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Molecular markers for sex determination in papaya (*Carica papaya* L.)

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Abstract We have developed molecular markers tightly linked to *Sex1*, the gene that determines plant sex in papaya (*Carica papaya* L.). Three RAPD products have been cloned and a portion of their DNA sequenced. Based on these sequences SCAR primers were synthesized. SCAR T12 and SCAR W11 produce products in hermaphrodite and male plants and only rarely in females. SCAR T1 produces a product in all papayas regardless of plant sex. SCAR T12 and SCAR W11 showed no recombination in a population of 182 F2 plants from a 'SunUp' by 'Kapoho' cross. Based on these results a PCR-based technique for rapidly and accurately determining the sex of papaya plants was developed using either W11 or T12 to detect the hermaphrodite or male allele and T1, which amplifies a product regardless of sex type, as a positive control. The sexing technique, using SCAR T12 and SCAR T1 as a positive control, was used to correctly predict hermaphrodite papaya plants in a population of seedlings with an overall accuracy of 99.2%.

Keywords Papaya · Plant-sex · Flower-type · Development · Genetic-mapping

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Introduction

Papaya (*Carica papaya* L.) is a polygamous species with three sex types: male, hermaphrodite and female. In most cases hermaphrodite plants are desired for commercial production. In papaya, plant sex is controlled by a single gene having at least three alleles; a dominant allele for male plants, a different dominant allele for hermaphrodite plants and a recessive allele for female plants (Hofmeyr 1938; Storey 1938, 1953, 1976; Sondur et al. 1996).

Despite the phenotypic observation of dominance, plant sex segregates with a 2:1 ratio when hermaphrodites are selfed, rather than the 3:1 ratio normally expected for a dominant/recessive gene system. It has been hypothesized that the dominant homozygotes are lethal thus leading to the 2:1 segregation ratio (Storey 1938, 1953). This interpretation is supported by the lack of true-breeding hermaphrodite or male plants. Hermaphrodite or male papayas crossed to female plants always produce one-half female plants; never the all-hermaphrodite or all-male population expected if the hermaphrodite or male parent was homozygous. Also, hermaphrodite plants that are self-pollinated always produce about one-third females.

The unusual segregation ratio and lack of homozygous plants carrying either of the dominant alleles led Storey (1953) to hypothesize that a recessive lethality factor is linked to the hermaphrodite and male alleles, which in the homozygous state causes seeds to be inviable. Further, he explained the observation that papaya sexes, in addition to differing in functionality of the androecium and gynoecium, also have several different co-segregating secondary sexual characteristics, such as ovary shape, degree of corolla fusion, flower number and peduncle length, by hypothesizing that sex in papaya is controlled by multiple tightly linked genes, the allelic forms of which constitute differential chromosome segments. Included in his hypothesis is a suppressor of recombination that is associated with this chromosomal region and keeps all of these genes linked as a simply inherited unit.

In the light of recent advances in our understanding of gene regulation and floral development, Sondur et al. (1996) suggested a model of sex type based on alternate alleles of a gene encoding a *trans*-acting factor. They proposed that the male allele of the sex gene, designated *SEX1-M*, encodes a *trans*-acting factor that induces male floral parts while inhibiting carpel development. The hermaphrodite allele, *SEX1-H*, is intermediate having the ability to induce male structures but only reducing carpel size. The female allele, *sex1-f*, is incapable of inducing male structures. The lethality of *SEX1-M* or *SEX1-H* homozygotes could result from an additional required function present only in the *sex1-f* allele or a tightly linked gene, although a lethal mutation tightly linked to the *SEX1-M* and *SEX1-H* alleles cannot, at this time, be ruled out.

The variability observed in the secondary sexual characteristics could result from environmental or allelic interaction effects on the expression or function of *Sex1*. For example, it is possible that the interaction between *SEX1-H* and its target, either a promoter sequence or another protein factor, could be less stable than the interaction between *SEX1-M* and the target. This could account for the difference in carpel size between males and hermaphrodites, and for the sex reversal sometimes seen in hermaphrodites but which is rare in males.

Sondur et al. (1996) produced a genetic linkage map of papaya using randomly amplified polymorphic DNA (RAPD) markers. This map includes a linkage group containing the phenotypically scored *SEX1* gene and RAPD markers in both phases that are fairly tightly linked to *SEX1*. One RAPD marker mapping 7.8 cM from *Sex1* in linkage with the female allele was uncovered, and two flanking markers both approximately 7 cM in linkage with the *SEX1-H* allele were found. Somsri et al. (1998) have reported DNA amplification fingerprinting markers linked to male and hermaphrodite alleles of *Sex1*. They were able to distinguish the male and hermaphrodite alleles in the cross of a male plant to a hermaphrodite plant. Parasnis et al. (1999) report a microsatellite sequence unique to males or hermaphrodites of several cultivars; however, they did not report detailed data on linkage. Additionally, Parasinis et al. (2000) and Urasaki et al. (2002) report SCAR markers that are specific for male and hermaphrodite plants. Again, these reports utilized a relatively small number of plants and no linkage data is given.

Molecular markers tightly linked to *Sex1* have several uses. The sex of papaya plants cannot be determined from morphological characteristics until flowering. In the case of some important commercial varieties, including the Hawaiian types such as 'Sunrise' and 'Kapoho', flowering is quite delayed. While molecular markers would probably be too expensive and technology intensive for routine use by farmers, it would be a valuable tool for researchers to employ in determining the sex of experimental material such as transgenic plants. Molecular markers, especially flanking markers, could also be used to clone *Sex1*. Although a number of genes involved in flower morphogenesis have already been cloned, the papaya sex system would be an interesting addition, especially in light of the multiple alleles with interesting and distinct phenotypes. Isolation of the three alleles of *Sex1*, female, hermaphrodite and male, would allow a detailed understanding of the distinct developmental differences among the sex types at the molecular level and could lead to development of true-breeding hermaphrodite papaya plants.

In this paper we report the cloning of RAPD fragments tightly linked to *Sex1* and the development of a PCR-based system for rapidly and reliably determining papaya plant sex from small tissue samples.

Materials and methods

Bulked-segregant analysis for the detection of *Sex1*-linked RAPD products

Equal amounts of DNA from 25 hermaphrodite plants and 25 female plants from the F_2 generation of a cross between the Hawaiian variety 'Sunrise' and UH Line 365 were bulked. This cross has been previously described (Sondur 1994; Sondur et al. 1996). DNA was extracted as previously described (Sondur et al. 1996). A total of approximately 500 random 10-base deoxyribonucleotides of the UH and Operon series (see Sondur et al. 1996) were used in RAPD-PCR reactions. The reaction products were analyzed by electrophoresis through 1.5% agarose gels. The RAPD-PCR and gel conditions have been previously described (Sondur et al. 1996).

Individual plants in the segregating populations were analyzed using the same DNA extraction, RAPD-PCR and gel-electrophoresis conditions to confirm linkage to plant sex.

Cloning and sequencing of RAPD products

After RAPD-PCR, as described above, the products were separated by electrophoresis in low-melting agarose. Three RAPD-PCR products were identified for cloning based on linkage to sex type and on their specificity and reproducibility. Two of these, T1 (previously designated T1C) and T12, were previously mapped near the *Sex1* locus (Sondur et al. 1996). One new RAPD product, W11, was also cloned. The nucleotide sequences of the RAPD

Table 1 RAPD-PCR products and primers used in this paper

Type	RAPD primer sequence	PCR primers deduced from the sequence of the RAPD product		PCR product size
T1	GGGCCACTCA	T1-F	TGCTCTTGATATGCTCTCTG	~1,300 bp
		T1-R	TACCTTCGCTCACCTCTGCA	
T12	GGGTGTGTAG	T12-F	GGGTGTGTAGGCACTCTCCTT	~800 bp
		T12-R	GGGTGTGTAGCATGCATGATA	
W11	CTGATGCGTG	W11-F	CTGATGCGTGTGTGGCTCTA	~800 bp
		W11-R	CTGATGCGTGATCATCTACT	

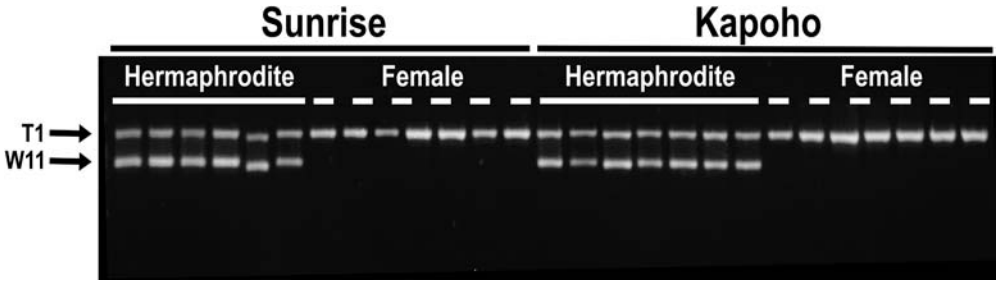

Fig. 1 Multiplex PCR showing segregation of SCAR W11 with flower sex. PCR was run on DNA from papaya varieties 'Sunrise' and 'Kapoho' using primers for both SCAR T1 and SCAR W11. Conditions for the multiplex PCR are as recommended for determination of plant sex and are described in the Materials and methods. SCAR T1 is present in all samples indicating a successful reaction. SCAR W11 is present in hermaphrodite plants but not in female plants. Plant sex was determined by the observation of flowers

primers, and the 20 and 21 base sequence-characterized amplified region (SCAR) primers (Paran and Michelmore 1993) developed from them, are shown in Table 1.

For cloning, sections of the RAPD gels containing polymorphic products were excised and the gel was digested with several units of agarase at 45 °C for 30 min. After digestion, approximately 200 µl of the recovered PCR product was ligated into pCR2.1 using conditions recommended by the manufacturer of the "TA Cloning Kit" (Invitrogen). White colonies were picked and analyzed by PCR using the appropriate RAPD fragment to confirm integration of the correct PCR product.

DNA was isolated from plasmids containing the correct product using the "Mini plasmid isolation kit" and procedures recommended by the manufacturer (Qiagen). DNA sequences were determined by automated DNA sequencing at the University of Hawaii Biotechnology Service Center. The universal forward and reverse primers homologous to the poly-linker region of pCR2.1 were used to prime the sequencing reactions.

PCR analysis of plant sex

Twenty and 21-base deoxyribonucleotides were synthesized based on the sequences of the cloned RAPD products (see above). Two primers, one forward and one reverse, were constructed for each RAPD fragment. The sequences of these primers are shown in Table 1. These primers were used in PCR reactions with template DNA (a 20 ng/25 µl reaction) from the F_2 population described above. The conditions of the PCR reaction were determined empirically by varying the annealing temperature and Mg^{+2} concentration so that the same conditions could be used for all primer sets allowing multiplex PCR to be carried out. Other components of the reaction were from the Promega PCR kit, and other reaction conditions were as recommended by the manufacturer. The conditions for a typical 25-µl reaction used in this research which we recommend for sex determination is:

Deoxyribonucleotide triphosphates (1.25 mM each)	3.0 µl	
10 × Promega Reaction Buffer	2.5 µl	
$MgCl_2$ (25 mM)	1.5 µl	
Primers (2 µM each)	2.2 µl each	(can use more than one set for multiplex reactions)
Taq DNA Polymerase (5 units/µl)	0.2 µl	(1 unit)
Papaya genomic DNA (5 ng/µl)	4.0 µl	(20 ng total DNA)
Water	to 25 µl	
Total volume:	25 µl.	

The reactions are incubated at 95 °C for 5 min and then run for 25 cycles of 1 min at 95 °C, 1 min at 58 °C and 1 min at 72 °C followed by a final extension period of 7 min at 72 °C.

Initial mapping population

A population of 182 F_2 plants from a cross between 'Sun Up' and 'Kapoho' were used to map SCAR T12 and SCAR W11. This population consisted of 123 hermaphrodites and 59 females.

Analysis of seedlings

In vitro-germinated seedlings of the papaya ringspot-resistant transgenic variety 'Rainbow', a hybrid between 'Kapoho' and 'SunUp', were analyzed for plant sex as described above. In vitro seedlings of 'Rainbow' by 'Kamiya' ('Laie Gold') and Rainbow by 'Kapoho' ('Poamoho Gold') crosses were also analyzed. In all, 1,937 seedlings of these crosses were analyzed prior to transfer to the field.

Results

Five hundred primers were tested and gave an average of about ten distinct RAPD products each, for a total of approximately 5,000 events examined. Twelve products (0.24%) were clearly specific to hermaphrodite plants. These 12 RAPD products were examined using RAPD-PCR and individual plants that contained a crossover between the previously characterized and mapped T1 and T12 RAPD products (Sondur et al. 1996). One of the 12 products, W11, showed no recombination in any of these plants indicating that it is closer to the *Sex1* gene than T1 and T12.

Three *Sex1*-linked RAPD products T1, T12 and W11 were cloned and analyzed in more detail. Based on the DNA sequence determined from the cloned RAPD T1 product, 20-base PCR primers were synthesized that were homologous to the ends of the fragment just internal to, but not including, the regions corresponding to the original RAPD 10-base deoxyriobnucleotide priming sites (Table 1). When these 20-base PCR primers were used to amplify DNA from individual hermaphrodite and female plants a product of approximately 1,300 base pairs was found in all plants regardless of flower sex.

The cloned T12 product was sequenced from each end and two 21-nucleotide sequences (that included ten bases of the original RAPD primer) were identified that were appropriate as SCAR priming sites. The product produced by these primers, designated T12F and T12R, is approximately 800 base pairs and shows linkage to

Table 2 Sex prediction in a diverse selection of papaya cultivars

Cultivar	Plant sex	W11 present	T12 present
Khaek Yellow	Hermaphrodite Female	5/5 0/5	
Khaek Nuan	Hermaphrodite Female	5/5 0/2	2/2 0/2
Khaek Dum	Hermaphrodite Female	3/3 0/3	
N94-93 (Australian)	Male Female	5/5 0/3	2/2 0/2
Mardi (Malaysian)	Hermaphrodite Female	5/5 2/5	2/2 0/1
Honey Dew (Indian)	Male Female	5/5 1/4	2/2 0/2
HCAR 159 (Honey Gold; South African)	Male Female		2/2 0/2
Pitsanulok (Thai)	Hermaphrodite Female		2/2 0/2
Correct sex prediction:		47/50 (94%)	23/23 (100%)

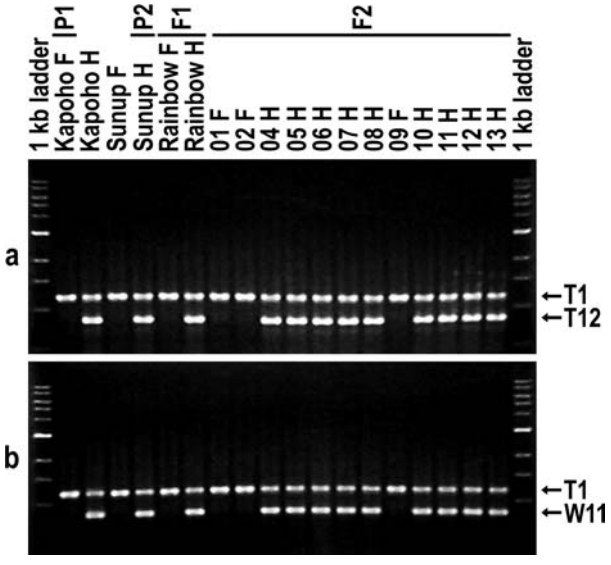

Fig. 2 Multiplex PCR showing segregation of SCAR T12 and SCAR W11 in a F_2 population of a cross between 'Sunup' and 'Kapoho'. Multiplex PCR was run as recommended for sex determination and as described in the Materials and methods. In addition to the F_2 population, the 'Kapoho' female patent, a hermaphrodite of the inbred 'Kapoho line, the male parent, a 'Sunup' hermaphrodite, a female of the inbred 'Sunup' line, and a female and hermaphrodite of the F_1 ('Rainbow'), is shown. SCAR T1 is present in all samples indicating a successful PCR reaction. SCAR T12 (*panel a*) and SCAR W11 (*panel b*) are present in all hermaphrodite plants

Sex1. It is present in hermaphrodite and male plants but is not found in female plants.

Based on the DNA sequence at the ends of the W11 RAPD fragment 20-base SCAR primers were constructed that included the ten bases of the original RAPD primer. As with T12, W11 is linked to *Sex1*, and the SCAR primers amplify a product approximately 800 base pairs in length in hermaphrodite and male plants but no product is produced in female plants. Figure 1 shows the segregation pattern for W11 in two Hawaiian cultivars, 'Sunrise' and 'Kapoho'.

An F2 population consisting of 182 plants derived from a cross between 'SunUp' and 'Kapoho' was used to examine the co-segregation pattern of sex, and the W11 and T12 SCARS produced, using the 20- and 21-base PCR primers described above. The T1 20-base SCAR primers, which produced a 1,300-bp product in all papaya samples tested, were used as positive controls to ensure that reactions were successful. W11 and T12 co-segregated with *Sex1* in all 182 plants from which DNA was successfully amplified. Figure 2 shows some of these results including parental and F1 plants.

Data from the F2 population were analyzed using MapMaker with the Kosambi Mapping function. SCAR T12 and SCAR W11 mapped at *Sex1*, as there were no crossovers detected.

A diverse variety of dioecious and gynodioecious papaya cultivars developed in different areas of the world were screened for linkage of W11 and T12 to sex type. Table 2 summarizes this data. In all varieties, the presence of SCAR T12 predicted the dominant hermaphrodite or male sex allele. Similarly, in the case of W11, all hermaphrodites or males, produced the SCAR W11 product; however, two of five females of the Malaysian Mardi variety and one of four females of the Indian Honey Dew variety also produced SCAR W11.

The utility of using the SCARs for plant sexing prior to planting in the field was investigated using the SCAR T12-SCAR T1 system. Thirty-five F1 hybrids from a cross between the transgenic PRSV-resistant 'Rainbow' and either 'Laie Gold' or 'Poamoho Gold' were examined before micropropagation. All 19 hermaphrodites were correctly predicted. A larger population of 1,937 seedlings was screened and those plants predicted to be hermaphrodites were planted in the field. Of the 750 surviving plants, 744 were hermaphrodite and six were female. This gives a successful prediction rate for hermaphrodites of 99.2% in a situation where this technology might be used to predict papaya plant sex.

Discussion

Three RAPD-PCR products showing linkage to the gene that determines flower sex, *Sex1*, in papayas have been cloned. Two of these RAPD products, T12 and T1, had been previously mapped to 7 cM flanking the *Sex1* gene. The third product, W11, was chosen because it did not show recombination in individual plants that did show recombination between *Sex1* and either T12 or T1, indicating that W11 is closer to *Sex1* than either T12 or T1. Although the three cloned sequences have not been com-

pletely sequenced in both strands, they show no significant homology to any sequences currently in public sequence databases and do not appear to encode proteins.

The RAPD-PCR fragment designated T1 shows linkage to *Sex1* at about 7 cM (Sondur et al. 1996). However, when 20-base SCAR primers were constructed based on the sequences within the cloned T1 fragment they amplified DNA from both hermaphrodites and females. The most-likely explanation for this is that one or more point mutations exist in the original 10-base primer site distinguishing the female parent of the original cross (UH breeding line 365) from the pollen donor hermaphrodite parent ('Sunrise'). The 20-base primers for SCAR W11 include the ten bases of the original RAPD primer and the next ten bases deduced from the sequence of the RAPD fragment. In the case of T12, the 21-base SCAR primers were produced based completely on sequences internal to the original T12 RAPD fragment. Both SCAR W11 and SCAR T12 produce products almost exclusively in males and hermaphrodites and not in females; however, it is not clear at this time whether the difference is the result of a limited number of base changes in the SCAR binding sites or more substantial alterations such as deletions of the binding sites or even the entire regions.

SCAR W11 and SCAR T12 were mapped in a 'SunUp' by 'Kapoho' cross. Both of these varieties are of the Hawaiian type. All Hawaiian types have previously been shown to be quite similar at the DNA level (Stiles et al. 1993). SCAR W11 and SCAR T12 showed linkage in all 182 plants, indicating these markers are within 0.3 cM of S*ex1*. There were no crossovers between SCAR T12 and *Sex1* in the 182 plants, indicating a linkage significantly closer than the 7.0 cM previously reported by Sondur et al. (1996). The difference in map distance between this study and the previous study of Sondur et al. (1996) could result from the use of different genetic backgrounds for construction of the papaya genetic maps. The parents of the mapping population previously used by Sondur et al. (1996) were more distant than those of the population used in this study. The difference could also result from the greater precision of the SCAR system.

The results presented here indicate that it is now possible to accurately and rapidly determine the sex of papaya plants using a PCR-based technique requiring only a minimal tissue sample taken, for example, from seedlings or tissue in culture. Detection of the male or hermaphrodite allele can be accomplished using either the SCAR T12 or SCAR W11 primer set in a multiplex reaction that includes the SCAR T1 primer set as a positive control. Tests on two populations of 37 and 1,937 hybrid papaya seedlings indicate that a success rate for identification of hermaphrodite plants of over 99% can be obtained using this system. Since the seedlings in this test population were used to develop potential virus-resistant hybrid lines for commercial production, the plants predicted to be female (that is, those that amplified SCAR T1 but not SCAR T12) were not planted and we cannot assess the accuracy of predicting female plants. However, based on the results from the mapping population a significant number of miss-classified females are not expected. Even so, caution should be observed in using this approach in cultivars with genetic backgrounds distant from the Hawaiian types, as some females were observed to amplify the W11 SCAR product (Table 2). The recommended PCR conditions for sex analysis are presented in the Materials and methods section.

SCAR W11 and SCAR T12 can also be used as starting points for positional cloning of the *Sex1* gene. A papaya BAC library has been constructed (Ming et al. 2001) and clones containing the W11 and T12 sequences have been isolated and are currently being characterized (H. Ma and R. Ming, personal communication). The cloning and molecular characterization of *Sex1* will greatly increase our understanding of the flowering process and of sex determination in papaya.

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