CROP BREEDING AND APPLIED BIOTECHNOLOGY

ARTICLE

Sex determination of papaya var. 'Maradol' reveals hermaphrodite-to-male sex reversal under greenhouse conditions

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Abstract: *Papaya is a nutritious fruit cultivated worldwide under suitable climate conditions. This plant is polygamous, bearing female, male, and hermaphrodite sex types determined by sex chromosomes XX, XY, and XY^h, respectively. In this paper, a molecular sex determination of papaya var. 'Maradol' was carried out based on PCR and specific primers. Specific molecular markers resulted in sixty-nine hermaphrodites and twenty-one female plants, matching 100% to the flower morphology. Nevertheless, since the summer stressing conditions rose in the greenhouse, sex reversal to male phenotype was observed in 43% of hermaphrodite plants due to high-temperature conditions. A specific male marker could not detect that change, supporting the proposal that sex reversal is caused by harsh environmental conditions aimed at epigenetic modification and genes related to hormones. Our study demonstrates the effectiveness of molecular sex determination and the importance of controlling the growing requirements of papaya to avoid sex reversal.*

Keywords: Carica papaya*, sex determination, sex reversal, molecular markers, hermaphrodite plant*

INTRODUCTION

Papaya (*Carica papaya* L.) is one of the most produced fruits worldwide. Although native to America, it is cultivated in tropical and subtropical areas of several countries such as India, the Dominican Republic, Brazil, Mexico, and Indonesia (Jiménez et al. 2014, Burns et al. 2022). Its nutritional composition is an added value for consumption due to providing vitamins, minerals, and antioxidants (Mehdipour et al. 2006, Santana et al. 2019). Papaya has three sex forms in its flowers: hermaphrodite, female, and male. Genetically, sex is determined by chromosomes XX, XY, and XY^h, for female, male, and hermaphrodite, respectively. Male and hermaphrodite sex are each one on a sex-determining region in chromosome 1 (LG 1), male-specific region (MSY), and hermaphrodite-specific region (HSY), being suppressed of recombination with X chromosome. For that reason, the sex ratio in a hermaphrodite self-pollination case is 2:1 (hermaphrodite-female), and cross-pollination between hermaphrodite and female gives 1:1 (Ma et al. 2004, Ming et al. 2007).

Papaya cultivation encompasses the sowing of three to five seedlings per hill and their management for three or four months before flowering. Consequently, growers must perform visual sex identification based on flower characteristics

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to eliminate male or female plants (Chaves-Bedoya and Nuñez 2007). This process is carried out every two years, which is the optimal life cycle of this crop (Duarte et al. 2020). Although both hermaphrodite and female plants produce fruits, consumers must prefer hermaphrodites because of their elongated shape and great pulp content (del Carmen et al. 2012). Strategies to sow only hermaphrodites encompass *in vitro* micropropagation (Araya-Valverde et al. 2019, Fitch et al. 2022) and grafting of lateral shoot scions of hermaphrodite plants to female seedlings (Chong et al. 2008). The molecular sex determination approach has been implemented to save time, money, and resources compared to conventional sexing during the vegetative stages. Leaves or seed samples are used for DNA extraction, and specific molecular markers for PCR analysis permit selection of hermaphrodite plants that improve the yields to growers. According to the literature, molecular sexing offers more than 99% accuracy from PCR results and flower morphology, making it a reliable tool for these purposes (Deputy et al. 2002, Saalau-Rojas et al. 2009, Aspeitia-Echegaray et al. 2014, Ruíz Ruíz et al. 2017, Araya-Valverde et al. 2019, Duarte et al. 2020). Molecular sexing based on molecular markers has been used for sex selection in commercial crops like date palm (*Phoenix dactylifera* L.), asparagus (*Asparagus officinalis* L.), and kiwifruit (*Actinidia chinensis* var. *deliciosa*) (Intha and Chaiprasart 2018, Chłosta et al. 2021, Drost 2023).

However, the sex fate of papaya flowers can be influenced by the environment, genetic and epigenetic background (Yu et al. 2008, Liao et al. 2017). The physiological effects of the environmental conditions on hermaphrodite flowers affect fruit production, such as carpeloidy, pentandry, and sex reversal. Carpeloidy results from a change of stamens to carpel-like structures; pentandry is characterized by the fusion of stamens at the ovary due to low temperatures. In turn, the sex reversal of hermaphrodite-to-male or vice versa is caused by high temperatures, water stress, and nitrogen scarcity (Moreira et al. 2019).

In the present work, we describe the molecular sex determination of 'Maradol' papaya using specific DNA markers during its vegetative stage. It was visually confirmed with the flower development in a greenhouse. We also report the influence of high temperature on the morphological characteristic of hermaphrodite flowers related to sex reversal.

MATERIAL AND METHODS

Plant material and seed germination

The papaya fruit variety 'Maradol', with characteristics proceeding from a hermaphrodite flowering, was obtained from the local market in the municipality of Irapuato, Guanajuato, Mexico. Seeds were disinfected with NaClO and fungicide, and the mucilage covering the seeds was removed manually. In March 2022, the seeds' germination was performed on polystyrene trays using peat moss (Sunshine Mix No. 3®), perlite, and vermiculite (3:1:1) as sterile substrate. During germination, the substrate was irrigated with water every three days under conditions of 28-30 °C and a photoperiod of 12 hours. Germination occurred after one month, in April.

Greenhouse conditions

The experiment was carried out in a greenhouse within the CINVESTAV-Unidad Irapuato facilities in Guanajuato (lat 20° 43' N, long 101° 19' W, alt 1724 m asl), Mexico. The greenhouse is a dome-shaped structure covered with plastic polyethylene on the top, with an anti-aphid mesh on three sides and only glass on the back. The greenhouse was 8 m in length and 8 m in width. The back side is adjacent to another greenhouse, which produces a gradient of light from the front to the inside of the greenhouse. The greenhouse was naturally ventilated. Conditions like temperature and relative humidity were not controlled; however, they were monitored at 2 m above the ground with a digital thermometer (Loriskors®) with a temperature precision of ± 1 °C and humidity of ± 2~3 % with 24 h record of minimum and maximum. Additionally, from June to December, the average climate temperatures of the municipality of Irapuato were obtained from the Comisión Nacional del Agua (CONAGUA 2022).

Ninety seedlings were transplanted to a greenhouse in 17 L plastic bags in June with solarized mix type substrate: leaf soil, loam soil, peat moss (Sunshine Mix No. 3®), vermiculite, and perlite in proportion 1:2:3:1:1, respectively. The seedlings were randomly distributed in three corridors. Fertilization, management, and pest control were applied as follows; at the beginning, the substrate was supplemented with NPK fertilizer (17-17-17) Vigoro®, and one week after transplanting, foliar applications were spread with Bayfolan Forte® for six months (from June to December). Aphids were controlled by foliar application using both Agrimec® and Knack®.

Genomic DNA extraction

Genomic DNA was extracted from leaves (n= 90) in July (after three months of seed germination, which occurred in April), according to the methodology of Amani et al. (2011), using 4 cm of leaf sample. The genomic DNA was measured on NanoDrop® 2000c spectrophotometer (Thermo Fischer Scientific) using the A260/ A280 ratio, and quality was checked by 1% agarose gel electrophoresis.

Papaya sex determination

Conventional sex determination

Visual determination of sex based on flower characteristics was carried out in September, according to Jiménez et al. (2014): hermaphrodite, with five petals, ten anthers, one ovary; female, five petals, and a rounded ovary without anthers. Due to the sex proportion of the 'Maradol' cultivar, only female and hermaphrodite flowers were expected.

Molecular sex determination

Molecular sex determination of papaya was performed by PCR at the end of July using three pairs of primers for each sex. SnapGene® v.1.1.3 was used to design primers and OligoAnalyzer® Tool (Integrated DNA Technologies) for their validation. For hermaphrodite sex, the W11 marker of 832 bp was designed from NCBI: accession AY850004.1 and named (W11Fw: 5´-CTGATGCGTGATCATCTACT-3´, W11Rv: 5´-CTGATGCGTGTGTGGCTCTA-3´). For male sex, the marker used was PMSM2 of 548 bp from Papaya Male Specific Marker 2 (NCBI accession: CP010988.1, region 3566–4113 bp) (PMSM2Fw: 5´-GCGATGCTTCAAGTGTTGAC-3´, PMSM2Rv: 5´-ACTATGAGCCTCACGCACTA-3´) (Liao et al. 2017). A chloroplast non-coding region corresponding to the intron of the TrnL gene (UAA) (NCBI accession: EU431223.1) was used as a control for the PCR reaction. This region is a molecular marker based on Taberlet et al. (1991), termed CpTrnL from *Carica papaya* TrnL of 603 bp in the present work. It amplifies in all three sexes (CpTrnLFw: 5´-GGGGATATGGCGAAATCGGT-3´, CpTrnLRv: 5´- TGGGGATAGAGGGACTTGAA-3´). Although hermaphrodite and male sex has specific primers to identify them, CpTrnL also serves as a marker to determine females as a discard. Thus, products of hermaphrodite sex amplification from PCR will have two bands (W11 and CpTrnL); male sex, three bands (W11, PMSM2 and CpTrnL), and for female sex products, only one band from CpTrnL. For the male sex, we evaluated the marker PMSM2 for the first time using leaf samples from a papaya male wild plant and searching for sex reversal phenomenon due to temperature variations in the greenhouse.

PCR reaction was carried out in a 25 µL reaction volume containing 1X buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer, 12.8-180 ng μL^{-1} of genomic DNA, and 0.625 units of Taq DNA Polymerase (5'Bio®, Cuernavaca, Mexico) according to the following conditions: initial denaturation at 95 °C; 3 min, denaturation at 95 °C; 30 sec, alignment at 58.2 °C (W11 and CpTrnL), 57.5 °C (PMSM2); 30 sec, extension at 72 °C; 1 min, final extension at 72 °C; 10 min, for 35 cycles. Amplified products were analyzed on 1% agarose gel and visualized with GelRed® staining under UV light.

Experimental data analysis

Fisher's exact test was applied to find the association between molecular markers W11 and CpTrnL and the hermaphrodite and female sexes using R Software v. 4.2.1 (R Core Team 2022).

RESULTS AND DISCUSSION

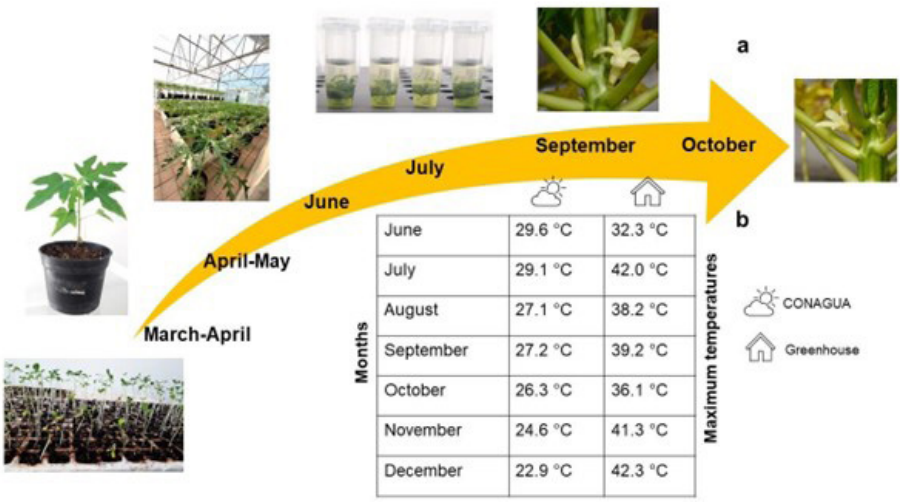
Papaya sex identification is a task that needs time, resources, and qualified personnel with the skills to discriminate between the types of flowers and their underlying changes in the field. Sex in papaya is determined by chromosomes X and Y/Y^h and influenced by the environment and phytohormones that trigger epigenetic changes (Ming et al. 2007, Liao et al. 2022). No specific genes are identified as responsible for sex in papaya, but efforts to find out are reported (Urasaki et al. 2012, VanBuren et al. 2015, Zerpa-Catanho et al. 2019). Recently, Short Vegetative Phase (*SVP*) gene has been proposed as a differentiation gene of hermaphrodite and male sexes since it is fully expressed in the MSY allele from male plants, the *SVP* gene bearing a complete MADS-box and K-box domains to suppress carpel development, and in hermaphrodites only K-domain is present, so their pistil is functional. Moreover, it is not expressed in the X chromosome (Urasaki et al. 2012, Ueno et al. 2015, Lee et al. 2018, Chae et al. 2021). For that reason, growers must sow a certain

number of plants for the hill, generally from three to five, and apply roguing at the flowering stage to eliminate male and female plants (Chaves-Bedoya and Nuñez 2007).

In the present work, we evaluated molecular markers for sex determination in papaya seedlings to identify hermaphrodites and females early in the vegetative stage. Visual sex determination of ninety papaya plants (Figure 1a) yielded 69 hermaphrodites (Figure 1b) and 21 females (Figure 1c) 90 days after transplanting (September). During the observation time, from October to December, thirty hermaphrodite plants out of sixty-nine (43%) showed male characteristics related to sex reversal, such as a sterile pistil in their flowers (Figure 1d). Occasional random temperature recordings in the greenhouse showed data above 35 °C from July through December, with relative humidity ranging from 10 to 35% (minimum) and 78 to 92% (maximum) in those months. There was more than 10 °C differences between external and internal (greenhouse) temperatures (Figure 2). Flower abortion was rarely observed, and male plants were absent. Damasceno Junior et al. (2018) mentioned that these events are regular; both types of flowers (hermaphrodite and male) in the same hermaphrodite plant, and even one or more hermaphrodite flowers have the capacity for fruit production.

Although many male flowers were present in the hermaphrodite plants, their yield was low. Therefore, these observations fit with sex reversal, also called summer sterility, a phenomenon characterized by ovarian atrophy or abortion with no fruit production. This sex reversal is due to stresses related to low or high temperatures, drought, or nitrogen scarcity. It can be male-to-hermaphrodite or hermaphrodite-to-male sex reversals (Ramos et al. 2011, Jiménez et al. 2014, Lin et al. 2016, Liao et al. 2017). In addition, it has been described that the hermaphrodite sex results from the sex reversal of males during papaya domestication. Thus, this sex is prone to reversal by environmental cues (VanBuren et al. 2015). Nevertheless, albeit *Carica papaya* grows well from 21 °C to 33 °C but no to less than 12 °C, the 'Maradol' cultivar is well-developed at temperatures not above 30 °C. Consequently, growing at temperatures less than 17 °C and above 35 °C promotes phenotypic variations, decrease yield and increase the number of unmarketable fruits (Gil and Miranda 2005, Hernández and Basulto 2014, Miranda-Ramírez et al. 2020, Hernández-Salinas et al. 2022).

Figure 1. A papaya plant and flowers according to sex type. a) Plant of papaya (*Carica papaya* L. var. 'Maradol'), Bar 15 cm. b) Hermaphrodite flower. c) Female flower. d) Hermaphrodite-to-male sex reversed flower. A sterile pistil can be observed. Petals were removed from flowers to show internal characteristics. The bar corresponds to 1.5 cm.



Months	CONAGUA	Greenhouse
June	29.6 °C	32.3 °C
July	29.1 °C	42.0 °C
August	27.1 °C	38.2 °C
September	27.2 °C	39.2 °C
October	26.3 °C	36.1 °C
November	24.6 °C	41.3 °C
December	22.9 °C	42.3 °C

Figure 2. Timeline of the papaya plant development. a) Seed germination from March to the beginning of April; transplanting to the pot room, end of April, remained until May; transplanting to the greenhouse in June; genomic DNA extraction and PCR experiments in July; flowering in September; first observations of sexual reversal in hermaphrodite plants in October. The plants remained in the greenhouse until December. b) Maximum temperatures reported from June to December in the municipality of Irapuato by the Comisión Nacional del Agua (CONAGUA) and in the greenhouse. The temperatures reported by CONAGUA (2022) represent the monthly average. In contrast, the reported temperatures in the greenhouse mean a random monthly record.

Likewise, net CO_2 assimilation is reduced gradually at temperatures above 35 °C depending on the cultivar; thus, the lower the photosynthesis rate, the lower the production (Jeyakumar et al. 2007). Our study observed increases in temperatures at the greenhouse in the summer and autumn seasons. Despite performing the transplant in June, where the temperature was adequate (32.3 °C, Figure 2b), at first flowering in September, all plants conserved the ratio of flowers genetically predicted; hermaphrodites and females. Subsequently, in October temperature-induced sex reversal was observed in thirty hermaphrodite plants. On the other hand, female flowers did not undergo any changes in their morphology.

These results are consistent with Salinas et al. (2020), who mentioned Autumn (September) as the worst season for fruit production of papayas due to functionally male flowers in all cultivars tested. They evaluated the seasonal frequency of different types of flowers of five cultivars of hermaphrodite papaya: 'BH-65', 'Sensation', 'Siluet', 'Intenzza', and 'Red Lady'. 'Sensation', 'Intenzza', and 'Red Lady' had high percentages of male flowers, 68%, 84%, and 67%, respectively. However, 'BH-5' and 'Red Lady' were the worst adapted to the growing conditions due to the high incidence of pentandric, carpelloid, and male flowers, which gave fruits without commercial value. In the same context, Salinas et al. (2022) reported up to 100% of male flowers from hermaphrodite plants of the 'Intenzza' cultivar in autumn (November) and predominantly in all seasons except in May (spring), recording a maximum temperature of 36.6 °C in July in a greenhouse with natural ventilation. Based on these findings, they recommend transplanting in February or March to achieve good yields.

Conversely, Honoré et al. (2019) reported a high temperature of 42.3 °C in July when producing papaya fruit using varieties such as 'Intenzza', Hermaphrodite 'Intenzza' onto female 'Intenzza', 'Sweet Sense', 'Vitale', 'Caballero', and 'Alicia'. Although no flowers were disturbed, varieties such as 'Intenzza' and 'Vitale' yielded low fruit production per plant. On the other hand, Damasceno Junior et al. (2018) stated that sex reversal and flower abnormalities such as carpeloidy and pentandry depend on the genetic background of genotypes, lines, or hybrids. They found their expression is predominantly in the warmer seasons of the year, like summer. For this, even if a good water and nutrition management program is applied, there will still be some floral abnormality depending on the cultivar and season of the year. Hence, papaya fruit production in Mexico in the year 2022 was not uniform since, in winter, monthly yield was less intense compared to spring and summer (SIAP 2022).

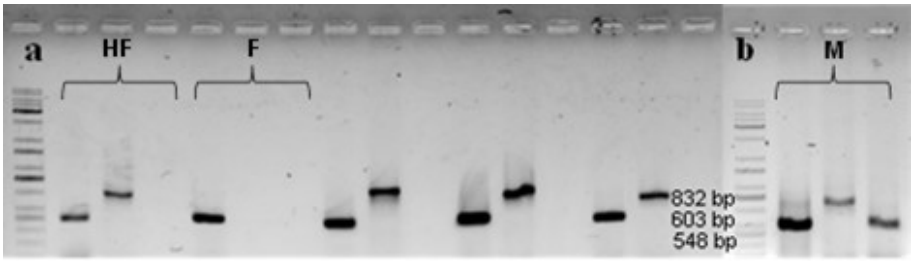


Figure 3. Representation of molecular sex identification of sex types from DNA samples of leaves using specific primers. a) Specific primers determined the sex of hermaphrodites and female plants, W11 amplifies male and hermaphrodites at 832 bp, and CpTrnL gives a band for all sexes at 603 bp. Two bands correspond to the hermaphrodite sex, and one is for the female sex. b) Male sex determination; only in male plants, the PMSM2 marker amplifies a 548 bp and the other bands as in hermaphrodites. The molecular weight marker corresponds to a 1 Kb DNA ladder. HF, hermaphrodite; F, female; and M, male.

'Maradol' is one of Mexico's most produced varieties, and floral abnormalities have also been reported. Alternatives such as the MSXJ hybrid (a cross between a wild papaya and a 'Maradol' cultivar), which tolerates temperatures above 35 °C, have been developed (Hernández and Basulto 2014). Although papaya is produced in open fields in Mexico, for example, greenhouse cultivation is an excellent option in Spain due to the subtropical climatic conditions, using natural or controlled ventilation. Cultivation of papaya in a greenhouse prevents the incidence of papaya ringspot virus, reduces water consumption, protects the plants from the wind, reduces growth time, and gives better yields (Galán Saúco and Rodríguez Pastor 2007).

With the use of specific primers for molecular sex determination, such as W11 for hermaphrodites, and CpTrnL for females, the sex was strongly associated with conventional determination in a greenhouse (p-value < 2.2e-16). It was a 100% prediction rate for both sexes. CpTrnL marker showed high specificity even with a low quantity of DNA (12.8 ng μL^{-1}). Also, CpTrnL amplifies in *Arabidopsis thaliana* genomic DNA (data not shown), which could be considered an internal control of the PCR reaction. These findings agree with Aspeitia-Echegaray et al. (2014) and Ruíz Ruíz et al. (2017). Although the W11 marker is specific to hermaphrodites and males since their DNA sequence corresponds to HSY and MSY sex-determining region (Deputy et al. 2002), some authors have reported more than 98% of the prediction rate (Deputy et al. 2002, Saalau-Rojas et al. 2009, Araya-Valverde et al. 2019). On the other hand, we tried the performance of the W11 marker as suitable for papaya var. 'Maradol' and, although faint amplification bands were observed, false negative results were not found (Figure 3a). Likewise, authors also reported false negative results (Deputy et al. 2002, Oliveira et al. 2007, Pirovani et al. 2018, Duarte et al. 2020). However, other markers related to hermaphrodite identification have been proposed yielding similar or better performance than W11 (Deputy et al. 2002, Urasaki et al. 2002, Chaves-Bedoya and Nuñez 2007, Urasaki et al. 2012). From DNA extraction and PCR analysis (July) to flower visualization (September), we were able to reduce by two months the sexing time by applying the molecular method.

After observing the hermaphrodite-to-male sex reversal phenomenon clearly in thirty plants, we conducted genomic DNA extraction. We performed a PCR assay to evaluate the functionality of the PMSM2 marker to understand whether the changes in floral morphology were genetic or induced by high temperatures. We used leaves of male wild papaya to amplify PMSM2 and carried out PCR, including W11 and CpTrnL markers. As a result, these pairs of primers (PMSM2) amplified a fragment of 548 bp only from male sex samples (wild type). No PCR band from the PMSM2 marker was obtained from the hermaphrodite-to-male sex reversal plants. Likewise, the W11 and CpTrnL markers continue amplifying in male samples (Figure 3b) as well as in hermaphrodite-to-male sex reversal samples. Some authors reported good efficiency in a similar male-specific marker (PMSM1) in male-to-hermaphrodite sex reversal events (Liao et al. 2017, Pirovani et al. 2018), confirming their male specificity. Moreover, this marker serves dioecious varieties of papaya, leaving aside that papaya fruit production is mainly based on gynodioecious cultivars (Burns et al. 2022).

Therefore, our results support the findings that the environment orchestrates sex reversal changes, for instance through temperature, which triggers a series of epigenetic modifications and genes related to hormones such as auxins like auxin efflux carrier (PIN-FORMED (*PIN*)) genes. *PIN1* and *PIN3* homologous genes are upregulated during male-to-hermaphrodite sex reversal due to low-temperature conditions, and these genes have a relevant role in gynoecium development because mutants revealed sterile pistil (Ramos et al. 2011, Lin et al. 2016). For this reason, it has been

proposed to apply plant growth regulators to disturb auxin homeostasis in hermaphrodite-to-male reversal cases because it is reported that, depending on auxin concentration, the gynoecium will develop and also can restore fertility in this type of sex reversal (Zhou et al. 2019, Zhou et al. 2022).

With these results, we support that the molecular identification of papaya sex could be an excellent alternative for having only hermaphrodite plants in the field, which would help increase yields, as reported by Salinas et al. (2018). In this regard, we must emphasize that even though sex segregation from the self-pollination of hermaphrodites is 2:1 or 1:1 from hermaphrodite to female pollination, eventually, phenotypic changes will appear from environmental cues. Hence, to avoid sex reversal in papaya var. 'Maradol' growers should take into account climatic conditions and consider inducing flowering in seasons when temperatures are not elevated, as well as considering planting two hermaphrodite plants to prevent losses in the case that one plant suffers from this phenomenon.

CONCLUSIONS

Sex determination in papaya var. 'Maradol' was successfully established in seedlings, achieving 100% of the prediction rate with specific molecular markers. This approach could be an excellent option for producers to ensure the planting of hermaphrodite or female plants in their fields. However, as papaya is gynodioecious, we also observed induced sex reversal from hermaphrodite-to-male flowers due to the high temperatures in our uncontrolled greenhouse. Male molecular markers failed to identify this phenomenon because it is possibly epigenetic and hormonal. With this work, we contribute to the knowledge about sex reversal caused by environmental factors not only for the male to hermaphrodite as it has already been reported in the literature, but also for the inverse. We have verified that the molecular marker CpTrnL can be used as an internal control for PCR. This marker also applies to other species, including *Arabidopsis*, as it is derived from a chloroplast gene region.

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