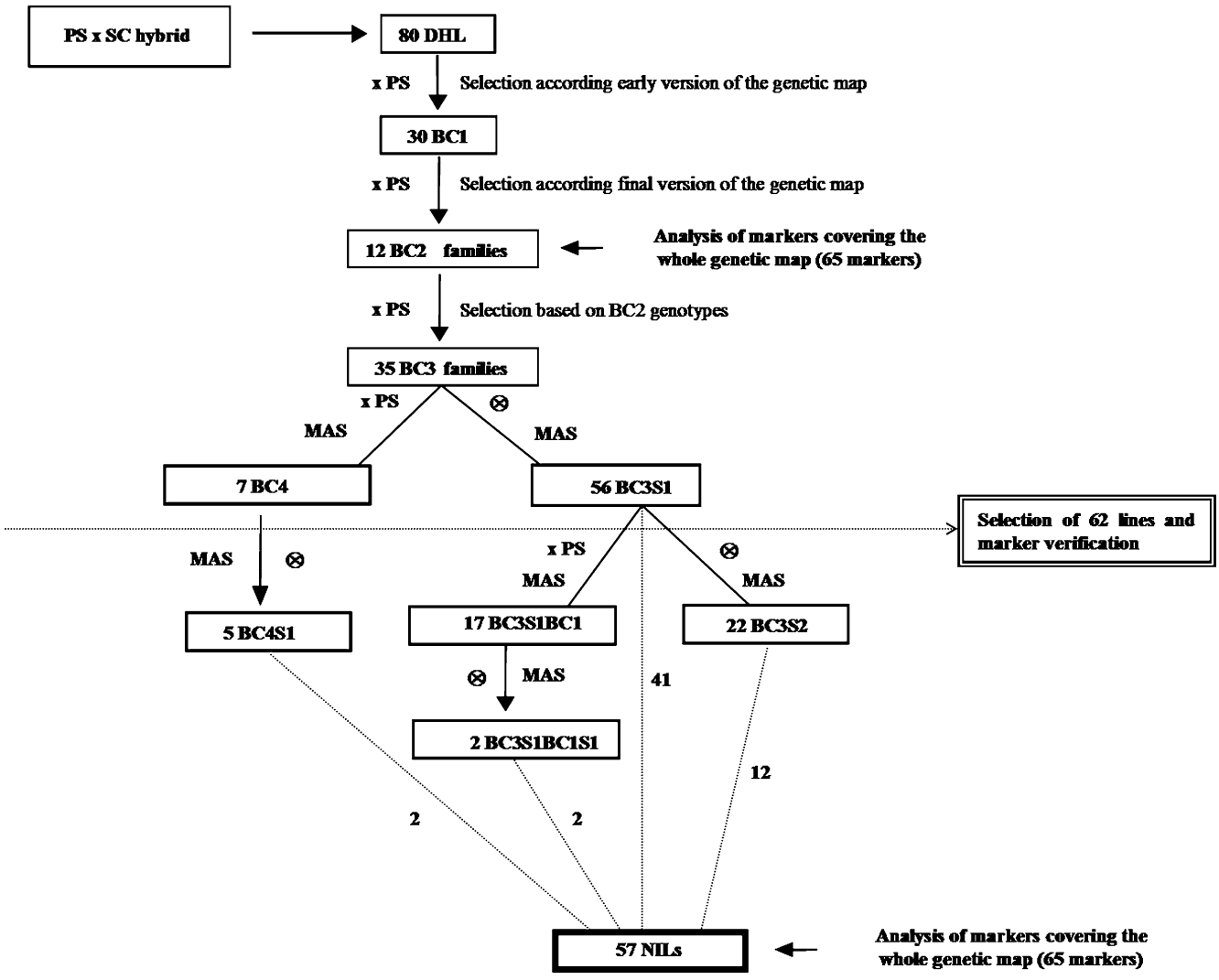

Fig. 1 Breeding scheme for NIL development. The different generations are included in *boxes*, linking successive generations with an *arrow*. Selfing rounds are depicted as "*encircled multiple symbol*" and backcrosses with the recurrent parent PS with "× PS". The selection steps carried out among generations are indicated on the *right*. Marker Assisted Selection (MAS) steps indicate marker screenings to fix candidate regions and/or remove other non-desirable regions. *Discontinuous lines* link the last generation where the definitive NILs were selected. *Numbers* alongside discontinuous lines show how many lines were obtained in each generation

A selection differential for reduction of SC allele frequency was calculated according to Falconer (1989) for each selection step:

$$S = 100\left(\frac{f_{(p)} - f_{(s)}}{f_{(p)}}\right);$$

where $f_{(p)}$ is the average frequency of SC alleles in the population under selection (DHL, BC_2) and $f_{(s)}$ the average frequency of SC alleles in the selected plants.

Twelve to two hundred seedlings from each BC_3 family were genotyped in order to select the target regions and discard other exotic introgressions. Several rounds of marker assisted selection (MAS), backcrossing with PS and selfing were performed to obtain a first set of NILs, with a single introgression from the donor genome. All these lines were genotyped with 65 molecular markers for verification. After verification, some plants showed undesirable introgressions and were substituted by other NILs to obtain a final set with single introgressions. These NILs were genotyped again with the 65 molecular markers for verification.

Molecular marker analysis

DNA was extracted as described by Klimyuk et al. (1993) and Garcia-Mas et al. (2000) from cotyledons and young leaves. Due to the fact that it was necessary to perform marker screenings of a relatively large number of plants in a short period of time, only codominant PCR-based markers were used. The set of molecular markers included 62 SSRs, one cleavage amplified polymorphic sequence (CAPS) and two sequence char-

acterised amplified regions (SCARs) selected from the map of Gonzalo et al. (2005). This set of markers covers more than 85% of the melon map and defines a map with one marker every 15.7 cM, an average number of 5.5 markers per linkage group (LG), and a maximum gap of 57 cM in LG IX (Fig. 2).

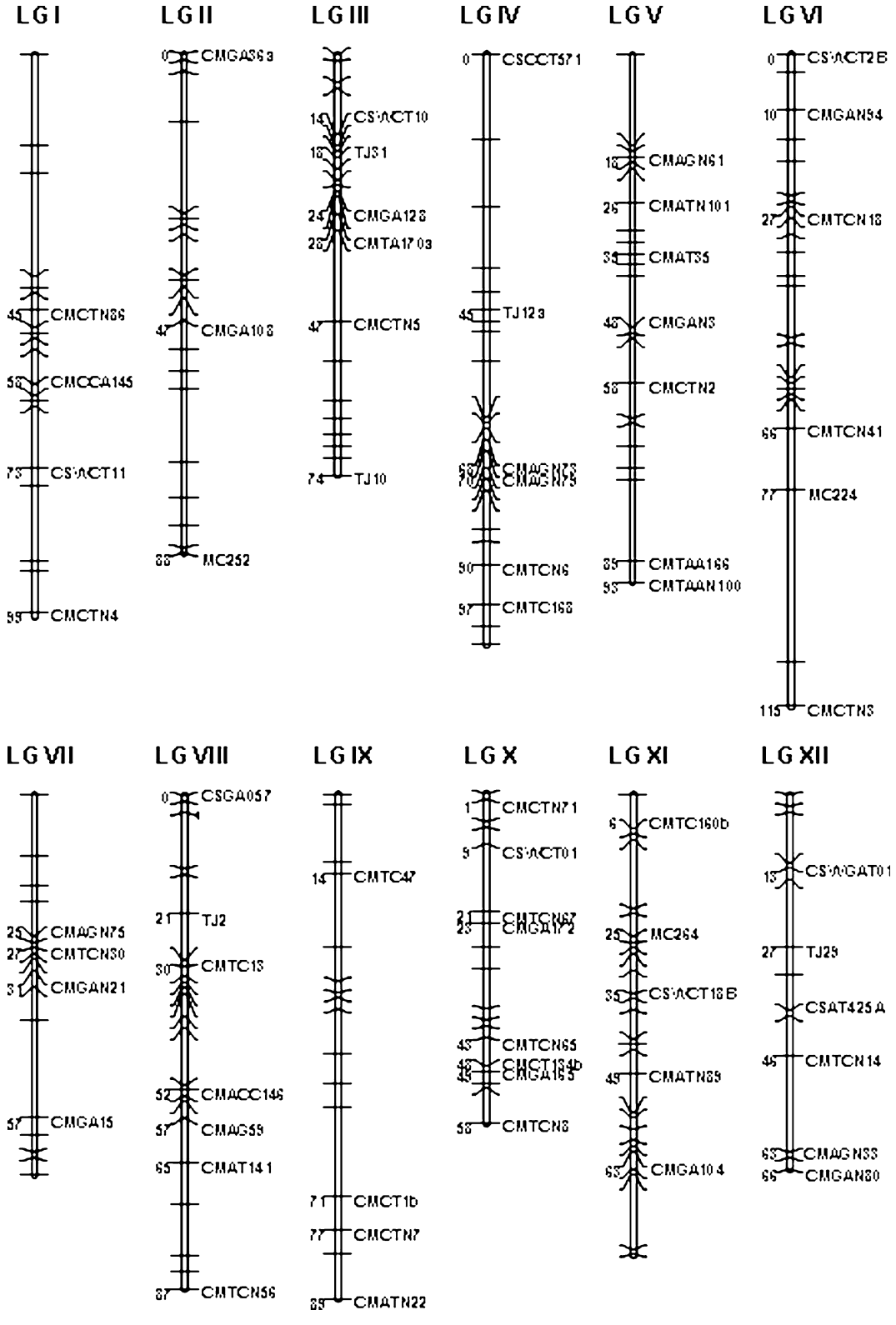

Fig. 2 Set of markers used in the construction of the NIL population on the skeleton of the genetic map according Gonzalo et al. (2005). Linkage group nomenclature is according Perin et al. (2002b)

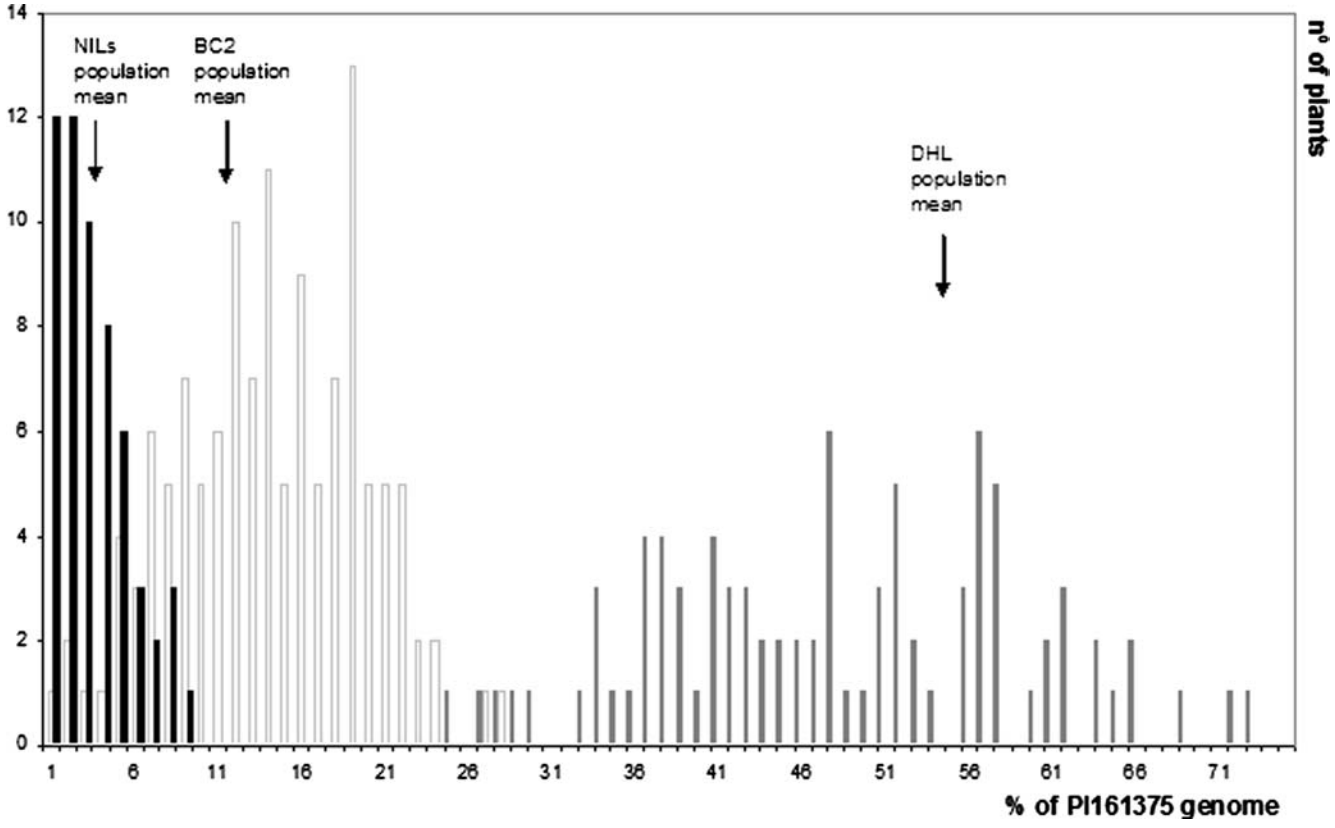

Fig. 3 PI161375 genome frequency among lines of the different populations. *Grey bars* indicate the DHL population, *boxed light gray bars* the BC_2 and *black bars* the NIL population. Means of the PI161375 genome frequency for the different populations are indicated with *arrows*

SSR were amplified in a total volume of 15 µl of 1 × SSR buffer (20 mM $(NH_4)_2SO_4$, 75 mM Tris–HCl pH 8.8, 0.01% (v/v) Tween 20), 2 mM $MgCl_2$, 166 mM dNTPs, 2 pmol of each primer (one labelled with IRD-800) and 2 U of Taq DNA polymerase (PE Applied Biosystems). Cycling conditions were as follows: an initial cycle at 94°C for 1 min followed by 35 cycles at 94°C for 30 s, the appropriate annealing temperature for 30 s and 72°C for 1 min and a final cycle at 72°C for 5 min. Loading buffer of 5 µl (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) were added to the PCR mix. Samples were denatured at 94°C for 3 min and 0.8 µl were loaded on to a LICOR IR2 sequencer (Li-Cor Inc, Lincoln, NE, USA) using 25 cm plates filled with 6% acrylamide gels in 1xTBE (90 mM Tris–borate, 2 mM EDTA pH 8.0 and 7.5 M urea) buffer. Electrophoresis was performed at 1,500 V, 35 mA and 31 W at 50°C. Bands were scored visually and the molecular weight of each band was estimated by comparing its migration on electrophoresis with the IRD-labelled STR molecular size marker (Li-Cor Inc, Lincoln, NE, USA).

The markers MC252 and MC224 were genotyped as described by Morales et al. (2004). Primers for the SCAR marker SMC264 were designed from the sequence of the melon cDNA clone MC264 (Oliver et al. 2001): SMC264-f: AGCATCATTGTTGTTAATGG AA and SMC264-r: CTGTCCATGTTACCTTGTCAGAA. DNA was amplified in 1 × PCR buffer (10 mM Tris–HCl pH 8.3, 2.5 mM $MgCl_2$, 50 mM KCl, 0.001% gelatine), 400 mM of each dNTP, 0.4 mM of each primer, 2 U of Taq polymerase and 50 ng of genomic DNA with an initial denaturation step for 2 min at 94°C, followed by 35 cycles at 94°C for 20 s, 53°C for 20 s and 72°C for 30 s, with a final extension step at 72°C for 5 min. The amplified band was 670 bp for SC and 700 for PS. The position of this marker in the map was verified using the DHL population (Gonzalo et al. 2005).

Genotype presentation and genome composition of DHL, backcross and NIL populations

Graphical genotypes for the DHL, backcrosses and NIL populations were obtained using the software program Graphical Genotyping (GGT, Van Berloo 1999, http://www.dpw.wau.nl/pv). The size and the number of introgressions, as well as the percentage of the donor genome, were determined with GGT. For the calculation of introgression size and genome percentages, the half-intervals flanking a marker locus were considered to be of the same introgression as

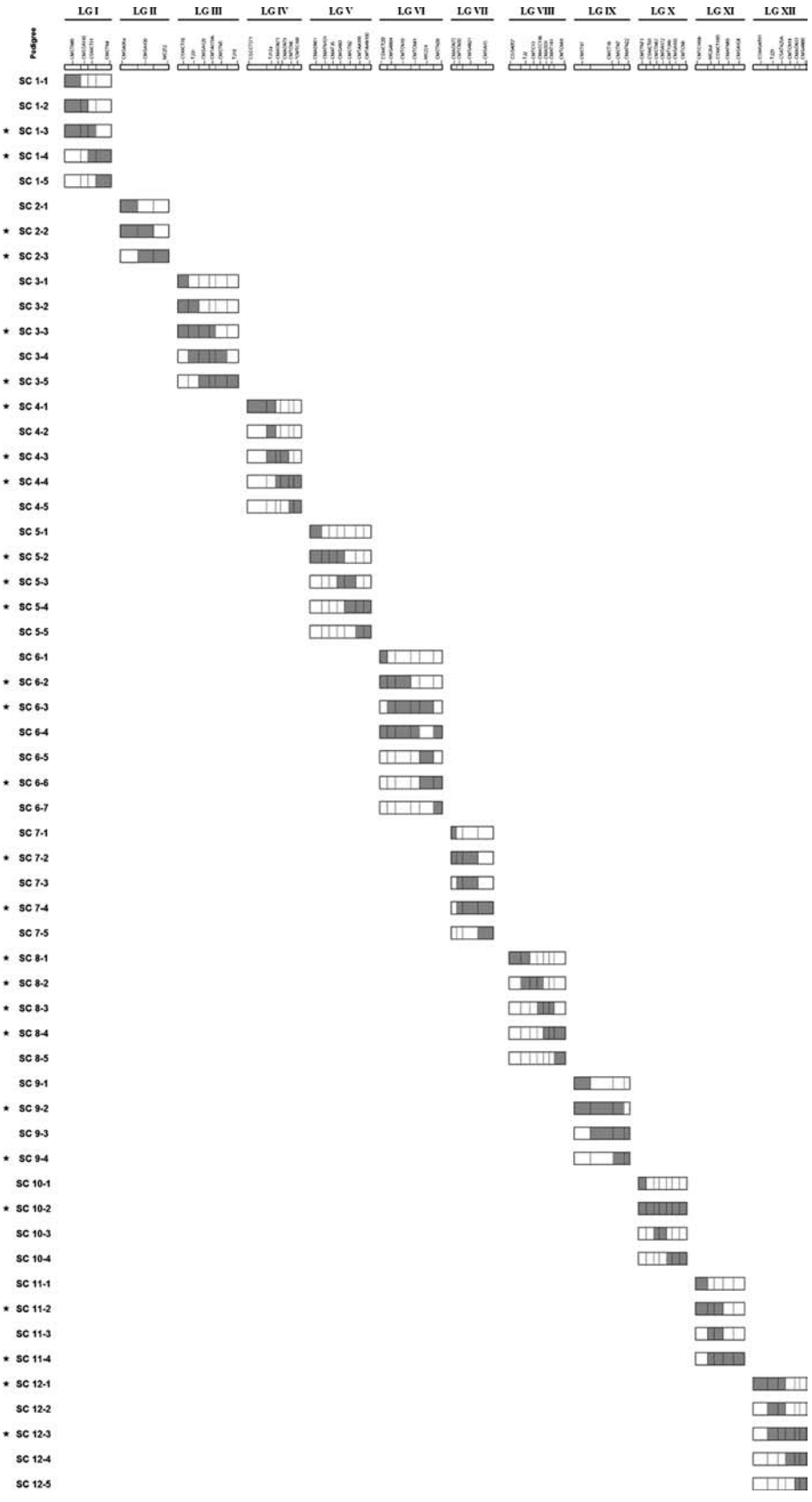

Fig. 4 Graphical genotypes of the NILs. Introgressions from SC are depicted in *grey*. Linkage groups (*LG*) are depicted above. The NIL names are indicated on the *right*. *SC* indicates the donor parental "Songwhan Charmi", the *first number* indicates the LG where the introgression maps and the *second number* indicates the order of the introgression within LG. The minimum set of 28 lines that cover all the PI161375 genome are marked with *asterisk* (*)

implemented by the GGT software. For missing marker data, plants were assumed to have the genotype of the two flanking markers, if these had identical genotypes for a line. However, if the two flanking markers had contrasting genotypes, then the data were recorded as missing.

Results

DHL, BC_1 and BC_2 genome composition

The average percentage of donor parent genome among the DHLs was 55% (Fig. 3). Thirty DHLs were selected to found the BC_1 population, and 12 of these BC_1 plants were selected for the BC_2 population. Ten BC_1 selected for low-donor genome and maximum coverage (mean donor genome percentage 25%, range 17–30%), and two for high-donor genome (mean 35%). From the 192 BC_2 plants, BC_3 seed was obtained from 128 BC_2 plants, and only these plants were genotyped. BC_2 plants had an average of six introgressions from SC, ranging from 2 to 10, with an average of 13.55% donor genome (Fig. 3). The entire donor genome was represented in the 128 BC_2 plants. The linkage groups with lower SC allele frequency were LG I and LG X (frequency of donor genome 9%), while LG VIII and LG VII had a higher level of donor genome representation (20%), as expected, because of their higher frequency in the DHL population (Gonzalo et al. 2005). Linkage groups LG XI, LG IX, LG II and LG VI were under-represented in the DHL population, but were more highly represented in the BC_2 population (with donor genome fragments in 20, 11, 12 and 15% of the plants, respectively).

During the NIL development process, data of marker segregation and recombination rates between linked markers were recorded for several families. Recombination rates corresponded very well with map genetic distances, and distorted segregations were not observed for the regions analysed (data not shown).

NILs development and characterization

Candidate introgressions were identified in 35 BC_2 plants with an average of 10% donor genome (range 2.6–20.0%), corresponding to a selection differential of 26% for the reduction of donor genome. Several rounds of backcrosses and selfing, monitored by appropriate MAS, were performed, to obtain a first set of 62 NILs for which the genotype was verified. Following this verification, 41 BC_3S_1, 12 BC_3S_2, 2 BC_4S_1 and 2 $BC_3S_1BC_1S_1$ (S_i, is the ith selfing generation) plants from the previous NIL set were selected for a definitive set of 57 NILs (Fig. 4). According to the map depicted in Fig. 2, and assuming no double crossing-overs, each NIL contains a single homozygotic introgressions, with an average introgression size of 41.0 cM (approximately 3.4% of the SC genome), ranging from 6.8 to 100.8 cM (0.6–8.9% of the donor genome). Each linkage group was represented by an average of 4.75 NILs with overlapping introgressions.

Taking into account the regions defined by these overlapping introgressions, the SC genome can be divided into identifiable genome fragments or bins. Each bin is defined as a unique genomic region defined by two linked molecular markers, with no recombination between them and with recombinations flanking the region, when taking the whole population genotypes into consideration. In this population a total of 49 bins were defined, with an average size of 18.9 cM per bin and a range of between 2 and 43.7 cM, respectively. If the objective is not to obtain the best resolution mapping, there is a subset of 28 NILs (Fig. 4) as the minimal number of lines with the same genome coverage as the whole population. This subset represents 41 bins with an average bin size of 24.6 cM ranging 5–74 cM and can be used to select candidate introgressions involved in the traits of interest when resources are limited and it is not possible to analyse the entire collection.

Due to the limited number of SSR markers placed in the molecular map (Fig. 2), some genomic regions were not well covered. For example, the distance of closest marker to the end of the linkage group in LG I and VII is 45 and 25 cM, respectively (involving NILs SC1-1, SC1-2, SC1-3, SC7-1 and SC7-2). It is possible that part of these linkages groups is not represented in the collection. Furthermore, gaps of more than 45 cM between SSR markers are also present in LG II, IV and IX (involving NILs SC2-1, SC2-2, SC4-1, SC9-2) where non-detected double cross-overs could have occurred. New SSR and PCR markers recently developed (Ritschel et al. 2004; Fukino et al. 2004; Puigdomènech et al. 2005) are currently used to verify the allelic state of those genomic regions.

Discussion

DHL, BC_1 and BC_2 genome composition

The average percentage of donor parent genome among the DHLs and the BC_2 population was 55 and 13.55%, respectively (Fig. 3), similar to the expected values for these type of populations estimated in 50 and 12.5%, respectively. There was no reduction of the frequency of the exotic alleles in the BC_2 compared with the allelic frequencies in the DHL population (Gonzalo et al. 2005), suggesting that, in general, distorted segregation is not expected in advanced populations derived from the current cross. Even though the objective of the present investigation was not the thorough study of distorted segregation or recombination rates, these results may be considered as a preliminary report of these phenomena. Nevertheless, these results contrast with the very common distorted segregation detected in advanced generations involving tomato wild species (Fulton et al. 1997; Chetelat and Meglic 2000; Monforte and Tanksley 2000a), and the recombination shrinkage observed after the introgression of small regions of exotic DNA into the cultivated genome background (Paterson et al. 1990; Monforte and Tanksley 2000b). These contrasting results may be due because the current population was created from an intraspecific cross in melon, avoiding the problems of self-incompatibility, unilateral incon-

gruity and gamete or zygote lower viability, which may arise after interspecific crosses (Bernacchi and Tanksley 1997; Monforte and Tanksley 2000a). Genomic regions with distorted segregation and/or recombination shrinkage are more difficult to dissect genetically because it is more complicated to generate and recover new recombinants (Pillen et al. 1996; Monforte and Tanksley 2000b). The fact that these problems were not detected in the current NIL population, indicates that intraspecific NIL collections such as the one developed in melon, will be good tools for QTL mapping and cloning.

NIL population characterization

The average bin size of the current population is 18.9 cM, which defines the genetic resolution to map new molecular markers or QTLs. This resolution will probably increase when a denser melon linkage map is available, allowing better definition of the recombination points limiting the introgressions, which would define more bins. Nevertheless, this resolution is sufficient to map QTLs with a higher precision than that of the standard mapping populations (Tanksley 1993; Hyne et al. 1995; Mackay 2001). Furthermore, the resolution can be easily increased by generating new recombinants from selected NILs, allowing fine mapping (Paterson et al. 1990) or map-based cloning of QTLs (Fridman et al. 2000; Frary et al. 2000; Liu et al. 2002).

Three NILs have already been used to verify the QTLs involved in several fruit traits: fruit shape (*fs1.1* and *fs9.1*), external colour (*ecol9.1*) and flesh colour (*gf1.1*) (Monforte et al. 2004a). The effects of these QTLs were verified using NILs with introgressions in the candidate regions (Eduardo et al. 2004). This preliminary work confirms that the present NIL collection will be very useful to dissect genetically complex traits in melon.

The quality of a NIL population can be defined by several factors: maximum coverage of the donor genome, minimum number of homozygous introgressions per NIL (ideally only one), type of markers used, preferably transferable codominant markers such as RFLPs (restriction fragment length polymorphisms) or SSRs and, to a lesser extent, the genetic resolution defined by average bin size. To the best of our knowledge, the NIL populations that best fulfill the above parameters are the tomato NIL populations of Eshed and Zamir (1994) and Monforte and Tanksley (2000a) and the melon NIL population reported here.

The NILs were developed from only 8 of the 12 selected DHLs: seven from DHLs selected for low-donor genome and one for high-donor genome. This result confirms that, theoretically, a NIL collection covering all the donor genome may be achieved with a starting set of individuals of very modest size, if a marker-characterised population is available to ensure complete donor genome coverage in the advanced populations.

Near isogenic line populations have been obtained with a highly variable number of generations (usually combining backcrosses and selfing). Eshed and Zamir (1994) needed 8–12 generations in tomato, whereas Pestova et al. (2001) needed only 4–5 in wheat. These differences are due to many factors, including the size of the genetic map in the recipient species, the donor genome (same species or wild relative), pre-existing information (markers, map, populations) or allocated resources (number of markers analysed per generation) and the resolution expected (average introgression size). For our melon NIL collection only six generations were required to obtain a reasonable genetic resolution (18.9 cM average bin size). Two factors were important for this. First, all BC_1 plants were genotyped for a selected set of markers covering most of the genome. This prevented the loss, in the early stages, of genomic regions of the donor genome that were under-represented due to skewed segregations which favour the recipient genome. Secondly, NIL extraction was started in the BC_3 generation, although this implied the screening of a large number of individuals for many markers at this stage.

Two important factors must be considered in the choice of when to start NIL extraction: the size of the recipient genome map and the length of the intergeneration period. For species with large maps and short generations such as lettuce (Jeuken and Lindhout 2004), starting at more advanced backcross generations may be a good strategy because there is the advantage of achieving a more homozygous genome over a relatively short-time period. In contrast, for species with short maps and long-generation periods, such as fruit trees of the *Prunus* genus (Aranzana et al. 2003), it would be clearly better to select as soon as possible, identifying plants with a low content of the donor genome from large BC_1 or BC_2 populations. Melon has an intermediate map size (1,021 cM) and relatively slow generation time (two generations per year), so the strategy of selecting in the BC_2 generation was efficient.

In this paper, we present a new genetic resource for melon genetics and genomics: a collection of 57 NILs with a single introgression from an exotic Korean melon (PI161375) into the genetic background of a PS cultivar. The analysis of this population will help to improve the current lack of knowledge of the genetic control of complex traits in melon, providing a better understanding of the high-phenotypic variability observed in melon germplasm. Furthermore, this population could be used to investigate QTL × environment and QTL × QTL interactions and to dissect the genetic basis of heterosis (Monforte et al. 2005). From the breeder's point of view, this population could be used to assess whether the new alleles provided by the exotic melon germplasm may be useful for the improvement of modern cultivars, and as a first systematic approach to incorporate exotic variability into elite germplasm.

Currently, as part of other molecular mapping projects, we are verifying the allelic state of some genomic

regions that were not completely covered by the map used to develop this population. After that verification, the collection will be released to international germplasm banks, for example, the "Centro de Conservación y Mejora de la Agrobiodiversidad Valenciana" (COMAV; http://www.comav.upv.es/comav_banco.html), for maintenance and distribution to interested researchers.

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