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ORIGINAL PAPER

A PCR-based marker for a locus conferring aroma in vegetable soybean (*Glycine max* L.)

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Abstract Vegetable soybean (*Glycine max* L.) is an important economic and nutritious crop in South and Southeast Asian countries and is increasingly grown in the Western Hemisphere. Aromatic vegetable soybean is a special group of soybean varieties that produce young pods containing a sweet aroma, which is produced mainly by the volatile compound 2-acetyl-1-pyrroline (2AP). Due to the aroma, the aromatic vegetable soybean commands higher market prices and gains wider acceptance from unfamiliar consumers. We have previously reported that the *GmAMADH2* gene encodes an AMADH that regulates aroma (2AP) biosynthesis in soybeans (Arikit et al. 2010). A sequence variation involving a 2-bp deletion in exon 10 was found in this gene in all investigated aromatic varieties. In this study, a codominant PCR-based marker for the aroma trait in soybeans was designed based on the 2-bp deletion in *GmAMADH2*. The marker was verified in five aromatic and five non-aromatic varieties as well as in F_2 soybean population segregating for aroma. The aromatic genotype with the 2-bp deletion was completely associated with the five aromatic soybean varieties as well as the aromatic progeny of the F_2 population with seeds containing 2AP. Similarly, the non-aromatic genotype was associated with the five non-aromatic varieties and non-aromatic progeny. The perfect co-segregation of the marker genotypes and aroma phenotypes confirmed that the marker could be efficiently used for molecular breeding of soybeans for aroma.

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Introduction

Vegetable soybean (*Glycine max* L.), called "Edamame" in Japan and "Mao Dou" in China, is one of the oldest vegetables cultivated by humans (Shurtleff and Lumpkin 2001). The immature seeds of this special kind of soybean are directly consumed as a vegetable or snack. Vegetable soybean is an important economic and nutritious food in Southeast Asian countries and is increasingly consumed in the Western Hemisphere. It is an excellent source of protein, carbohydrates, fiber, vitamins, minerals and phytoestrogens (Song et al. 2003).

Aromatic vegetable soybean, also called "Chamame," is a group of vegetable soybean varieties that contain a sweet aroma similar to that of aromatic rice, such as the jasmine and basmati types (Fushimi and Masuda 2001). Aromatic vegetable soybean has been popular in southern Taiwan for decades and has gained wider acceptance in Japan, the United States and Europe (Wu et al. 2009). This kind of vegetable soybean commands higher market prices than

non-aromatic varieties (Statistics Department, Ministry of Agriculture, Forestry and Fisheries 2009). Moreover, the aromatic varieties have been the preferred choice for promoting the vegetable soybean to unfamiliar consumers because the aroma can increase the acceptance of this crop (AVRDC 2003).

The aroma of aromatic vegetable soybean varieties has been associated with increased levels of 2-acetyl-1-pyrroline (2AP) (Arikit et al. 2010; Fushimi and Masuda 2001; Plonjarean et al. 2007; Wu et al. 2009). 2AP is thought to be the volatile compound responsible for the "popcorn-like" aroma found in a large variety of cereal products and in vegetable and animal food products (Adams and De Kimpe 2006). It plays a critical role in the value of food products due to consumer preference. The aroma trait is considered one of the most challenging traits for plant breeders to select for, because it is difficult to evaluate the aroma phenotype (Bradbury et al. 2005). In many breeding programs for aroma, for example in rice, many subjective sensory methods have been applied, including boiling seeds in a vial and noting the aroma (Wanchana et al. 2005). In soybeans, a similar aroma evaluation method has been reported (AVRDC 2003). However, there are limitations to these methods, such as the inability to process large numbers of samples. Another objective method, in which 2AP levels in seeds are analyzed using gas chromatography, has also been applied and widely used due to its robustness and quantitative results. Although it is highly efficient, the assay requires large tissue samples and is time-consuming (Fushimi and Masuda 2001; Plonjarean et al. 2007; Wu et al. 2009).

In most molecular breeding programs, especially those in which the phenotypes are difficult to evaluate, tightly linked or gene-specific markers are very important and necessary to select the desired plants. We recently reported that the synthesis of 2AP in soybeans is controlled by a recessive allele of the *GmAMADH2* gene, which is a homolog of the rice *Os2AP* gene (Arikit et al. 2010). According to that study, a 2-bp deletion in exon 10 of *GmAMADH2* was found in the aromatic varieties. Here, we report the development of a perfect PCR marker for aroma in soybeans that can be efficiently applied in marker-assisted selection (MAS) breeding programs.

Materials and methods

Plant materials

Ten varieties of aromatic and non-aromatic vegetable soybeans were used to verify the Gm2AP marker, whose design is described below. The five varieties of aromatic soybean were "Chamame" (Tohoku Seed Co. Ltd., Utsunomiya, Japan), "Kouri" (Marutane Corporation, Kyoto, Japan), "Kaori Hime" (Mikado Kyowa Seed Co. Ltd., Tokyo, Japan), "Yuagari Musume" (Kaneko Seeds Co. Ltd., Maebashi, Japan) and "Fukunari" (Takii Co. Ltd., Kyoto, Japan). The five varieties of non-aromatic soybean were "Okuhara Wase" (Sakata Seed Corporation, Yokohama, Japan), "Oishi Edamame" (Tohoku Seed Co. Ltd., Utsunomiya, Japan), Shirono Mai (Sakata Seed Corporation, Yokohama, Japan), "Chiang Mai 60 (CM60)" (Chiang Mai Field Crops Research Center, Department of Agriculture, Thailand) and "Jack" (provided by Dr. Randall L. Nelson, United States Department of Agriculture, Agricultural Research Service, IL, USA). In addition, 60 F_2 progenies derived from a single F_1 plant from the cross between Kaori Hime × Jack were used to validate the efficiency of the marker. Kaori Hime was used as aromatic female parent and Jack was used as non-aromatic male parent. The crossing was performed during February–March 2009. F_1 plants were grown in April 2009, and one of these lines was selected to develop the F_2 population. F_2 seeds derived from the selected F_1 plant were harvested in June 2009, and 60 F_2 plants were grown in July 2009 to produce F_3 seeds. The F_3 seeds were harvested at the 85% pod fill stage from 60 F_2 plants in September 2009 for 2AP evaluation. All soybean plants in this study were grown indoors in pots at Kasetsart University, Nakhon Pathom, Thailand.

Chi-square test for phenotypic and genotypic segregations

A Chi-square test was applied to determine the segregation of the aromatic and non-aromatic phenotypes, as well as the segregation of the genotypes in the F_2 population. The expected ratio for the aromatic to non-aromatic phenotypes was 1:3, and the expected ratio of the homozygous (Kaori Hime) to heterozygous to homozygous (Jack) genotypes was 1:2:1. Both ratios for phenotypes and genotypes were tested to validate the goodness-of-fit to the Mendelian model of a single recessive gene that controls the trait. The phenotypes of the F_2 individuals were classified as aromatic and non-aromatic by determining the level of 2AP present in the F_3 seeds. The genotypes were characterized with the Gm2AP marker.

2-Acetyl-1-pyrroline analysis

The young soybeans were manually shelled and lyophilized. Five seeds from each line were bulked and ground in a Waring blender. Portions (0.2 g) of ground samples were extracted with 0.75 ml ethanol containing 200 ppb 2-acetyl-(^{13}C-methyl)-1-pyrroline prepared as described by Yoshihashi et al. (2002) in a 12 × 32 mm glass vials.

Polypropylene screw caps with polytetrafluoroethylene (PTFE) septa (part model 5182-0725; Agilent Technology, Wilmington, DE, USA) were used as vial covers. They were then extracted at room temperature for 2 h. After centrifugation, the supernatant was subjected to gas chromatography–mass spectrometry–selected ion monitoring (GC–MS–SIM) analysis (Raina and Hall 2008; Yoshihashi 2002). Two microliters of the extract were injected onto a DB-WAXetr 60 m × 0.25 mm i.d. × 0.25-μm film thickness, fused silica capillary column (Agilent Technology, Wilmington, DE, USA) installed in a Shimadzu GCMSQP-2010 GC–MS (Kyoto, Japan). Helium gas (99.99% pure), passed through a molecular sieve and an oxygen trap at a carrier velocity of 41.2 cm s^{-1}, was used as the carrier gas. The injector and the GC–MS interface temperatures were set at 150 and 250°C, respectively. The following temperature program was used: the column temperature was isothermally maintained at 40°C for 2 min and increased at a rate of 10°C min^{-1} to 100°C, at a rate of 5°C min^{-1} to 140°C, and then at a rate 20°C min^{-1} to 250°C; the column temperature was then maintained isothermally at 250°C for 10 min. The mass detector was used in the electron ionization mode with the ion source temperature set at 200°C and ionization energy at 70 eV. The SIM was set up to monitor m/z 111 for 2AP and at m/z 112 for the ^{13}C labeled analog as the internal standard. Under these conditions, the retention times of 2AP and the ^{13}C labeled analog were both 12.51 min. Quantification was performed by measuring the area ratios between m/z 111 and 112, corresponding to 2AP and the ^{13}C labeled analog, respectively. Each extract was analyzed three times by GC–MS–SIM to obtain an average peak area and SD. The amount of 2AP was calculated from a calibration curve.

Primer design for the Gm2AP marker

The genomic sequences for the non-aromatic allele of the *GmAMADH2* gene were obtained from the gene model Glyma05g01770 of the shotgun genome sequence database for soybeans (Phytozome, http://www.phytozome.net/soybean) as well as from the gene sequences of five non-aromatic varieties: Okuhara wase, Oishi Edamame, Shirono Mai, Chiang Mai 60, and Jack (Arikit et al. 2010). The sequences for the aromatic allele were the gene sequences of the five aromatic vegetable soybean varieties: Chamame, Kouri, Kaori Hime, Fukunari and Yuagari musume (Arikit et al. 2010). Two oligonucleotide primers, Gm2AP Forward (5′ GGTCAGATATGCAGTGCAAC 3′) and Gm2AP Reverse (5′ TTGACCCATTTCACAATCCTAT 3′), were designed using Primer 3 Version 0.4.0 (Rosen and Skaletsky 2000) to flank the 2-bp deletion in exon 10 of the *GmAMADH2* gene.

DNA extraction, polymerase chain reaction (PCR) and PAGE analysis

Genomic DNA was extracted from young leaves of the ten soybean varieties, F_1 plant and F_2 progenies using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). PCR was performed in 25 μl reaction mixtures containing 50 ng of genomic DNA template, 0.1 mM dNTPs, 0.25 mM each of the forward and reverse primers, 0.25 units of Taq DNA polymerase, 2.0 mM $MgCl_2$ and 1× thermophilic DNA polymerase buffer (Promega, Madison, WI, USA). PCR amplification was performed using the Gene Amp PCR system 9700 thermal cycler (Perkin Elmer, Foster City, CA, USA). After pre-heating at 94°C for 2 min, the PCR reaction was carried out for 30 cycles of 94°C denaturation for 30 s, 55°C annealing for 30 s and 72°C extension for 45 s, with a final extension at 72°C for 5 min. PCR products were analyzed by 6% denaturing polyacrylamide gel electrophoresis (PAGE) and stained with silver. A lambda phage/*Hin*fI molecular weight standard (MBI Fermentas, Vilnius, Lithuania) was used to estimate the PCR fragment sizes.

Results

Development of the PCR marker specific to soybean aroma

The Gm2AP marker for the aroma trait in soybeans was designed based on the genomic sequences of *GmAMADH2* that flank the two base thymine (TT) deletion in exon 10 (Fig. 1). The marker was verified by amplifying the genomic DNA isolated from five varieties of aromatic soybeans (Chamame, Kouri, Kaori Hime, Yuagari Musume and Fukunari) and five varieties of non-aromatic soybeans (Okuhara Wase, Oishi Edamame, Shirono Mai, Jack and Chiang Mai 60). The two groups of aromatic and non-aromatic soybeans were classified according to the 2AP contents of seeds, as previously reported (Arikit et al. 2010). As expected, the marker clearly separated the two groups of soybean varieties. The sizes of the PCR products amplified by the Gm2AP marker were 191 bp for aromatic varieties and 193 bp for non-aromatic varieties.

Evaluation of the effectiveness of the Gm2AP marker in an aroma segregating population

The F_1 plant and the parents, Kaori Hime and Jack, were genotyped with the Gm2AP marker. The results clearly showed three expected types of genotypes (Fig. 2). The genotypes of the 60 F_2 plants analyzed with the Gm2AP marker showed that 14 lines carried the allele of Kaori

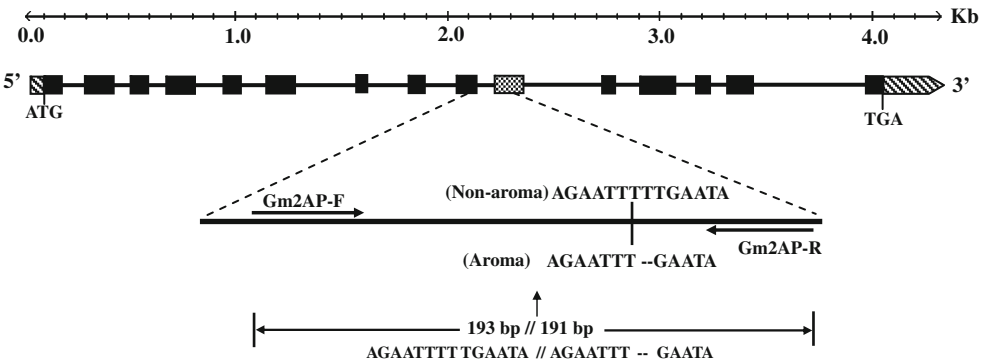

Fig. 1 The locations of the forward and reverse primers of Gm2AP in the *GmAMADH2* gene. The sequence variation, a 2-bp deletion, on chromosome 10 and expected sizes of the PCR products are provided

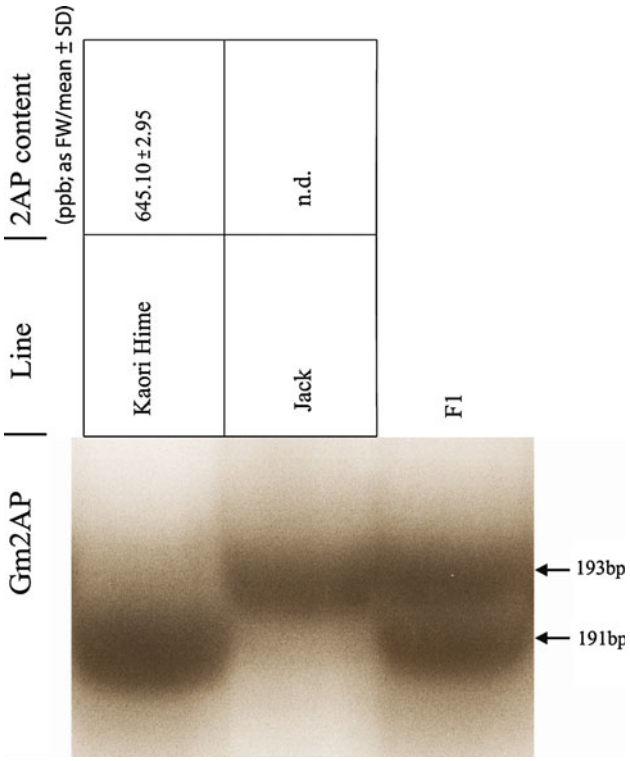

Fig. 2 Genotyping patterns of the Gm2AP marker among the two parents, Kaori Hime (aromatic) and Jack (non-aromatic), and their F_1 progeny. The 2AP contents in the parent seeds are provided, with *n.d.* not-detectable

Hime, 30 lines were heterozygous and 16 lines carried the allele of Jack (Fig. 3). The segregation ratio of the three genotypes in the F_2 agreed perfectly with the expected ratio of 1:2:1 ($\chi^2 = 0.133$; $P = 0.9356$), corresponding to the Mendelian segregation of a single gene (Table 1). For phenotyping, the immature F_3 seeds from individual F_2 plants were harvested, and the 2AP contents of the seeds were analyzed. As a result, 2AP was detected in 14 lines and was not detected in the seeds of the remaining 46 lines (Fig. 3). The numbers of the aromatic and non-aromatic F_2 lines showed a ratio of 1:3 ($\chi^2 = 0.089$; $P = 0.7654$). The segregation of phenotypes also agreed with the Mendelian segregation ratio for a single recessive gene that controls a trait (Table 1). According to these results, the phenotypes and genotypes of all 60 F_2 progenies were correlated.

Discussion

The aroma trait in vegetable soybean is thought to be recessive (AVRDC 2003). However, the location of the aroma gene/QTL in the soybean genome has not been reported. Although aroma might be thought of as a quantitative trait, only one gene has been reported to influence this trait in rice (Bradbury et al. 2008; Vanavichit et al. 2008). Our previous study also showed that the *GmAMADH2* gene is likely the key gene responsible for 2AP biosynthesis in soybeans (Arikit et al. 2010). The inactivation of this gene likely caused an accumulation of 2AP. This would support the hypothesis that a single recessive gene controls the aroma trait. The segregation analysis of aroma in the F_2 population in the present study confirmed that this trait is probably controlled by a single recessive gene. The Gm2AP marker can be described as a "perfect marker" because it showed a polymorphism that is perfectly associated with a phenotype. This was verified by genotyping a range of selected soybean varieties of known phenotypes. The marker was designed to be a co-dominant marker so that the heterozygous allele could be detected.

It is noteworthy that the 2AP content of F_3 seeds of the heterozygous F_2 plants was not detected in this study. In theory, the phenotypes of F_3 seeds from the heterozygous F_2 plants segregate at a ratio of 1:3 of aromatic to non-aromatic seeds. However, due to the small-scale method employed for 2AP analysis used in our study, the 2AP content of one-third of the aromatic seeds in heterozygous families might not be enough amounts to validate the presence of 2AP detected by GC–MS (Fig. 3). Variations in 2AP levels were observed in the F_3 seeds of the aromatic lines quantified by the GC–MS in this study (Fig. 3). This might suggest that one or more other genes associated with 2AP levels are segregating in the

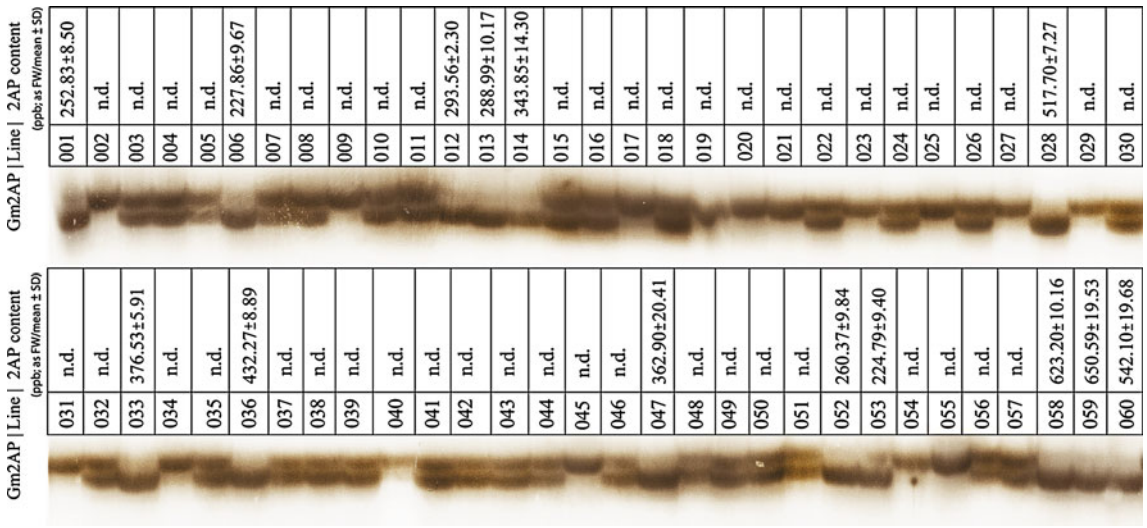

Fig. 3 Genotyping patterns of 60 F_2 plants using the Gm2AP marker. 2AP contents in the F_3 seeds are provided, with *n.d.* not-detectable

Table 1 Chi-square test for phenotypic and genotypic segregations in the F_2 plants derived from the cross Kaori Hime × Jack

	Number		χ^2	*P* value
	Observed	Expected		
Phenotype (expected ratio is 1:3)				
Aromatic	14	15	0.067	
Non-aromatic	46	45	0.022	
Total	60	60	0.089	0.7654
Genotype (expected ratio is 1:2:1)				
Kaori Hime	14	15	0.066	
Kaori Hime/Jack	30	30	0.000	
Jack	16	15	0.066	
Total	60	60	0.133	0.9356

population. Because *GmAMADH2* is involved in downstream pathway of the polyamine metabolism (Arikit et al. 2010), it is possible that a series of genes, i.e., genes in the upstream pathway or genes that enhance or regulate polyamine metabolism, can affect the level of precursor(s) of 2AP. The Gm2AP marker would be of value as a marker for determining the aromatic and the non-aromatic phenotypes, but it could not be used as a marker for determining the quantitative levels of 2AP.

It is also worth noting that other types of mutations in the gene *GmAMADH2* could exist in other aromatic soybeans. This is based on the knowledge that in the case of rice, besides the major mutation type of 8-bp deletion, some other types of mutations were also found in the gene *Os2AP* (*BADH2*) of aromatic varieties (Kovach et al. 2009). Therefore, the Gm2AP marker might not cover the other types of mutations, if they exist.

In summary, the Gm2AP marker could be efficiently used for molecular breeding of soybeans for aroma. The assay for genotyping soybeans using the Gm2AP marker in this study is a simple and robust method for screening soybean populations for aroma. The PCR products can be analyzed easily and inexpensively in a polyacrylamide gel electrophoresis (PAGE) with silver staining or, alternatively, by more sophisticated high-throughput experiments.

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