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ORIGINAL PAPER

A single base substitution in *BADH/AMADH* is responsible for fragrance in cucumber (*Cucumis sativus* L.), and development of SNAP markers for the fragrance

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Abstract

Key message **Sequence analysis revealed that an SNP (A1855G) in *CsBADH* of cucumber accession PK2011T202 causes amino acid change in a highly conserved motif, Y163C. Gene mapping showed association between the SNP and the fragrance.**

Abstract Pandan-like fragrance is a value-added trait in several food crops such as rice, vegetable soybean and sorghum. The fragrance is caused by the volatile chemical 2-acetyl-1-pyrroline (2AP). Mutation(s) in *betaine aldehyde dehydrogenase2* (*BADH2*; also known as *aminoaldehyde dehydrogenase2*) gene causes defective BADH2 and results in biosynthesis of 2AP. Recently, cucumber cultivars possessing pandan-like fragrance were discovered in Thailand. In this study, we report an association between *CsBADH* and the fragrance in cucumber accession "PK2011T202". Gene expression analysis of *CsBADH* in leaves of PK2011T202 and "301176" (non-fragrant) at various growth stages revealed that *CsBADH* was expressed in both accessions. Sequence comparison of *CsBADH* showed that PK2011T202 possesses a single base substitution (A1855G) in exon 5 which causes an amino acid change in a highly conserved motif of BADH, Y163C. Single nucleotide-amplified polymorphism markers were developed to detect the SNP polymorphism between the wild-type and fragrance alleles. Since *CsBADH* is located on chromosome 1, quantitative trait locus (QTL) mapping was conducted for this chromosome using an F_2 and a backcross populations developed from the cross between PK2011T202 and 301176. QTL analysis in both populations showed that the major QTL for fragrance, *qFgr*, was co-localized with the *CsBADH*. We concluded that the defect function of CsBADH is responsible for fragrance in cucumber PK2011T202.

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Introduction

Cucumber (*Cucumis sativus* L.; *Cucurbitaceae*) ($2n = 2x = 14$) is one of the most economically important vegetable crops in the world with the total production of more than 60 Mt per annum. Cucumber fruits are consumed in fresh or cooked or pickled forms. China is the largest producer followed by Iran and Turkey with the annual production of about 47.31, 1.82, and 1.75 Mt, respectively (FAOSTAT 2013). Traits determining cucumber fruit quality include stripes, spine color, fruit color, weigh, length, diameter and firmness.

India is the center of origin and domestication of cucumber, where cultivated (*C. sativus* var *sativus* L.) and wild (*C. sativus* var. *hardwickii*) forms coexist. Thailand, especially the northern region, is a part of the center of diversity where *C. sativus* var. *hardwickii* and primitive

cucumber cultivars are found (de Wilde and Duyfjes 2010). Recently, a few cucumber cultivars with fragrance fruits were found in Thailand. Their fruits and leaves have pandan (*Pandanus amaryllifolius* L.)-like fragrance (Pramnoi et al. 2013) which is the same as those possessed by fragrant rice (*Oryza sativa* L.), soybean [*Glycine max* (L.) Merr.] and sorghum [*Sorghum bicolor* (L.) Moench]. The pandan-like fragrance in the latter three crops is caused by the presence of the volatile chemical compound 2-acetyl-1-pyrroline (2AP) (Buttery et al. 1983; Fushimi and Masuda 2001; Yundaeng et al. 2013). Genetic studies demonstrated that the fragrance in these crops is controlled by a single recessive gene (AVRDC 2003; Ayyangar 1938; Berner and Hoff 1986; Murty et al. 1982; Sood and Siddiq 1978). Gene mapping and molecular genetic studies in these crops demonstrated that mutation(s) in *betainealdehyde dehydrogenase 2* (*BADH2*) (also known as *aminoaldehyde dehydrogenase*; *AMADH2*) gene results in the null and reduced function of BADH2 protein and causes accumulation of 2AP which is responsible for the fragrance (Bradbury et al. 2005; Wanchana 2005; Chen et al. 2008; Kovach et al. 2009; Juwattanasomran et al. 2011, 2012; Yundaeng et al. 2013). In rice, insertion, deletion, or substitution in *OsBADH2* that causes premature stop codon or amino acid change is responsible for fragrance (Bradbury et al. 2005; Shi et al. 2008; Kovach et al. 2009). In case of soybean, deletion or single base substitution in *GmBADH2* results in premature stop codon and amino acid change that associates with the fragrance (Juwattanasomran et al. 2011, 2012). For sorghum, deletion in *SbBADH2* causes premature stop codon and gives rise to the fragrance (Yundaeng et al. 2013). Apart from *OsBADH2*, mutation in *OsBADH1* also associates with fragrance in rice, although the effect is far less than that of *OsBADH2* (Amarawathi et al. 2008).

Genetic control of fragrance in cucumber has been reported recently (Pramnoi et al. 2013). The authors found that the fragrance in fruits and leaves is controlled by a single recessive gene, proposed as *fgr* with no xenia effect for the fragrance. However, molecular basis of the fragrance in cucumber has not yet been determined. Since *BADH* is responsible for the fragrance in rice (Bradbury et al. 2005), soybean (Juwattanasomran et al. 2011), and sorghum (Yundaeng et al. 2013), this gene is hypothesized as the candidate gene responsible for the fragrance in cucumber.

In this paper, we reported a molecular genetic basis of fragrance in cucumber accession PK2011T202. The objectives of this study were (1) to determine the association between the candidate gene *BADH* and the fragrance in cucumber, and (2) to develop a functional marker for the fragrance. Information on the causal gene and the functional marker(s) will be useful for breeding new fragrant cucumber cultivars.

Materials and methods

Plant materials and DNA extraction

Two cucumber accessions, "PK2011T202" and "301176", were used as parents for population development. PK2011T202 is a local cultivar from Thailand which possesses strong pandan-like fragrance; while 301176, an inbred line from Clover Seed Company, Hong Kong possesses no fragrance. These two accessions were the same parents used in inheritance study of fragrance reported earlier by Pramnoi et al. (2013). Quantification of 2AP content in fruit by head-space gas chromatography revealed that PK2011T202 had high 2AP while 301176 had no 2AP (Wongpornchai and Somta, unpublished data). PK2011T202 (as female parent) was hybridized by 301176 to develop F_1 seeds. F_1 plants were grown in a crossing block in which one plant was self-pollinated to produce an F_2 population comprising 123 individuals. Other F_1 plants were backcrossed as female parent to PK2011T202 to develop a BC_1F_1 population comprising 138 individuals. The F_2 plants and 10 plants from each of the parents were grown in an experimental field of Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand during August to October 2013. Similarly, the BC_1F_1 plants and 10 plants from each of the parents were grown in pots (one plant per pot) at Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand during February to April 2014. The materials were used for DNA extraction and fragrance evaluation by olfactory test.

Total genomic DNA of the parental, BC_1F_1 and F_2 plants was extracted from young leaves as per Lodhi et al. (1994) with minor modification. DNA concentration was estimated on 1 % agarose gel electrophoresis by comparing with a known concentration λ DNA, and adjusted to10 ng/μl for DNA marker analysis.

Evaluation for fragrance

The fragrance was evaluated by olfactory test following Sood and Siddiq (1978). At 30 days after planting (DAP), young leaves of parental, F_2, and BC_1F_1 plants were excised and used for the test. The leaves from each plant were weighted for three grams and cut into pieces of about 0.5 × 0.5 cm, put in a 25-ml tube and added with 15 ml of 1.7 % KOH (w/v) at 40 °C for 10 min. Subsequently, the tube was opened and smelled directly by 5 volunteers. The fragrance was scored as 1 (no fragrance), 2 (mildly to moderately fragrance), or 3 (highly fragrance). Average scores of the 5 volunteers were used for segregation and QTL analyses.

Chi-square (χ^2) analysis

Chi-square (χ^2) analysis for segregation of fragrance scores in the F_2 and BC_1F_1 populations was performed using the *R*-Program 2.10.0 (R Development Core Team 2010). The plants with score higher than 1.5 were classified as fragrant, while those with score equal to or lower than 1.5 were classified as non-fragrant.

Expression analysis of the *CsBADH*

PK2011T202 and 301176 were grown in pots in a greenhouse under natural condition during May to June 2012. At 15, 30 and 45 DAP, 0.5 g of young fresh leaves from three plants of each accession were harvested for extraction of total RNA using Concert™ Plant RNA Reagent (Invitrogen). Each RNA sample was derived from a single different plant. The RNA was treated with DNA-free™ DNaseI (Ambion) and converted to cDNA using RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas) according to manufacturer's instruction. cDNA concentration was quantified using ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., DE, USA).

A complete cDNA sequence of *BADH* from melon (*Cucumis melo* L.) (Genbank accession number JN091961), a closely related species to cucumber, was used for BLAST search against reference genome sequences of cucumber lines "Gy14" and "9930" in Phytozome v9.1 database (http://www.phytozome.net/cucumber) and Cucumber Genome Database (http://cucumber.genomics.org.cn), respectively, to identify the candidate gene(s) for BADH in cucumber. Annotated gene sequences identified as *BADH* (*Cucsa.197230* in Gy14 and *Csa005363* in 9930) were used in designing primers for real-time PCR analysis using Primer3 (Rozen and Skaletsky 2000) (Table S1). Primers were also designed for the *ACTIN* based on sequence of Genbank accession number AB698859 (Table S1). Real-time PCR analysis was carried out following Yundaeng et al. (2013). Three analyses (three biological replicates) were conducted at each growing stage. Expression of *CsBADH* was quantified with *ACTIN* normalization using Livak method (Livak and Schmittgen 2001).

Sequencing of the *CsBADH* gene and development of allele-specific markers

Eleven primer pairs (Table S2) were designed to amplify a genomic region of 5.068 kb covering the *CsBADH*. PCR was performed in a total volume of 25 µl containing 20 ng genomic DNA, 1 × *Taq* buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 U *Taq* DNA polymerase (Fermentas), and 0.5 µM each of forward and reverse primers. Amplification was performed in a GeneAmp PCR9700 System (Applied Biosystems) programmed as follows: 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min, and 72 °C for 5 min. PCR products were then electrophoresed on agarose gel and purified using E-gel® CloneWell™ Agarose Gels (Invitrogen). The purified PCR products were sequenced by Macrogen Co. Ltd. (South Korea) using BigDye® Terminator v3.1 Cycle Sequencing Kit on an ABI 3730xl DNA Analyzer (Applied Biosystems). The individual sequences were assembled using software Sequencher 5.0 (Gene Code Corporation). The complete sequences of *CsBADH2* of PK2011T202 and 301176 were submitted to GenBank as the accession numbers KJ577629 and KJ577628, respectively.

The *CsBADH* sequences from PK2011T202, 301176, Gy14 and 9930 were aligned using ClustalW (http://www.ebi.ac.uk/clustalw) to identify polymorphic sites among them. Predicted protein sequences encoded from these genes were also aligned using the same software.

Upon identification of a single nucleotide polymorphism (SNP) that may cause the fragrance, single nucleotide-amplified polymorphism (SNAP) markers specific to wild-type and mutant alleles were designed using WebSNAPPER (Drenkard et al. 2000; http://pga.mgh.harvard.edu/cgi-bin/snap3/websnaper3.cgi). The SNAP markers were tested in the parents by PCR amplification. The amplification conditions included 94 °C for 3 min, followed by 28 cycles at 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and 72 °C for 5 min. The PCR products were run on 1 % agarose gel electrophoresis, stained with ethidium bromide and visualized under ultraviolet light. The markers were then analyzed in the F_2 and BC_1F_1 plants.

Linkage map and QTL analyses

Forty-three simple sequence repeat (SSR) markers evenly covering chromosome 1 of cucumber (Ren et al. 2009) were screened for polymorphism between PK2011T202 and 301176. In addition, 11 SSR markers developed from sequence information of *CsBADH* gene (*Cucsa.197230*) or nearby genomic regions (Table 1) were also used. PCR amplification, electrophoresis and DNA band visualization were performed as described by Somta et al. (2008) with an exception that PCR contained 10 ng of genomic DNA. Polymorphic markers were used to analyze the F_2 and BC_1F_1 populations.

A linkage map was constructed using MapMaker3.0 (Lander et al. 1987) with the LOD score of 3, the maximum recombination frequency (*r*) of 4.0 and Kosambi's mapping function (Kosambi 1944). QTL analysis was conducted to locate the gene controlling fragrance in the F_2 and BC_1F_1 population by composite interval mapping (CIM) using Windows QTL Cartographer 2.5 (Wang et al. 2012). Significant LOD threshold for the QTL was determined by 10,000 runs of permutation test at $P = 0.01$.

Table 1 PCR primers designed for real-time PCR analysis of *CsBADH* and the reference *ACTIN* genes. The annealing temperature for all primers is 58 °C

Primer name	Target gene	Primer sequence	PCR product size (bp)
Cs_BADH	*BADH*	F: GGCTGTTCGTGCCAAGTATT	149
		R: AGTAATCGAAGCACCCAGCA	
Cs_ACTIN	*ACTIN*	F: AGGCCGTTCTGTCCCTCTAC	150
		R: CAGTAAGGTCACGACCAGCA	

Phylogenetic analysis

BADH/AMADH protein sequences of plants [*Arabidopsis thaliana* (AAM13070.1 and AAK44148.1), *Amaranthus hypochondriacus* (AAB58165.1 and AAB70010.1), *Atriplex hortensis* (ABF72123.1 and P42757.1), *Avicennia marina* (AAK55121.1 and BAB18544.1), *Beta vulargis* (BAE07176.1), *Cucumis melo* (AEK81574.1), *C. sativus* (Cucsa.197230), *G. max* (BAG09376.1 and BAG09377.1), *Gossypium hirsutum* (AAR23816.2), *Hordeum vulgare* (BAB62846.1 and BAB62847.1), *Lycium barbarum* (ACQ99195.1), *Leymus chinensis* (BAD86757.1 and BAD86758.1), *O. sativa* (NP_001053016.1_and NP_001061833.1), *Picea sitchensis* (ACN40431.1), *Pisum sativum* (CAC48392.3/CAC48393.2), *Populus trichocarpa* (XP_002322147.2 and DR448910), *Solanum lycopersicum* (AAX73303.1 and ACI43573.1), *Solanum tuberosum* (PGSC0003DMT400083025 and PGSC0003DMT400063205), *Sorghum bicolor* (06g019200.1 + 06g019210.1 and AGZ15751.1), *Spinacia oleracea* (AAA34025.1), *Vitis vinifera* (XP_002283690.1 and XP_002281984.1), *Zea mays* (ACS74867.1 and ACS74868.1), *Zoysia tenuifolia* (BAD34953.1 and BAD34949.1) and *Physcomitrella patens* (EDQ78577.1)] were obtained from GenBank or Phytozome (www.phytozome.net), or Sol Genome (http://solgenomics.net) databases. Phylogenetic analysis was performed using Phylogeny.fr (Dereeper et al. 2008) in which the sequences were subjected to multiple sequence alignments by MUSCLE 3.7, and then to phylogenetic tree construction by maximum likelihood with 1000 bootstraps.

Results

Fragrance variation in the F_2 and BC_1F_1 populations and inheritance of the fragrance

Fragrance scores evaluated by olfactory test of leaves in the F_2 population ranged from 1.0 to 2.8 with the mean of 1.45. Similarly, the fragrance scores in the BC_1F_1 population varied from 1 to 3 with the mean of 1.64. The scores for PK2011T202 and 301176 were 3 (highly fragrant) and 1 (non-fragrant), respectively. Frequency distribution of fragrance scores of both populations in Fig. 1 showed a skewed distribution toward the non-fragrant parent.

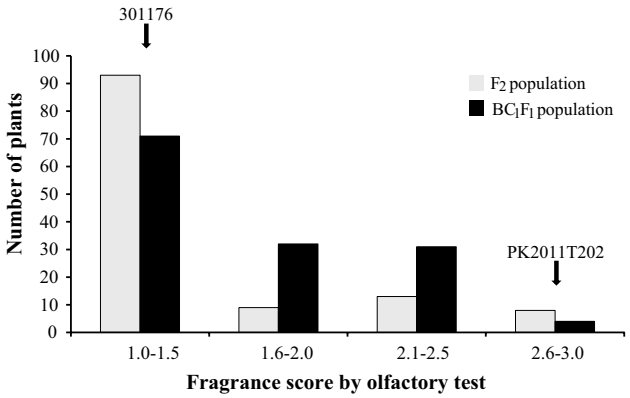

Fig. 1 Frequency distribution of fragrance scores in the F_2 (301176 × PK2011T202) and BC_1F_1 [(301176 × PK2011T202) × PK2011T202] populations evaluated by olfactory test

Inheritance of the fragrance was determined based on olfactory test in the F_2 and BC_1F_1 populations. In the F_2 population, 30 and 93 F_2 plants were classified as fragrant and non-fragrant, respectively. In the BC_1F_1 population, 71 and 67 plants were classified as fragrant and non-fragrant, respectively. The segregation in the F_2 population fitted well with a 3 (non-fragrant):1 (fragrant) ratio ($\chi^2 = 0.0244$, $P = 0.8759$), while that in the BC_1F_1 population fitted a 1 (non-fragrant):1 (fragrant) ratio ($\chi^2 = 0.1159$, $P = 0.7335$). These indicated that the fragrance in cucumber accession PK2011T202 is conditioned by a single recessive gene.

Identification and expression analysis of *CsBADH*

BLAST search using *CmBADH* against cucumber reference genome sequences at Phytozome and Cucumber Genome databases identified one annotated locus in each database as *CsBADH* gene; *Cucsa.197230* (scaffold01357:699724..704791) in the former and *Csa005363* (chromosome01:1164184..1168771) in the latter databases. CDS comparison revealed that *Cucsa.197230* and *Csa005363* are the same loci. Based on *Cucsa.197230*, *CsBADH* has genomic sequence of 5068 nucleotides, transcript sequence of 2003 nucleotides, coding sequence

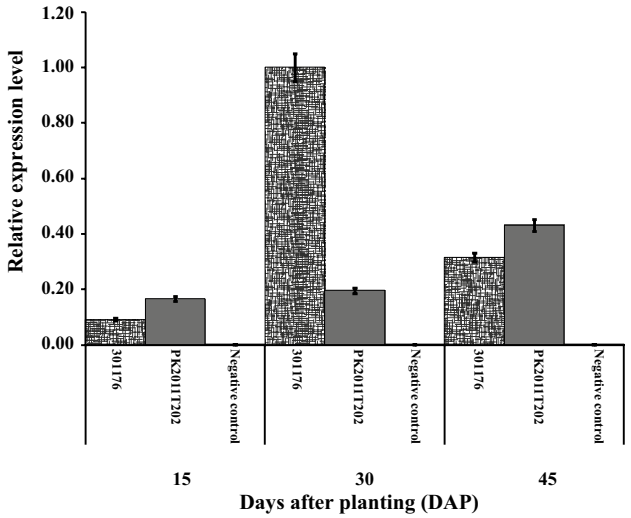

Fig. 2 Expression of *CsBADH2* in fully expanded leaves of PK2011T202 and 301176 at 15, 30 and 45 days after planting (DAP)

(CDS) of 1512 nucleotides from 15 exons, and protein sequence of 503 amino acids.

Expression of *CsBADH* in PK2011T202 and 301176 was determined in leaves at 15, 30 and 45 DAP using real-time PCR analysis. The *CsBADH* showed constitutive expression at all three growth stages of both accessions (Fig. 2). 301176 showed the highest expression level at 30, followed by 45, and 15 DAP, respectively. PK2011T202 had the greatest expression level at 45, followed by 30, and 15 DAP, respectively. The χ^2 test for homogeneity revealed that expression levels of *CsBADH* at all three growth stages in both cucumber varieties were not different ($\chi^2 = 0.43$, P value $= 0.81$). A t test at $P = 0.01$ for *CsBADH* expression level of the two varieties at each growth stages showed that they were not different ($t = 0.5384$ and P value $= 0.6189$; $t = -3.1884$ and P value $= 0.03327$; and $t = 0.4819$ and P value $= 0.6550$, respectively).

Sequence and allele-specific markers of *CsBADH*

Sequences of *CsBADH* in both PK2011T202 and 301176 were determined and compared with the reference sequences to identify mutation(s). The sequence length of *CsBADH* in 301176 was 4594 bp, while that of PK2011T202 was only 4577 bp (Fig. 3). When the sequences of PK2011T202 and 301176 were aligned against the reference sequences, 14 mutations, 6 single base substitutions, and 8 indels were observed. All the substitutions were in exons (exons 1, 2, 4, 5, 8 and 9), while all the indels were in introns (four 1-bp deletion each in intron 1, 4, 8, and 11, a 3-bp deletion in intron 10, a 4-bp deletion in intron 4, a 5-bp deletion in intron 13, and a 6-bp deletion in intron 2). The substitutions in exons 1, 4, 5 and 8 caused amino acid changes. However, only the mutation in exon 5 is unique to the fragrant cucumber PK2011T202. This mutation is the nucleotide change from adenine (A) in 9930, Gy14 and 301176 to guanine (G) in PK2011T202 at the position 1855. The SNP introduced an amino acid change from tyrosine (Y) to cytosine (C) at the position 163 (Y163C) of CsBADH (Fig. 4).

Six SNAP markers were developed to detect the SNP in exon 5; three each for the wild-type (A) and mutant (G) alleles in 301176 and PK2011T202, respectively (Table 2). All three SNAP markers for the wild type amplified DNA band in 301176, while two SNAP markers for the mutant, CsBADH_G1 and CsBADH_G3, amplified DNA band in PK2011T202 (Fig. 5a). DNA band patterns amplified by SNAP markers CsBADH_A1 and CsBADH_G1 in some F_2 plants are shown in Fig. 5b.

Linkage map and QTL analyses

QTL mapping was conducted in the F_2 and BC_1F_1 populations to determine the association between fragrance and *CsBADH*. Marker screening revealed that 11 out of 43 SSR markers gave polymorphism between the parents. The 11 polymorphic SSR markers and the marker CsBADH-AG were used to construct linkage group. The linkage groups in the F_2 and BC_1F_1 populations spanned 61.0 and 152.2 cM in length, respectively (Fig. 5).

Single marker regression analysis in both populations revealed that 10 markers associated with the fragrance. The markers explained 9.74 to 58.83 % of the fragrance variation in the F_2 population and 6.26 to 55.14 % in the BC_1F_1 population (Supplementary Table S3).

LOD thresholds for QTL determined by permutation test of fragrance score in the F_2 and BC_1F_1 populations were 6.1 and 5.7, respectively. CIM identified single major QTL for fragrance score, *qFgr*, in the F_2 and BC_1F_1 populations with LOD scores of 44.45 and 19.96, respectively (Fig. 6). In the F_2 population, the *qFgr* was located 1.1 cM away from the SNAP marker CsBADH-AG and accounted for 80.85 % of the fragrance score variation. The *qFgr* showed additive and dominant effects of non-fragrant (301176) allele at the olfactory scores of −0.34 and −0.58, respectively. In the BC_1F_1 population, the QTL was 1.0 cM from the SNAP marker and explained 43.27 % of the fragrance variation. Additive effect of the non-fragrant allele in this population was −0.72. In both populations, alleles from PK2011T202 increased the fragrance score.

Phylogenetics of CsBADH

Phylogenetic analysis was performed to determine the relationship of CsBADH with other monocot and dicot BADHs/AMADHs. The phylogenetic tree revealed two

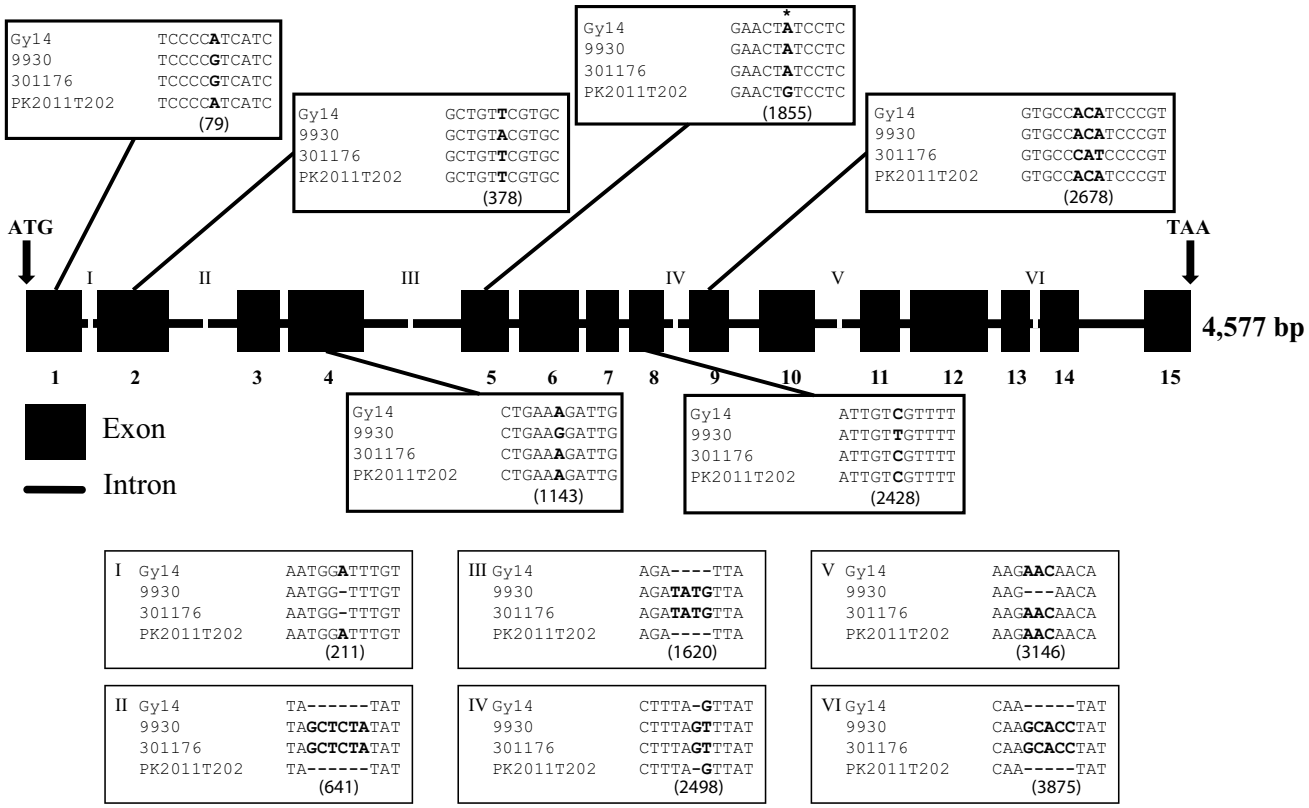

Fig. 3 A scheme depicting the location of SNPs and indels in *CsBADH* among cucumber accession PK2011T202, 301176, and the reference sequences. Start codon (ATG), 15 exons (*solid boxes*), 14 introns (*solid line*) and stop codon (TAA) are illustrated. SNPs and indels are bolded. *Asterisk* indicates the SNP that caused amino acid change specific to PK2011T202. Roman numbers indicate mutations in introns

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9930          MAISIPTRQLFIDGEWREPVLKKRIPVINPTTEETIGSIPAATAEDVELAVDAARKALARNKGKDWRSASGAVRAKYLRAIAAKITERKSELAKLEAIDCGKPLEEAAWDMDDVAGCFDY 120
Gy14          MAISIPTRQLFIDGEWREPVLKKRIPIINPTTEETIGSIPAATAEDVELAVDAARKALARNKGKDWRSASGAVRAKYLRAIAAKITERKSELAKLEAIDCGKPLEEAAWDMDDVAGCFDY 120
301176        MAISIPTRQLFIDGEWREPVLKKRIPVINPTTEETIGSIPAATAEDVELAVDAARKALARNKGKDWRSASGAVRAKYLRAIAAKITERKSELAKLEAIDCGKPLEEAAWDMDDVAGCFDY 120
PK2011T202    MAISIPTRQLFIDGEWREPVLKKRIPIINPTTEETIGSIPAATAEDVELAVDAARKALARNKGKDWRSASGAVRAKYLRAIAAKITERKSELAKLEAIDCGKPLEEAAWDMDDVAGCFDY 120

                                                                                *
9930          YADLAEGLDAKQKAPVDVPMDTFKSYVLKEPIGVVGLITPWNYPLLMCAWKVAPALAAGCAAILKPSELASVTSLELAEICKDVGLPSGILNVLTGYGPEAGAPLASHPHVDKVAFTGSS 240
Gy14          YADLAERLDAKQKAPVDVPMDTFKSYVLKEPIGVVGLITPWNYPLLMCAWKVAPALAAGCAAILKPSELASVTSLELAEICKDVGLPSGILNVLTGYGPEAGAPLASHPHVDKVAFTGSS 240
301176        YADLAERLDAKQKAPVDVPMDTFKSYVLKEPIGVVGLITPWNYPLLMCAWKVAPALAAGCAAILKPSELASVTSLELAEICKDVGLPSGILNVLTGYGPEAGAPLASHPHVDKVAFTGSS 240
PK2011T202    YADLAERLDAKQKAPVDVPMDTFKSYVLKEPIGVVGLITPWNCPLLMCAWKVAPALAAGCAAILKPSELASVTSLELAEICKDVGLPSGILNVLTGYGPEAGAPLASHPHVDKVAFTGSS 240

9930          ATGSKIMASAAQLVKPVTMELGGKSPIVVFDDVDLDKAAEWAIFGCFWTNGQICSATSRLLVHENIADEFLDKIVQWCKNIKISDPLEEGCRLGPVVSAGQYEKVLKFVSTAEQEGAKIL 360
Gy14          ATGSKIMASAAQLVKPVTMELGGKSPIVVFDDVDLDKAAEWAIFGCFWTNGQICSATSRLLVHENIADEFLDKIVQWCKNIKISDPLEEGCRLGPVVSAGQYEKVLKFVSTAEQEGAKIL 360
301176        ATGSKIMASAAQLVKPVTMELGGKSPIVVFDDVDLDKAAEWAIFGCFWTNGQICSAHPRLLVHENIADEFLDKIVQWCKNIKISDPLEEGCRLGPVVSAGQYEKVLKFVSTAEQEGAKIL 360
PK2011T202    ATGSKIMASAAQLVKPVTMELGGKSPIVVFDDVDLDKAAEWAIFGCFWTNGQICSATSRLLVHENIADEFLDKIVQWCKNIKISDPLEEGCRLGPVVSAGQYEKVLKFVSTAEQEGAKIL 360

9930          YGGVRPKHLKKGYFVEPAIITNVTTSMQIWKEEVFGPVLCVKTFSSEDEAIELANDTIYGLGAAVISNDLERCDRVAKAFQAGIVWINCSQPCFTQAPWGGIKRSGFGRELGEWGLDTYL 480
Gy14          YGGVRPKHLKKGYFVEPAIITNVTTSMQIWKEEVFGPVLCVKTFSSEDEAIELANDTIYGLGAAVISNDLERCDRVAKAFQAGIVWINCSQPCFTQAPWGGIKRSGFGRELGEWGLDTYL 480
301176        YGGVRPKHLKKGYFVEPAIITNVTTSMQIWKEEVFGPVLCVKTFSSEDEAIELANDTIYGLGAAVISNDLERCDRVAKAFQAGIVWINCSQPCFTQAPWGGIKRSGFGRELGEWGLDTYL 480
PK2011T202    YGGVRPKHLKKGYFVEPAIITNVTTSMQIWKEEVFGPVLCVKTFSSEDEAIELANDTIYGLGAAVISNDLERCDRVAKAFQAGIVWINCSQPCFTQAPWGGIKRSGFGRELGEWGLDTYL 480

9930          TVKQVTQYISDEPWGWYKSPSKL 503
Gy14          TVKQVTQYISDEPWGWYKSPSKL 503
301176        TVKQVTQYISDEPWGWYKSPSKL 503
PK2011T202    TVKQVTQYISDEPWGWYKSPSKL 503
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Fig. 4 Alignment of the amino acid sequences of betaine aldehyde dehydrogenase protein encoded by *CsBADH* from fragrant cucumber PK2011T202, non-fragrant cucumber 301176, and the reference sequence. *Asterisk* indicates amino acid change unique to PK2011T202

Table 2 Characteristics of SNAP primer pairs specific to A/G polymorphism in the exon 5 of the *CsBADH* gene. The annealing temperature for all primers is 60 °C

Primer name	Primer sequence	PCR product size (bp)
CsBADH2-A1	F: GCTGATGTCTATCTTTTATTGAAGGAACAA	155
	R: CACAATAAATAAAGTTCATAAAATCCATAATCAC	
CsBADH2-A2	F: TGCTGATGTCTATCTTTTATTGAAGGAAATA	156
	R: CACAATAAATAAAGTTCATAAAATCCATAATCAC	
CsBADH2-A3	F: TGCTGATGTCTATCTTTTATTGAAGGAATTA	156
	R: CACAATAAATAAAGTTCATAAAATCCATAATCAC	
CsBADH2-G1	F: TGATGTCTATCTTTTATTGAAGGAACCG	153
	R: CACAATAAATAAAGTTCATAAAATCCATAATCAC	
CsBADH2-G2	F: GCTGATGTCTATCTTTTATTGAAGGAACAG	155
	R: CACAATAAATAAAGTTCATAAAATCCATAATCAC	
CsBADH2-G3	F: GCTGATGTCTATCTTTTATTGAAGGAATTG	155
	R: CACAATAAATAAAGTTCATAAAATCCATAATCAC	

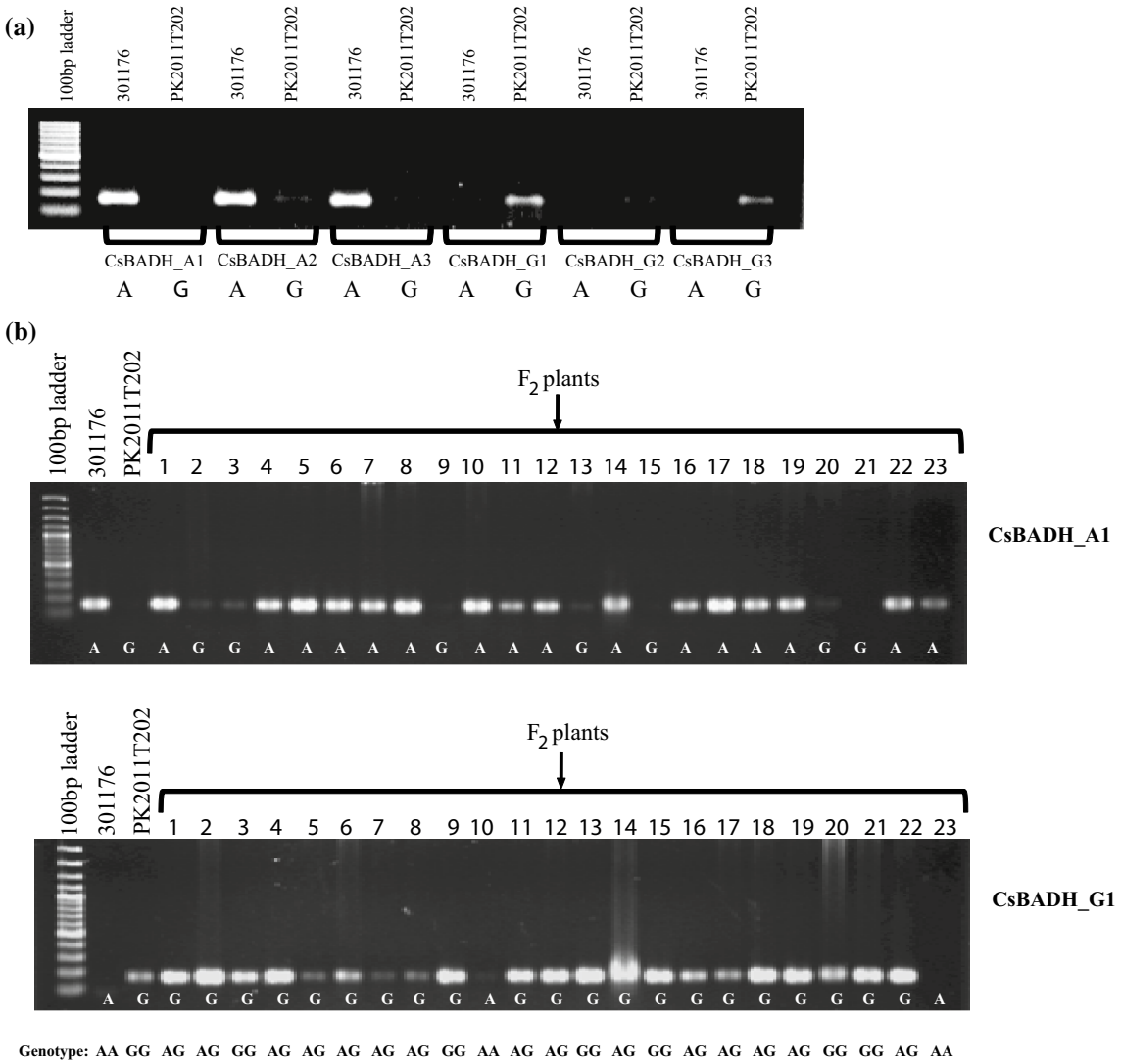

Fig. 5 DNA bands amplified by SNAP markers specific to single nucleotide polymorphism in exon 5 of *CsBADH* in cucumber PK2011T202 and non-fragrant cucumber 301176 (**a**). Segregation of the SNAP marker CsBADH-A1 and CsBADH-G1 in 23 F_2 progenies from the cross between 301176 and PK2011T202 is also shown. Allele detected by each marker in each individual plant is shown at the bottom of the gel (**b**). Genotypes inferred by the two markers are shown at the bottom of the figure

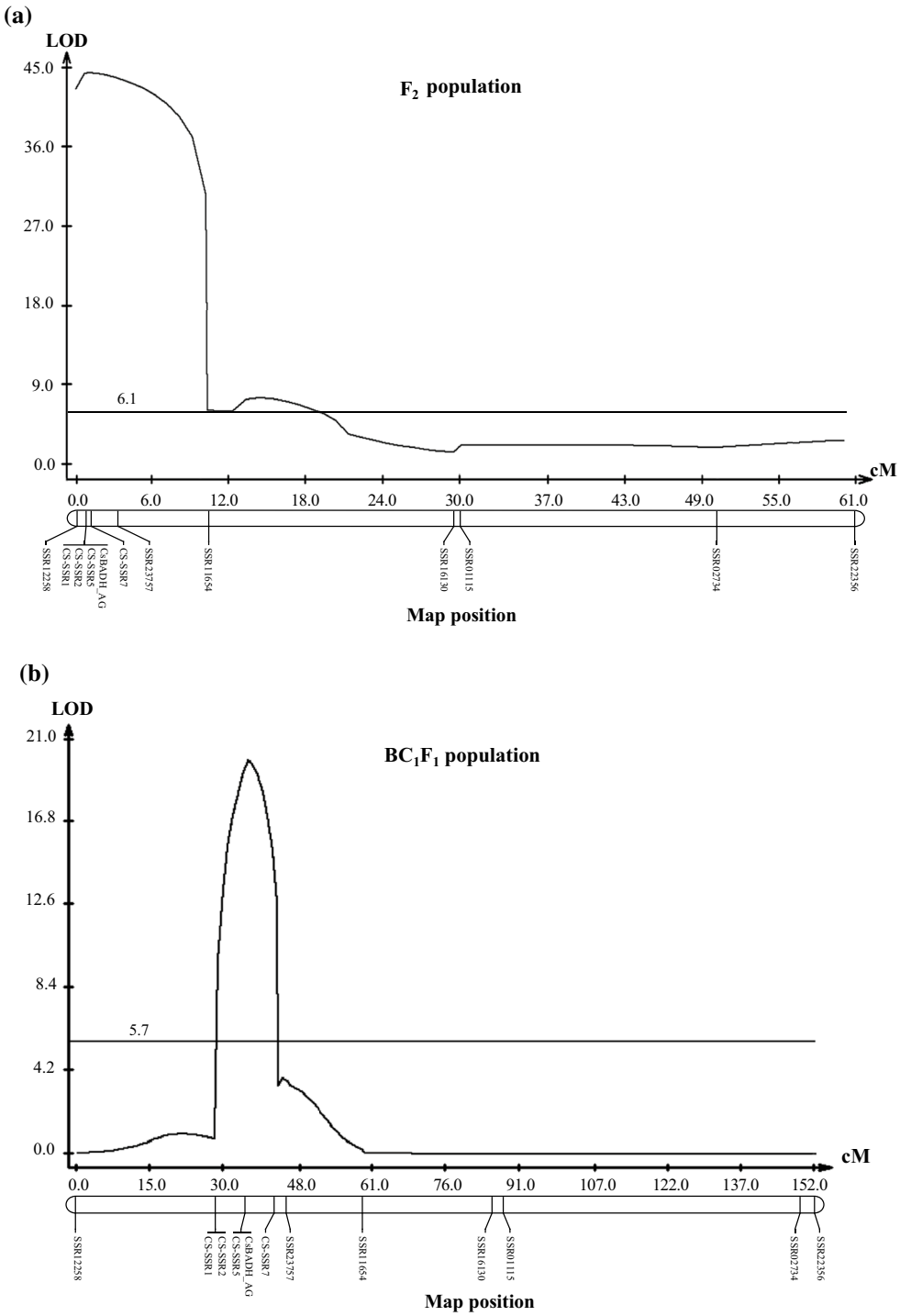

Fig. 6 LOD graph for the major QTL controlling fragrance score (*qFgr*) detected on chromosomes 1 by composite interval mapping in the F_2 (301176 × PK2011T202) and BC_1F_1 [(301176 × PK2011T202) × PK2011T202] populations

major clusters of plant BADHs/AMADHs, each cluster belonged to the respective monocot and dicot groups (Fig. 7). In the dicot group, *P. patens* and *Picea sitchensis* were clustered in this group. Each species of both groups have two homologous sequences of BADHs/AMADHs, with exception in *C. sativus*, *C. melo*, *G. hirsutum*, *L. Barbarum*, *S. oleracea*, *B. vulgaris*, and *P. patens* which have no homologous sequence, and in *Z. mays* which has three homologous sequences. There were two clear orthologous subclusters in the monocots, while there was no clear subcluster in the dicots. The CsBADH identified from this study was closely related to CmBADH, GhBADH and PtBADH2/PtAMADH2 (Fig. 7).

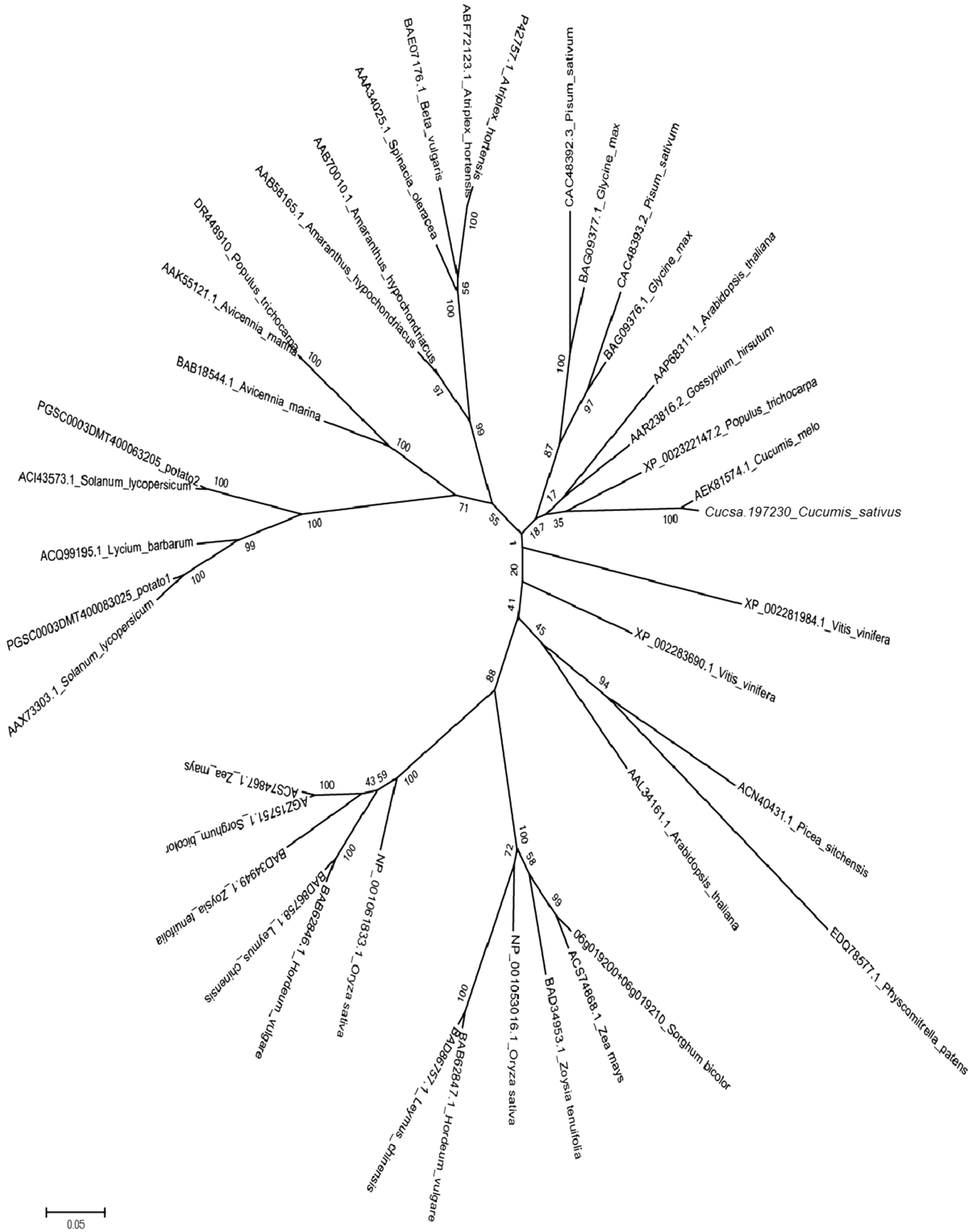

Fig. 7 A phylogenetic tree reconstructed from BADHs/AMADHs sequences of several plant species including cucumber. Bootstrap frequencies are labeled at the dichotomous points

Discussion

Fragrance is an important value-added trait of many edible crops. Cultivars with pandan-like fragrance are more preferable to consumers than the non-fragrant ones. Breeding for fragrance is a major objective in rice, vegetable soybean and sorghum breeding programs. The fragrance is mainly the result of the presence in volatile compound 2AP. In this study, segregation analysis confirmed the result of Pramnoi et al. (2013) that the fragrance in cucumber "PK2011T202" is controlled by a single recessive gene. The recessiveness of the fragrance suggests that this trait is created by a loss in gene function.

Studies on fragrance in field crops including rice (Bradbury et al. 2005; Shi et al. 2008; Shao et al. 2011; Amarawathi et al. 2008; Kovach et al. 2009), soybean (Juwattanasomran et al. 2011, 2012) and sorghum (Yundaeng et al. 2013) demonstrated that the fragrance and 2AP synthesis are associated with defected function of BADH2. Silencing *BADH2* in non-fragrant rice and soybean cultivars by RNAi resulted in 2AP production and the fragrance (Niu et al. 2008; Arikit et al. 2010), thus confirming the association between *BADH2* and the fragrance. In this study, we demonstrated that an SNP in gene coding for BADH in cucumber associates with the fragrance. This is the first report on molecular basis of the fragrance in vegetable crops. The SNP A1855G in *CsBADH* is present only in the fragrant cucumber PK2011T202 and results in Y163C change in BADH2. The mutation appears to involve in the fragrance in this cucumber. BADHs (AMADHs) enzymes belong to the aldehyde dehydrogenase families 9 and 10 (Kirch et al. 2005). The substrates of BADHs include betaine aldehyde, 3-aminopropionaldehyde, 3-dimethylsulfoniopropionaldehyde, γ-guanidinobutyraldehyde, and γ-aminobutyraldehyde (GABald) (for details see Fitzgerald et al. 2008). Enzymatic studies in pea (Kopečný et al. 2011), spinach (Díaz-Sánchez et al. 2012), tomato and maize (Kopečný et al. 2013) revealed that Y163 (equivalent to Y160 in spinach; Díaz-Sánchez et al. 2012) is a critically active residue responsible for the specificity of plant BADHs. Y163 maintains overall geometry of the substrate channel that allows appropriate orientation of the substrate, which results in optimal reaction rate (Kopečný et al. 2011). Kopečný et al. (2011) and Kopečný et al. (2013) showed that Y163A mutation of BADH in pea (PsAMADH2 in the original paper) and spinach (SoBADH) results in weak binding to its substrates including GABald, as compared to the wild-type protein. In a proposed pathway for biosynthesis of 2AP (Bradbury et al. 2008), functional BADH2 inhibits the biosynthesis of 2AP by converting GABald to γ-aminobutyric acid, whereas non-functional BADH2 leads to an accumulation of GABald which is then acetylated to form 2AP. Thus, the Y163C mutation in cucumber accession PK2011T202 possibly impairs the binding between BADH/AMADH and GABald, leading to 2AP production and fragrance. A similar finding was reported by Juwattanasomran et al. (2011) that an SNP in *GmBADH2* causes an amino change in a conserved region, G334D, of GmBADH2 that produces the fragrance in vegetable soybean cultivar "Kaori".

In this study, we considered the fragrance as a quantitative trait and evaluated it by sensory test. The QTL detected for the fragrance, *qFgr*, accounted for 80.85 and 43.27 % of the variation of fragrance scores in the F_2 and BC_1F_1 populations, and its position corresponded to a genome region containing *CsBADH2* gene. This supports that the A1855G mutation in *CsBADH2* that causes Y163C change of CsBADH2 is responsible for the fragrance in cucumber accession PKT2011T202. However, the moderate fragrance variation (43.27 %) explained by *qFgr* in the BC_1F_1 population suggests that there may be other genetic factor(s) involving the fragrance in cucumber. We examined association between genotypes detected by the SNAP marker CsBADH-AG and fragrance scores in the F_2 and BC_1F_1 populations and found imperfect association between the marker and the score in both populations. In the F_2 population, one plant showing homozygous alleles from PKT202 had fragrance score of only 1.2 and one plant showing heterozygous alleles had fragrance score higher than 1.5. In the BC_1F_1 population, six plants possessing homozygous alleles from PKT202 had fragrance score lower than 1.5, while eight plants possessing homozygous alleles had fragrance score higher than 1.5. Similar observations have been reported for the fragrance in rice (Amarawathi et al. 2008) and soybean (Juwattanasomran et al. 2011, 2012). The imperfect association between the functional marker and the fragrance score may also arise from the inaccuracy of the sensory test used for evaluation of the fragrance. However, it suggests that apart from *BADH* gene which is the principal cause of the fragrance, there may be also other genetic factor(s) affecting the fragrance in cucumber. Since 2AP is the chemical that is responsible for the fragrance and the biosynthesis pathway of 2AP involves several other chemicals such as proline (Bradbury et al. 2008) which is highly influenced by environmental factors, genetic factor(s) controlling proline can affect fragrance level in cucumber. Moreover, in another pathway proposed for 2AP biosynthesis that is independent of BADH enzyme, Huang et al. (2008) demonstrated that Δ^1-pyrroline-5-carboxylic acid synthetase (P5CS) may react directly with methylglyoxal (MG) leading to 2AP formation in rice, while Wu et al. (2009) showed that P5CS may degrade to 1-pyrroline and interact with MG to produce 2AP. Genetic factor(s) controlling these chemicals may also affect fragrance level in cucumber. In addition, QTL mapping studies for fragrance in rice revealed that the fragrance is controlled

by one major QTL (*BADH2* gene) on chromosome 8 and three minor QTLs each on chromosomes 3, 4 and 12 (Amarawathi et al. 2008; Lorieux et al. 1996).

Although fragrance is controlled by a single recessive gene and its evaluation can be achieved by sensory test, improvement of the trait requires repeated evaluations of selfed progenies after each generation of backcross breeding (Juwattanasomran et al. 2011). Using MAS for selection of fragrance cucumber can shorten such steps. Functional marker(s) for a trait is highly demanded for MAS as they perfectly associate with the trait. In this study, an SNP in *CsBADH* gene associated with fragrance in cucumber was identified and enabled us to develop SNAP markers as functional markers for detecting *CsBADH* polymorphism. SNAP is a preferable marker for SNP detection because it is simple, rapid and inexpensive to analyze (Drenkard et al. 2000). The SNAP markers developed in this study can facilitate marker-assisted selection for fragrant cucumber genotypes in a large-scale breeding program using PK2011T202 or other cucumber germplasm that possesses the same fragrance alleles as the gene donor.

Previous phylogenetic analyses showed that flowering plant species possess two homologous BADHs/AMADHs and that there are two orthologous BADHs/AMADHs subgroups in the monocot plants (Arikit et al. 2010; Kopečný et al. 2013). Our phylogenetic analysis gave similar result as the previous reports. Arikit et al. (2010) postulated that the two homologous BADHs/AMADHs in flowering plants had evolved through a duplication of the ancestral gene after the divergence of monocots and dicots, and that the two *BADHs/AMADHs* in each monocot were duplicated in its common ancestor before the divergence, while they were independently duplicated in each species after the divergence of the dicots. Kopečný et al. (2013) found that maize, which is among plants with large genome size (2500 Mbp), has three homologous sequences of BADHs/AMADHs. The authors also noted that number of homologous sequences of BADHs/AMADHs can be higher in species with genome duplications. In our study, however, duplication of *BADH/AMADH* was not detected in cucumber genome, as BLAST search against the two reference genome sequences of cucumber identified only *CsBADH*. Cucumber has a small genome size of only about 367 Mb (Arumanagathan and Earle 1991). Whole genome sequencing revealed that cucumber does not experience recent whole genome duplication and has a few tandem duplications in the genome (Huang et al. 2009). This supports the absence of *BADH/AMADH* duplication in cucumber found in our study.

Author contribution statement C.Y. carried out major part of the experiments and analyzed the data. C.Y., S.C., P. Somta and P. Srinives prepared plant materials. S.T. participated in gene expression analysis and sequencing. P. Somta initiated and coordinated the study, and analyzed the results. C.Y., P. Somta, S.T. and P. Srinives wrote the manuscript. All authors approved the manuscript.

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Ethical standards and conflict of interest All the experiments performed in this study comply with the current laws of Thailand. The authors declare that they have no conflict of interest.

References

Amarawathi Y, Singh R, Singh AK, Singh VP, Mohapatra T, Sharma TR, Singh NK (2008) Mapping of quantitative trait loci for basmati quality traits in rice (*Oryza sativa* L.). Mol Breed 21:49–65

Arikit S, Yoshihashi T, Wanchana S, Uyen TT, Huong NTT, Wongpornchai S, Vanavichit A (2010) Deficiency in the amino aldehyde dehydrogenase encoded by *GmAMADH2*, the homologue of rice *Os2AP*, enhances 2-acetyl-1-pyrroline biosynthesis in soybeans (*Glycine max* L.). Plant Biotechnol J 9:75–87

Arumanagathan K, Earle ED (1991) Nuclear DNA content of some important plant species. Plant Mol Biol Rep 9:229–241

AVRDC (2003) AVRDC progress report 2002. AVRDC-The World Vegetable Center, Shanhua

Ayyangar GNR (1938) Studies in sorghum. J Madras Univ 11:131–143

Berner DK, Hoff BJ (1986) Inheritance of scent in American long grain rice. Crop Sci 26:876–878

Bradbury LMT, Fitzgerald TL, Henry RJ, Jin QS, Waters DLE (2005) The gene for fragrance in rice. Plant Biotechnol J 3:363–370

Bradbury LMT, Gillies SA, Brushett DJ, Waters DLE, Henry RJ (2008) Inactivation of an aminoaldehyde dehydrogenase is responsible for the fragrance in rice. Plant Mol Biol 68:443–449

Buttery RG, Ling LC, Juliano BO, Turnbaugh JG (1983) Cooked rice aroma and 2-acetyl-1-pyrroline. J Agric Food Chem 31:823–826

Chen S, Yang Y, Shi W, Ji Q, He F, Zhang Z, Cheng Z, Liu X, Xu M (2008) *Badh2*, encoding betaine aldehyde dehydrogenase, inhibits the biosynthesis of 2-acetyl-1-pyrroline, a major component in rice fragrance. Plant Cell 20:1850–1861

de Wilde WJJO, Duyfjes BEE (2010) *Cucumis sativus* L. forma *hardwickii* (Royle) W.J. de Wilde & Duyfjes and feral forma *sativus*. Thai For Bull (BOT) 38:98–107

Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F, Dufayard JF, Guindon S, Lefort V, Lescot M, Claverie JM, Gascuel O (2008) Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Res 36:465–469

Díaz-Sánchez ÁG, González-Segura L, Mújica-Jiménez C, Rudiño-Piñera E, Montiel C, Martínez-Castilla LP, Muñoz-Clares RA (2012) Amino acid residues critical for the specificity for betaine aldehyde of the plant ALDH10 isoenzyme involved in the synthesis of glycine betaine. Plant Physiol 158:1570–1582

Drenkard E, Richter BG, Rozen S, Stutius LM, Angell NA, Mindrinos M, Cho RJ, Oefner PJ, Davis RW, Ausubel FM (2000) A simple procedure for the analysis of single nucleotide polymorphisms facilitates map-based cloning in Arabidopsis. Plant Physiol 124:1483–1492

Fitzgerald TL, Waters DLE, Henry RJ (2008) The effect of salt on betaine aldehyde dehydrogenase transcript levels and 2-acetyl-1-pyrroline concentration in fragrant and non-fragrant rice (*Oryza sativa*). Plant Sci 174:539–546

Food and Agriculture Organization (2013) FAOSTAT. http://faostat.fao.org. Accessed 5 March 2014

Fushimi T, Masuda R (2001) 2-acetyl-1-pyrroline concentration of the vegetable soybean. In: Lumpkin T, Shanmugasundaram S (eds) Proceeding of the 2nd international vegetable soybean conference. Washington State University, Pullman, p 39

Huang TC, Teng CH, Chang JL, Chuang HS, Ho CT, Wu ML (2008) Biosynthetic mechanism of 2-acetyl-1-pyrroline and its relationship with Δ^1-pyrroline-5-carboxylic acid and methylglyoxal in aromatic rice (*Oryza sativa* L.) callus. J Agric Food Chem 56:7399–7404

Huang S, Li R, Zhang Z, Li L, Gu X, Fan Lucas WJ, Wang X, Xie B, Ni P, Ren Y, Zhu H, Li J, Lin K, Jin W, Fei Z, Li G, Staub J, Kilian A, van der Vossen EAG, Wu Y, Guo J, He J, Jia Z, Ren Y, Tian G, Lu Y, Ruan J, Qian W, Wang M, Huang Q, Li B, Xuan Z, Cao J, Asan WZ, Zhang J, Cai Q, Bai Y, Zhao B, Han Y, Li Y, Li X, Wang S, Shi Q, Liu S, Cho WK, Kim J, Xu Y, Heller-Uszynska K, Miao H, Cheng Z, Zhang S, Wu J, Yang Y, Kang H, Li M, Liang H, Ren X, Shi Z, Wen M, Jian M, Yang H, Zhang G, Yang Z, Chen R, Liu S, Li J, Ma L, Liu H, Zhou Y, Zhao J, Fang X, Li G, Fang L, Li Y, Liu D, Zheng H, Zhang QN, Li Z, Yang G, Yang S, Bolund L, Kristiansen K, Zheng H, Li S, Zhang X, Yang H, Wang J, Sun R, Zhang B, Jiang S, Wang J, Du Y, Li S (2009) The genome of the cucumber, *Cucumis sativus* L. Nat Genet 41:1275–1281

Juwattanasomran R, Somta P, Chankaew S, Shimizu T, Wongpornchai S, Kaga A, Srinives P (2011) A SNP in *GmBADH2* gene associates with fragrance in vegetable soybean variety ''Kaori'' and SNAP marker development for the fragrance. Theor Appl Genet 122:533–541

Juwattanasomran R, Somta P, Kaga A, Chankaew S, Shimizu T, Sorajjapinun W, Srinives P (2012) Identification of a new fragrance allele in soybean and development of its functional marker. Mol Breed 29:13–21

Kirch HH, Schlingensiepen S, Kotchoni S, Sunkar R, Bartels D (2005) Detailed expression analysis of selected genes of the aldehyde dehydrogenase (ALDH) gene superfamily in *Arabidopsis thaliana*. Plant Mol Biol 57:315–332

Kopečný D, Tylichová M, Snegaroff J, Popelková H, Šebela M (2011) Carboxylate and aromatic active-site residues are determinants of high-affinity binding of ω-aminoaldehydes to plant aminoaldehyde dehydrogenases. FEBS J278:3130–3139

Kopečný D, Končitíková R, Tylichová M, Vigouroux A, Moskalíková H, Soural M, Šebela M, Moréra S (2013) Plant ALDH10 family: identifying critical residues for substrate specificity and trapping a thiohemiacetal intermediate. J Biol Chem 288:9491–9507

Kosambi DD (1944) The estimation of map distances from recombination values. Ann Eugen 12:172–175

Kovach MJ, Calingacion MN, Fitzgerald SR McCough (2009) The origin and evolution of fragrance in rice (*Oryza sativa* L.). Proc Natl Acad Sci USA 106:14444–14449

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newberg LA (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25:402–408

Lodhi MA, Ye GN, Weeden NF, Reisch BI (1994) A simple and efficient method for DNA extraction from grapevine cultivars and *Vitis* species. Plant Mol Biol Rep 12:6–13

Lorieux M, Petrov M, Huang N, Guiderdoni E, Ghesquiere A (1996) Aroma in rice: genetic analysis of a quantitative trait. Theor Appl Genet 93:1145–1151

Murty DS, Nicodemus KD, House LR (1982) Inheritance of basmati and dimpled seed in sorghum. Crop Sci 22:1080–1082

Niu X, Tang W, Huang W, Ren G, Wang Q, Luo D, Xiao Y, Yang S, Wang F, Lu BL, Gao F, Lu T, Liu Y (2008) RNAi-directed downregulation of *OsBADH2* results in aroma (2-acetyl-1-pyrroline) production in rice (*Oryza sativa* L.). BMC Plant Biol 8:100. doi:10.1186/1471-2229-8-100

Pramnoi P, Somta P, Chankaew S, Juwattanasomran R, Srinives P (2013) A single recessive gene controls fragrance in cucumber (*Cucumis sativus* L.). J Genet 92:147–149

R Development Core Team (2010) A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna. ISBN 3-900051-07-0

Ren Y, Zhang Z, Liu J, Staub JE, Han Y, Cheng Z, Li X, Lu J, Miao H, Kang H, Xie B, Gu X, Wang X, Du Y, Jin W, Huang S (2009) An integrated genetic and cytogenetic map of the cucumber genome. PLoS ONE 4:e5795

Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics methods and protocols: methods in molecular biology. Humana Press, Totowa, pp 365–386

Shao GN, TangA Tang SQ, Luo J, Jiao GA, Wu JL, Hu PS (2011) A new deletion mutation of fragrant gene and the development of three molecular markers for fragrance in rice. Plant Breed 130:172–176

Shi WW, Yang Y, Chen SH, Xu ML (2008) Discovery of a new fragrance allele and the development of functional markers for the breeding of fragrant rice varieties. Mol Breed 22:185–192

Somta P, Musch W, Kongsamai B, Chanprame S, Nakasathien S, Toojinda T, Sorajjapinun W, Seehaluk W, Tragoonrung S, Srinives P (2008) New microsatellite markers isolated from mungbean (*Vigna radiata* (L.) Wilczek). Mol Ecol Resour 8:1155–1157

Sood BG, Siddiq EA (1978) A rapid technique for scent determination in rice. Indian J Genet 38:268–275

Wanchana S (2005) Identification of genes controlling grain aroma and amylose content for positional cloning and marker-assisted selection program in rice (*Oryza sativa* L.). Ph.D. Thesis, Kasetsart University, Thailand

Wang S, Basten CJ, Zeng ZB (2012) Windows QTL Cartographer 2.5. Department of Statistics. North Carolina State University, Raleigh

Wu ML, Chou KL, Wu CR, Chen JK, Huang TC (2009) Characterization and the possible formation mechanism of 2-acetyl-1-pyrroline in aromatic vegetable soybean. J Food Sci 74:192–197

Yundaeng C, Somta P, Tangphatsornruang S, Wongpornchai S, Srinives P (2013) Gene discovery and functional marker development for fragrance in sorghum (*Sorghum bicolor* (L.) Moench). Theor Appl Genet 126:2897–2906