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SHORT COMMUNICATION

Rapid sex identification method of spinach (*Spinacia oleracea* L.) in the vegetative stage using loop-mediated isothermal amplification

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Abstract
Main conclusion **A LAMP-mediated, simple and rapid method for sex identification in spinach was developed. Nutrient compositional analysis showed a higher iron content in male than female plants.**

Spinach (*Spinacia oleracea* L.) is a dioecious plant with its sex determined by the XY system. Male and female floral organs differ morphologically, but plants do not differ in the vegetative stage before flowering. PCR with Y chromosome markers has been used to determine the sex of dioecious plants before flowering. In this study, we developed a genotype-specific loop-mediated isothermal amplification (LAMP) for sex identification of individual vegetative-stage spinach plants, using primers designed for the genomic region flanked by male-specific markers. LAMP could specifically detect spinach males. The method was further modified to omit DNA purification and use just an aliquot of crude leaf extract homogenized in water. We compared the nutrient composition of males and females, finding higher amounts of iron in the males. Our method could therefore be used for rapidly discriminating male plants in the field, which is useful for efficient hybrid breeding.

Keywords LAMP · Plant genomic DNA · Iron · Dioecious plant

Introduction

Spinach (*Spinacia oleracea* L.), a largely dioecious organism with separate male and female plants, is a highly nutritious leafy vegetable crop. Cultivars grown in Japan, the world's third largest producer of spinach, fall into three groups based on the provenance of seeds: Japanese, Western and Japanese–Western hybrid (hybrid). F1 (first filial generation) hybrid offspring between a variety of spinach cultivars are in demand. Since spinach is dioecious, sex identification of individual plants is important for efficient hybrid breeding.

Sex in spinach is largely determined genetically, with an active "Y chromosome" determining maleness (Janick and Stevenson 1954, 1955; Ellis and Janick 1960; Sugiyama and Suto 1964), suggesting that male-determining gene(s) of spinach exists on this non-heteromorphic chromosome. Sexual dimorphism is manifested in the flowers (Sather et al. 2010), and Okuse and Saga (1995) reported differences in nutrient composition between males and females at the flowering stage. It is not known whether these nutrient differences are also found at the vegetative stage, when the crop is used. Spinach leaves contain an abundance of nutrients including vitamins, potassium and

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iron. Therefore, sex differences in nutrient compositions at the edible vegetative stage would be of agricultural importance. It will also be interesting to know whether genes other than those associated with flower phenotypes, possibly located near the male-determining gene, contribute to differences between male and female spinach.

However, it is impossible to identify sexes in the seeding or early growth stages, because there are no clearly defined morphological differences. Because of the absence of chromosome heteromorphism, sexing spinach plants in the vegetative stage is therefore currently possible only using molecular markers, generally using PCR-based detection of fully sex-linked DNA markers (Milewicz and Sawicki 2013; Akamatsu et al. 1998). LAMP is a target sequence detection method with high amplification efficiency and specificity compared to PCR and has been used for the diagnosis of plant pathogens in the field (Maejima et al. 2010, 2011). It is suitable for on-site use because it can amplify the target sequence in approximately 1 h using a water bath at a constant temperature of 60–65 °C and amplification of target sequence is easily visualized through green fluorescence emitted by calcein as the reaction indicator. The LAMP method has recently been used for sex identification in other dioecious plants without major sex chromosome heteromorphism, including papaya and asparagus, and in some animals (Shiobara et al. 2011; Hsu et al. 2012; Centeno-Cuadros et al. 2016).

In this study, we show that the LAMP system can identify males in multiple spinach cultivars. We further improved and simplified the method by omitting DNA purification steps, leading to a rapid sex screening for fresh spinach plants. We call this method “Sex-LAMP assay”, and used it to compare nutrient contents of vegetative-stage male and female spinach plants, and found that male spinach plants contain higher iron content than females.

Materials and methods

Plant materials and DNA extraction

Six commercial spinach cultivars, Nippon (Japanese group; Kaneko seeds Co., Ltd., Maebashi, Japan), Viroflay (Western group; Kaneko seeds), Hoyo (Japanese group; Takii & Co., Ltd., Kyoto, Japan), Alright (Japanese-Western hybrid group; Takii), Hambourg (Western group; Tohoku Seed Co., Ltd, Utsunomiya, Japan) and Wase-salad Akari (Japanese–Western hybrid group; Tohoku Seed), were grown in the field or in a controlled room under artificial fluorescent lights (FHF32EX-N-H; Panasonic Co. Ltd., Osaka, Japan) at 15–21 °C under short-day conditions (8 h light, 16 h dark) in an advanced plant factory in Tokyo University of Agriculture and Technology, Tokyo, Japan (Ogiwara and Arie 2010). Total DNA was extracted from 10 mg of leaf tissue using the standard CTAB method. Ten nanograms of extracted DNA was used for the LAMP reaction. For nutrient component analysis, plants grown in a greenhouse located in Kiyose, Tokyo, Japan, were used for the first test of the seven nutrient compositions. Sample plants were grown under the same conditions and harvested at the same time. The average iron contents are shown in Fig. 3 ($P < 0.05$, Student’s t test).

LAMP primer design

LAMP primers were designed from the male-specific sequences V20A (1.3 kbp) and T11A (1.7 kbp) (Akamatsu et al. 1998), which are located on the Y chromosome and completely co-segregated with a potential male-determining gene (Yamamoto et al. 2014), using PrimerExplorer V4 software (http://primerexplorer.jp/). A set of LAMP primers, which includes two inner primers [FIP (F1c + F2) and BIP (B1c + B2)], two outer primers (F3 and B3), and two loop primers (Loops F and B), which yielded positive reactions in male plants was used in this study. The LAMP sequences and positions are shown in Fig. S1. Details of the primers are provided in Table S1.

LAMP assay

The LAMP reaction was performed as described previously (Komatsu et al. 2015), except that extracted DNA was used as the template instead of total RNA. Unless otherwise stated, the mixture was incubated at 64 °C for 60 min followed by incubation at 80 °C for 5 min to inactivate the DNA polymerase. For real-time monitoring of LAMP reactions, a fluorescent detection reagent (Eiken Kagaku, Tokyo, Japan) was added to the reaction mix, and fluorescence was detected using the Genie II instrument (OptiGene, Horsham, UK). DNA amplification was also detected based on the fluorescence intensity observed under a 254-nm ultraviolet light. LAMP reaction using a toothpick sampling method was carried out as described previously (Komatsu et al. 2015). For LAMP reaction using an aliquot of crude extract of a leaf sample directly homogenized in water, 1 μL of the homogenate of 10 mg fresh leaf tissue in 200 μL of water was used. Results of the LAMP assay were confirmed by visual inspection of the flowers or by conventional PCR-mediated sex identification (Akamatsu et al. 1998).

Nutrient component analyses

To screen for differences in nutrients between the male and female plants, 32 spinach plants (*Spinacia oleracea L.* ca. Hunter; Kaneko seeds Co., Ltd.) grown under greenhouse

conditions were freshly harvested and grouped into males and females (16 plants each) by LAMP. The number of plants in a group was determined based on the minimum sample size required for a test of seven nutrients (16 in this case). Because we found an appreciable difference in iron content in the first test, we repeated the test with 40 plants and individually tested them for their iron content. The analyses were repeated twice, and the average amount of each nutrient was compared between males and females. All nutrient composition analyses were carried out in the Japan Food Research Laboratory (Tama, Japan) according to its standard methods.

Results and discussion

Sex-LAMP assay showed clear sex differences in fluorescence emission

To develop a sex-LAMP assay, we first designed seven primer sets from either the V20A or T11A marker sequences (Akamatsu et al. 1998), using a standard Web-based program. Preliminary tests using these seven sets failed to detect the male-linked marker sequences; the reactions resulted in either no amplification or non-sex-specific amplification. This result was consistent with the data shown in Akamatsu et al. (1998), which showed that the male-linked PCR amplification is highly dependent on primer combinations even though primers were designed from the V20A or T11A sequences. To increase male specificity of the LAMP primer sets, the forward or reverse primer of V20A or T11A marker was employed as external LAMP primers (E3 and B3). One of these primer sets, denoted as V20A ID5 (Fig. S1), used the IN101-3 sequence (Akamatsu et al. 1998) as its B3 primer (Table S1). Sex-LAMP reactions with this primer set specifically detected the male-linked marker sequences after 60 min, using 10 ng of male spinach DNA, whereas female spinach DNA template produced no green fluorescence (Fig. 1a). Real-time monitoring of the fluorescence found the highest intensity with reaction times of 25–30 min (Fig. 1b).

The sex-LAMP assay could be applied to different cultivars of spinach

The LAMP reaction with V20A ID5 was then tested in six different spinach cultivars, two each from the three provenance groups, Japanese, Western and Japanese–Western hybrid. High fluorescence intensity was obtained specifically with DNA from male samples from all cultivars tested. Reaction times required for the detection varied between 20 and 60 min between the cultivars and between individual plants from the same cultivar (Fig. 1c, d). In the Japanese–Western hybrids, which are commercially popular in Japan, the time of highest fluorescence was less varied, being approximately 35 min for all four male samples tested from cultivars ‘Alright’ and ‘Wase-salad-Akari’ (Fig. 1e).

The sex-LAMP assay was optimized for field use

DNA sample preparation with purification steps represents a bottleneck in rapid spinach sex determination in the field. To simplify the DNA extraction process and to shorten the operation time for the sex-LAMP assay in spinach, we tested whether the DNA purification steps could be omitted. First, we tested simply using a toothpick for sample collection. We poked a fresh spinach leaf with a toothpick and dipped it into the LAMP reaction mix prepared in a PCR tube. Amplification from male spinach plants was observed in about 60 min, similar to reactions using purified DNA, but the success rate was only 60–70%, even with extending reaction time of 90 min (Fig. 2a), suggesting that the toothpick method yields too little DNA. We next tested using a microwave treatment before poking with a toothpick, a technique often used in simple DNA extraction methods (Saini et al. 1999; Tendulkar et al. 2003). Microwave treatment for only 10 s (500 W) distinctly increased success rates, with amplification from all male samples within 30 min. However, the non-specific amplification in female samples occurred after 60 min (Fig. 2b). Together with the difficulty of using the microwave method in the field, we rejected this method. We next tried to use an aliquot of crude extract of a leaf sample directly homogenized in water as template for LAMP reactions. This yielded positive results only from the male samples, and no non-specific amplification from female samples was detected after 90 min reactions (Fig. 2c). This method therefore appears the best approach for DNA preparation for rapid sex identification by LAMP, although longer reaction times are required for detecting all males (60 min for purified DNAs; 90 min for crude sample in water). Therefore, to shorten the reaction time, we tested increased amounts of crude extract added as template in the LAMP reaction mixture, from 1 to 6.5 μL. However, contrary to our expectation, this caused inhibition of the LAMP reaction, with no amplification in either male or female samples, suggesting that spinach contains substances that inhibit LAMP reactions.

Nutritional differences between male and female spinach

Using the sex-LAMP assay described above, we tested whether nutrient compositions differ between vegetative-stage male and female spinach plants. A first test with 16 male and 16 female plants found no significant differences for six of seven nutrients tested, except for non-

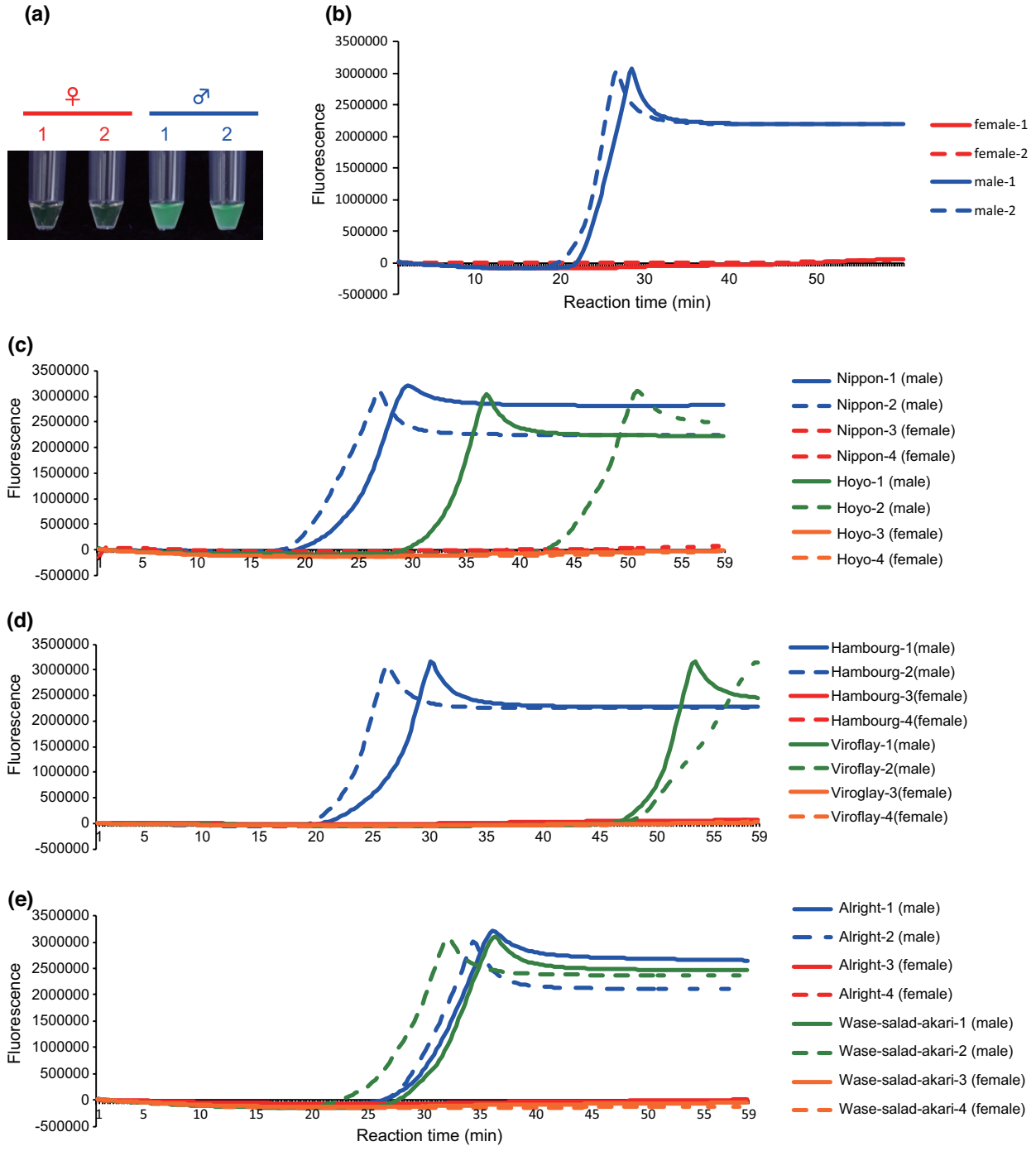

Fig. 1 Male-specific LAMP amplification (**a**–**b**), shown by visual detection of the amplified LAMP product using UV light after 60 min of reaction (**a**), or LAMP amplification curves of the 60 min reaction (**b**). Representative results from three independent experiments are shown. Cultivar sensitivity test of the LAMP assay using DNA extracted by CTAB method (**c**–**e**). Amplification curves of the LAMP reaction using DNA extracted from two Japanese cultivars, Nippon and Hoyo (**c**), two Western cultivars, Hambourg and Viroflay (**d**), and two cross-bred cultivars, Alright and Wase-salad-akari (**e**), as templates. For each cultivar, two female/male biological replicates were used for the assay. *Blue* and *green lines* indicate males and *red* and *orange lines* indicate females

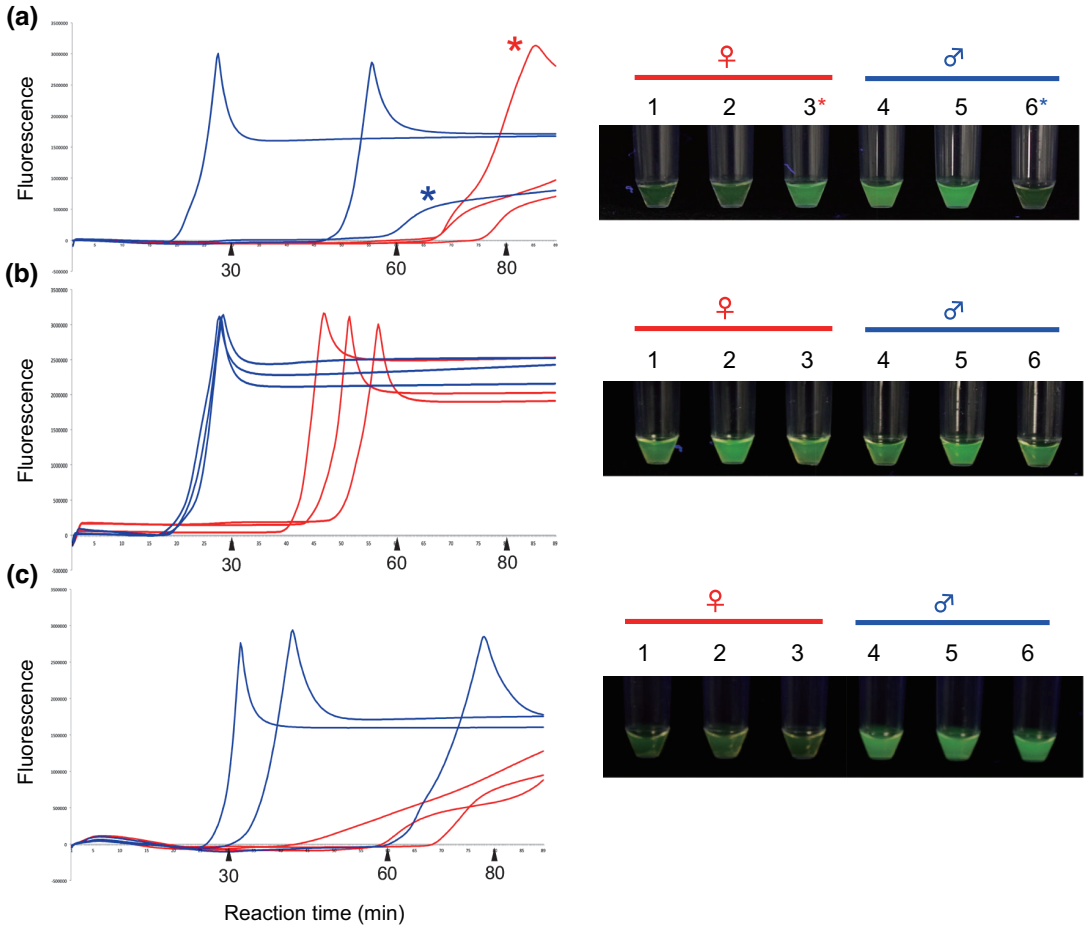

Fig. 2 Three different LAMP assays without DNA purification steps. LAMP amplification curves are shown on the *left* and visual detections of the amplified LAMP product using UV light after 90 min of reaction are shown on the *right*. The LAMP assay was performed using a fresh spinach pricked by a toothpick (**a**), spinach plants heated in a microwave for 10 s before being pricked by a toothpick (**b**) or fresh spinach homogenized in water (**c**) as a template. *Red* and *blue asterisks* indicate nonspecific female and undetected male amplifications, respectively

significantly higher iron content in male plants (Table S2). However, nutrient composition in spinach can vary according to light intensity, temperature and possibly other environmental factors (Chang et al. 2013; Yoon et al. 2017). We then tested 40 additional spinach plants from a local grocery (whose cultivars and growth conditions were unknown), sexing them by the sex-LAMP assay (there were 23 males and 17 females). The male plants had significantly higher amounts of iron than females ($P < 0.05$, Student's t test; average iron contents are shown in Fig. 3).

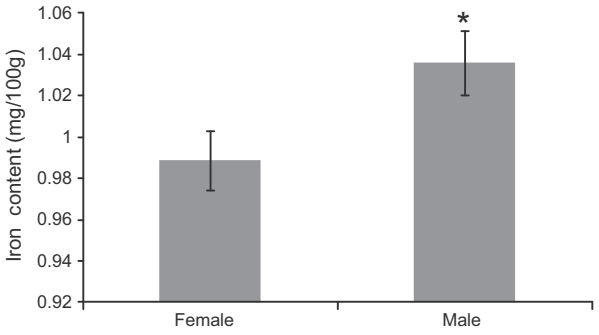

Fig. 3 Iron contents of whole edible parts compared between female and male spinach plants. *Error bars* represent the standard errors of the mean for 17 female and 23 male samples. *Asterisk* (*) indicates a significantly higher iron content in male spinach than in female spinach ($P < 0.05$; Student's t test)

Conclusion

The LAMP method targeting plant genomic DNA is in demand especially for the detection of transgenic plants (Li et al. 2014; Zhou et al. 2014; Wang et al. 2016). However,

LAMP assay without DNA purification is often difficult due to the small mole number of a target genomic DNA. In this study, we developed the simple method, only homogenizing samples in water, which yielded a 100% success rate. This method can be applied for the detection of transgenic plants, as well as extended to the field use of the recently reported sex identification LAMP in asparagus and papaya (Shiobara et al. 2011; Hsu et al. 2012).

Spinach is well known to be a good source of iron. Interestingly, our data demonstrate the higher iron content in male spinach than in the female plants. In the sex-LAMP method, male and female are discriminated by detecting the male-specific region linked to a male-determining gene. Therefore, it further implies the possibility that gene(s) that affects iron content is also linked to the male-specific region. Alternatively, nutrient such as iron content may be affected by sexual development events in plants. Although its biological significance is unknown, our finding uncovers an interesting aspect of the correlation between sex and nutrient content in a dioecious plant.

Author contribution statement NF and KK conceived and designed the research. TT, KW and TA evaluated the designs from the application perspective in the field. NF, YA and MF performed the research. NF and KK wrote the manuscript. All authors read and approved the manuscript.

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