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SHORT COMMUNICATION

A non-synonymous SNP in homolog of *BADH2* gene is associated with fresh pod fragrance in dolichos bean (*Lablab purpureus* var. lignosus (Prain) Kumari)

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Abstract Fresh pods are harvestable and marketable economic product in dolichos bean. Fresh pod fragrance is one of the 'farmers' and 'consumers' preferred traits in dolichos bean varieties. The pods with high fragrance fetch a premium price in the market. In breeding programmes, pod fragrance is routinely assessed by organoleptic (sensory) means, which is highly relative and subjective. Identification of linked DNA markers not only offer an objective means but also enable selection of fragment genotypes at seedling stage itself. Betaine aldehyde dehydrogenase (*BADH*) is known to be the key gene responsible for fragrance in other legumes such as vegetable soybean and mung bean. Taking cues from highly conserved domains in proteins coded by *BADH* genes, we isolated dolichos bean homolog (*LpBADH2*) of soybean *GmBADH2* gene using reported degenerate primers designed to conserved domains. Analysis of the translated amino acid sequence of *LpBADH2* showed high degree of similarity (97.30%) with those of soybean homolog (*GmBADH2*). Conserved amino acid sequence of aldehyde dehydrogenase-super family were also identified in *LpBADH2*. Multiple sequence alignment of nucleotide sequences of *LpBADH2* with those of related legumes using "ClustalW" revealed the presence of a single non-synonymous single nucleotide polymorphic (SNPs) and three synonymous SNP sites in *LpBADH2*. The substitution of the amino acid tyrosine in (fragrant genotypes) with phenyl alanine (non-fragrant genotypes) in protein coded by *LpBADH2* appeared to be the cause for switch over from fragrance to non-fragrance in dolichos bean. These results are discussed in relation to strategies to breed dolichos bean cultivars with desired level of pod fragrance.

Keywords *BADH2* · Fragrance · Functional marker · Homologs · Non-synonymous SNP

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Introduction

Dolichos bean var. lignosus is one of the important and ancient food legumes in India (Ramesh and Byregowda 2016). It is commonly known as 'hyacinth bean', 'field bean', 'Indian bean', etc. It is a self-pollinated crop with 2n = 22 chromosomes (Goldblatt 1981) and genome size of 367 Mbp (Iwata et al. 2013). It is believed that dolichos bean is originated in India (Nene 2006). It is predominantly grown as a rainfed crop for fresh beans for use as a vegetable in districts of southern Karnataka state and adjoining districts of neighbouring Indian states such as Andhra pradesh and TamilNadu. Fresh dolichos beans are one of the most important sources of protein (22 to 28%) to a large number of people, especially

those who depend on vegetarian diets (Ramesh and Byregowda 2016). Recent research finding indicate that dolichos bean extracts impede infections of viral diseases such as influenza and SARS-CoV-2 which have been declared as world pandemics by world health organization (Liu et al. 2020). It contributes to food security and better nutrition and increased income to rural poor (Ramesh and Byregowda 2016). Though fresh beans are the consumable products fresh pods are the harvestable and marketable economic products. A few genotypes produce fresh pods which emit characteristic fragrance, attributed to volatile exudates. The pod exudates are known to contain predominantly two types of unsaturated fatty acids, namely trans-2-dodecenoic acid and Trans-2-Tetradecenoic acid (Fernandes and Nagendrappa 1979). The varieties with high fresh pod fragrance are highly preferred by farmers and consumers. Hence, development of varieties with high fresh pod yield and high pod fragrance is one of the major objectives of dolichos bean breeding (Udaykumar et al. 2016). Phenotyping pod fragrance is expensive, time consuming and inaccurate. Emittance of pod fragrance is also environment sensitive. Hence, conventional phenotype-based selection of genotypes with desired level of pod fragrance in breeding populations is ambiguous and rather difficult (Udaykumar et al. 2016; Ramesh et al. 2018). This necessitates the use of environment neutral, easily assayable and highly heritable surrogates such as DNA markers linked to loci controlling fresh pod fragrance. However, linkage disequilibrium (LD)-based DNA markers are germplasm dependent and very often they are not reliable due to linkage drag associated with them (Addison et al. 2020). Gene-based markers are considered as more effective than LD-based markers. Identification and characterization of candidate genes controlling pod fragrance is a step-towards this. However, non-availability of genomic sequence information in dolichos bean necessitates the use of comparative genomics to explore candidate genes using genome sequence information from model crops.

Aroma as a consumer preferred trait has been reported in several crops such as rice (Vanavichit et al. 2010; Veerabhadraswamy et al. 2022), vegetable soybean (Arikit et al. 2011; Qian et al. 2022), sorghum (Yundaeng et al. 2013; Monkhan et al. 2021; Zhang et al. 2022), cucumber (Yundaeng et al. 2015), winter melon (Ruangnam et al. 2017), mung bean (Attar et al. 2017), coconut (Dumhai et al. 2019) and sponge gourd (Saensuk et al. 2022). The volatile compound, 2-Acetyl-1-Pyrroline (2-AP) is generally believed to be responsible for aroma in these crops (Wakte et al. 2017; Qian et al. 2022). The gene, betaine aldehyde dehydrogenase (*BADH2*) is reported to play a major role in biosynthesis of 2-AP in these crops. The major source of 2-AP is 4-amino butyraldehyde (*GABald*), catalyzed by betaine aldehyde dehydrogenase (*BADH*) (Zhang et al. 2022). *BADH2* was first identified in rice (*OsBADH2*) (Bradbury et al. 2005). Subsequently, *GmBADH1* and *GmBADH2* genes homologous to *OsBADH2,* which are responsible for aroma in vegetable soybean were identified, cloned and characterized (Juwattanasomran et al. 2010). Based on a very high amino-acid sequence similarity of *OsBADH2* with *GmBADH1* and *GmBADH2* genes, we hypothesize that homologs of these genes also control fresh pod fragrance in dolichos bean and it is possible to isolate and characterize them. This hypothesis is based on the rationale that high degree of similarity of nucleotide and amino acids sequences among *OsBADH2*, *GmBADH1* and *GmBADH2* genes provide opportunity for PCR amplification of homologs of these genes in dolichos bean. To test our hypothesis, the present study was carried out to isolate the candidates of genes controlling fresh pod fragrance in dolichos bean and characterize them using bioinformatic tools.

Material and methods

Material consisted three non-fragrant (HA 3, Arka Vijay, Namdari) and three fragrant (HA 4, HA 5, HA 10–8) genotypes (Table 1).

Genomic DNA isolation and quantification

Leaf sample from the 35 days old seedling of the six genotypes collected and DNA was isolated by Cetyl-tri-methyl ammonium bromide (CTAB) method (Doyle and Doyle 1990) with minor modifications. The integrity of isolated purified DNA was determined by gel electrophoresis and DNA was quantified

Table 1 Dolichos bean genotypes used to isolate and characterize candidate genes controlling fresh pod fragrance

Genotype	Pod fragrnce	Responses to photoperiod	Pedigree	References
HA-4	Fragrant	Insensitive	HA 3 × Magadi local	Mahadevu and Byregowda, (2005)
HA-5	Fragrant	Insensitive	HA 4 × GL 153	Ramesh et al. (2018)
HA-10–8	Fragrant	Insensitive	HA 4 × GL 153	Shivkumar et al. (2016)
HA-3	Non-Fragrant	Insensitive	HA 1 × EUS 67–31	Shivashankar et al.(1975)
Arka Vijay	Non-Fragrant	Insensitive	Unknown	–
Namdari	Non-Fragrant	Insensitive	Unknown	–

using Nanodrop (Thermo scientific) and diluted accordingly to final concentration.

Isolation of dolichos bean homologs of *OsBADH2, GmBADH1* and *GmBADH2*

A total of 11 degenerate primers (Table 2) designed to conserved domains of *GmBADH1, GmBADH2* and *OsBADH2* genes are used to isolate the sequences of these genes in dolichos bean. 25 µL PCR mixture was prepared and tubes were spinned briefly and inserted into the wells of the thermal cycler.

Table 2 List of degenerate primers used to PCR-amplify priming regions corresponding to vegetable soybean (GmBADH2) and rice (OsBADH2) candidate genes controlling fragrance

Sl. no.	Primer sequence	Gene	References	Expected product size (bp)
1	F:GGAAGAAGGTTGCAGACCAGG R:AAAGCA TACCTGCCCTTTACTTTAGAA	*GmBADH2*	Juwattanasomran et al. (2010)	250–300
2	F:TGGAAGAAGGTTGCAGACAAGG R:AAA GCATACCTGCCCTTTACTTTAGAA			250–300
3	F: CCTTGGAAGAAGGTTGCAGACTAAG			
	R: AAAGCATACCTGCCCTTTACTTTAGAA			250–300
4	F:TGGAAGAAGGTTGCAGACGAGA			
	R: AAAGCATACCTGCCCTTTACTTTAGAA			250–300
5	F: TGGAAGAAGGTTGCAGACCAGA			
	R: AAAGCATACCTGCCCTTTACTTTAGAA			250–300
6	F: TCCCGCCTTATTGTACATGC			
	R: TTTTGACCCATTTCACAATCC			250–300
7	F: CTCTTCCCATGGACACATTC			
	R: AAACATGTCACAGATGCCAA			250–300
8	F:TGCCAAGTATCCCCTGATTCC			
	R: TTTGTGCCTCCTTTGCAGATTC			250–300
9	F:GATCTCACTCCAAGTAAACTCTGAC			
	R: ACTGCCATTGCTTCTGTTCTC			250–300
10	F:GGTTCATTCAAGCCTCCAGC			
	R; TTTTCCCACCAGCCAAACAT	*OsBADH2*	Cordeiro et al. (2002)	250–300
11	F: AGGTGCCTTAGATCGAACAG			
	R:CAAAATCGTACTTACCTTGC			250–300

Genotyping fragrant and non-fragrant genotypes

The degenerate priming genomic regions of six genotypes were amplified using PCR with Taq DNA polymerase. PCR mixtures contained approximately 1.5 µl of DNA (50 ng per µl), 0.2 µl Taq polymerase (5 units per µl), 1.5 µl 10X TE buffer, 1.5 µl DNTPs (2 mM) and 0.5 µl each of forward and reverse primer in a total of 15 µl solution. The PCR cycle consisted of 5 min at 95 °C (hot start), 0.30 min at 95 °C (denaturation), 1 min at respective annealing temperature of primer, 1 min at 72 °C (extension), 8 min at 72 °C (final extension) followed by infinite time at 4 °C for holding. The denaturation, annealing and extension step were carried out for 40 cycles.

Separation, visualization and sequencing of amplified products

The PCR products was loaded on 3% agarose gel in 1X TBE buffer stained with ethidium bromide and bromophenol blue as loading dye. Amplicons were separated in an electrophoresis unit at 80 V for three hours using 1X TBE buffer. PCR amplification was successful in isolating only *GmBADH2* priming regions (hereafter referred as *LpBADH2*). The *LpBADH2* amplicons of fragrant and non-fragrant genotypes were sequenced (both forward and reverse sequencing approach) at Eurofins Genomics India Pvt. Ltd., Bengaluru using Sanger sequencing method.

Bioinformatics analysis

Homology of nucleotide sequences of *LpBADH2*, with those of model species such as soyabean, cowpea and mung bean available at the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) we explored using BLASTn (nucleotide database using a nucleotide query) tool. To detect sequencing errors if any, both forward and reverse sequence of *LpBADH2* amplified from both fragrant and non-fragrant genotypes were aligned using ClustalW2 tool. The non-homologous sequences were trimmed between forward and reverse nucleotide sequences of *LpBADH2* using BioEdit software version 7.2.5. Only homologous nucleotide sequences were used for further bioinformatical/statistical analysis. To characterize and identify presence of functional domain, sequence of amplified products were subjected to conserved domain (cd) search tool (www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The number and type of conserved domain were identified.

To identify corresponding homology of amino acid sequences of *LpBADH2* amplicons, BLASTx (protein database using a translated nucleotide query) analysis was performed against the genome sequences of model species such as soyabean, Medicago, cowpea, and other related legume species such as mungbean, ricebean available at the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/).

Criteria to identify dolichos bean homologs *LpBADH2* of *GmBADH2* gene

The amino acid sequence homology (~97%) of *LpBADH2* with those of soybean *GmBADH2* and of other legumes, and presence of conserved domains of Aldehyde dehydrogenase super family (ALDH-SF) in BADH-encoded protein sequences was considered as a criterion to determine if the *LpBADH2* amplicon is a candidate of *GmBADH2* (Juwattanasomran et al. 2010).

Phylogenetic analysis

To explore phylogenetic relationship to identify closest legume species for candidate genes controlling fresh pod fragrance in dolichos bean, we aligned amino acid sequences of *LpBADH2* with those of model species and other related legume species by Neighbour joining clustering method with a bootstrap value of 10,000 using Phylogeny.fr (https://ngphylogeny.fr/) tool.

Results and discussion

The PCR amplification was successful in isolating only *LpBADH2* amplicons with expected product size (250–300 bp) from all the six genotypes (Fig. 1; Table 3). BLASTx analysis showed high degree of similarity (97.30%) of translated amino acid sequences of *LpBADH2* amplicons with those of soybean (*GmBADH2*) (Table 4). Further, conserved amino acid sequence of ALDH-SF were

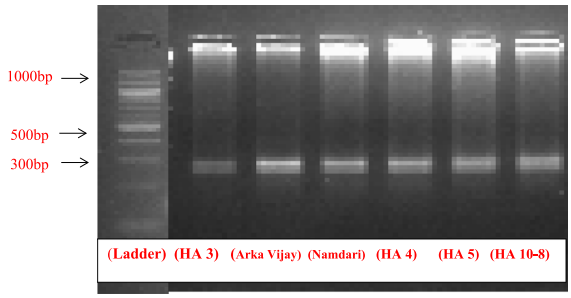

Fig. 1 Agarose gel showing PCR amplification of dolichos bean homolog (*LpBADH2)* of *GmBADH2*

identified in *LpBADH2* amplicons (Fig. 2). Phylogenetic analysis resulted in grouping of *LpBADH2* and *GmBADH2* into a single clade (Fig. 3). These results provide preliminary evidence that *LpBADH2* could be the putative dolichos bean homolog of *GmBADH2*. However, expression analysis of *LpBADH2* in non-fragrant genotypes is essential for confirmatory role of *LpBADH2* in controlling fresh pod fragrance in dolichos bean. Molecular genetic investigations in rice, vegetable soybean, sorghum, cucumber and coconut demonstrated that 2-AP biosynthesis is principally controlled by a single recessive gene (Saensuk et al. 2022). In all these crops, loss of function mutation in *BADH2* leads to the accumulation of *GABald*, which is subsequently converted into 2-AP (Qian et al. 2022).

Non-synonymous SNP between fragrant and non-fragrant genotypes

An examination of nucleotide sequences of *LpBADH2* revealed differentiation of fragrant and non-fragrant genotypes at four nucleotide base pairs. Bioinformatic analysis of translated amino acid sequences of *LpBADH2* suggested that one of these four polymorphic nucleotide base pair sites contributed to substitution of the amino acid tyrosine with phenylalanine. Thus, a non-synonymous single nucleotide polymorphic (SNP) site is responsible for fragrance in dolichos bean (Table 5). Juwattanasomran et al (2011) in vegetable soybean and Saensuk et al. (2022) in sponge gourd have also reported the role of non-synonymous SNP (A > G) in *GmBADH2* and *LcBADH2*, respectively for enhanced biosynthesis of 2-AP, resulting in increased aroma. Non-synonymous SNPs at the positions between 223 (corresponding to exon 2) and 1156 (corresponding to exon 12) in *SbBADH1*, (a homolog of *BADH2*) is reported to be cause for fragrance in sorghum (Monkhan et al. 2021). Substitution of amino acid glycine with aspartic acid at 334th position of *GmBADH2*-encoded protein is responsible for enhanced aroma in vegetable

Table 3 Polymorphisms in dolichos bean homolog (*LpBADH2*) of soybean candidate gene (*GmBADH2*) controlling fresh pod fragrance

Sl. no.	Gene to which primers are designed	Expected amplicon size	Amplification obtained in						Sequence polymorphism
			HA 4	HA 5	HA 10–8	HA 4	Arka Vijay	Namdari	
01	*BADH2*	250–300	Yes	Yes	Yes	Yes	Yes	Yes	SNP

Table 4 Amino acid sequence similarity of dolichos bean homologue (*LpBADH2*) with those of other legumes

Dolichos bean homologue	Nucleotide sequence similararity to those of other legumes		E value	Maximum identity
LpBADH2	ADN03180.1	*Glycine max*	1e-30	97.18
	RZC10783.1	*Glycine soja*	6e-30	97.18
	QHO43618.1	*Arachis hypogaea*	7e-27	83.10
	KOM24838.1	*Vigna angularis*	2e-26	90.14
	XP_027921078.1	*Vigna unguiculata*	3e-26	90.14
	KEH35085.1	*Medicago truncatula*	3e-26	91.55
	XP_020203480.1	*Cajanus cajan*	3e-26	91.55

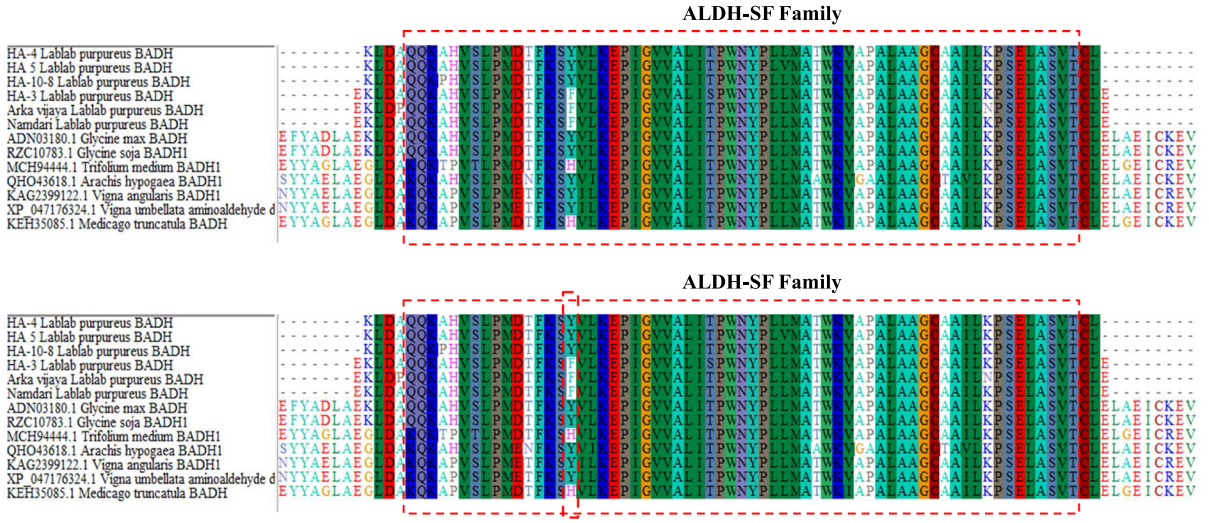

Fig. 2 Multiple alignment of the predicted coding domain amino acid sequences of dolichos bean homolog *LpBADH2* corresponding to homologs those of vegetable soybean, cowpea, rice bean and *Medicago*. Highly conserved amino acids are in dark color depending on the level of identity (darker = higher level)

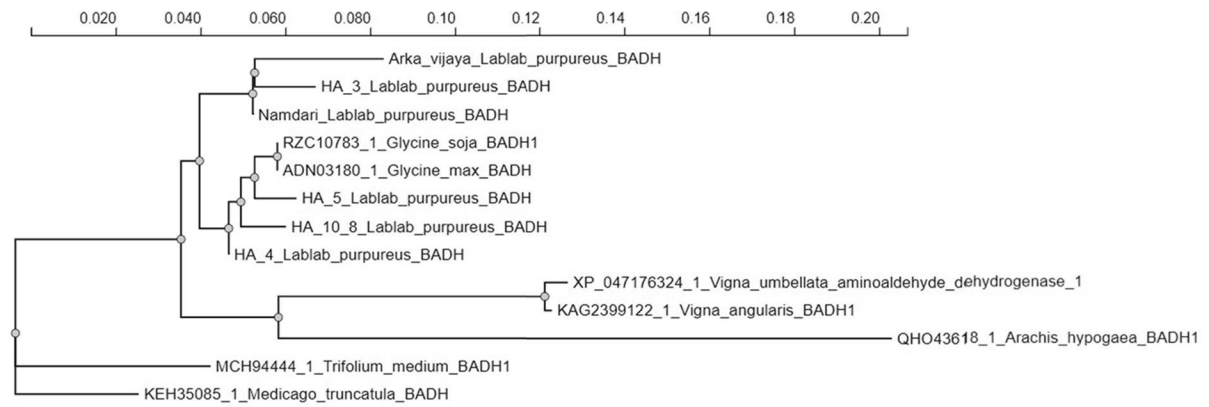

Fig. 3 Phylogenetic tree depicting clustering of dolichos bean homolog (*LpBADH2)* with those of different model crops and legume crops

Table 5 Nucleotide changes in *LpBADH2* between fragrant and non-fragrant dolichos bean genotypes and their amino acid substitution

Sl. no.	Dolichos bean homolog of *BADH2*	Nucleotide in fragrant genotypes	Nucleotide in non-fragrant genotypes	Amino acid substitution
01	*LpBADH2*	A	T	Tyrosine to Phenyalanine
02		G	T	Synonymous mutations
03		C	G	
04		C	T	

soybean (Juwattanasomran et al. 2011). Substitution of methionine by valine 9 at the position 75 in one genotype and that of isoleucine by valine at position 386 in a different genotype in *SbBADH2*-coded protein has been reported as the cause for fragrance in sorghum (Monkhan et al. 2021). Similarly, substitution of amino acid tyrosine to cysteine at position 163 of *LcBADH2* is responsible for aroma in sponge gourd (Saensuk et al. 2022). Addison et al (2020) identified perfect association of one haplotype (Hap6) with aromatic phenotype in rice. Thus, our reports and those reported by others indicated that substitution of different amino acids in *BADH2*-coded proteins cause fragrance in different crops, though economic product which exhibit fragrance differ with the crop.

Breeding implications

As is practiced in vegetable soybean (Wu et al. 2009), fresh pod fragrance in dolichos bean is routinely assessed through organoleptic (sensory) means using a panel of analysts. Phenotyping pod fragrance by sensory means is highly relative and subjective. Also, sensory methods can only qualitatively characterize fragrance a 'presence' or 'absence' (Udaykumar et al. 2016). Further, analyst's sensitivity to sensory reduces if there exist subtle differences among the test genotypes. Efficiency of breeding dolichos bean varieties with high pod fragrance hinges on high throughput large scale relatively less expensive objective method of phenotyping fragrance in breeding populations. Candidate gene (*LpBADH2)* identified in the present study serve as potential genomic resources for developing diagnostic markers to identify genotypes with high pod fragrance in the seedling stage itself after validation. Gene-based (functional) markers will facilitate rapid screening for fragrance controlling genes and linkage-drag-free introgression of genes controlling pod fragrance from donor parents to recipient genotypes through marker assisted selection (Qian et al. 2022). In several crops such as vegetable soybean (Arikit et al. 2011; Juwattanasomran et al. 2011; Qian et al. 2022), sorghum (Yundaeng et al. 2013; Zanan et al. 2016), cucumber (Yundaeng et al. 2015), winter melon (Ruangnam et al. 2017), coconut (Dumhai et al. 2019), rice (Addison et al. 2020; Li et al. 2020) and sponge gourd (Saensuk et al. 2022), functional markers for aroma controlling candidate genes have been developed for use in breeding programme.

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Declarations

Conflict of interest Author of this manuscript declare that we have no confict of interest.

Consent for publication All the authors have provided the consent for publication of this manuscript.

Ethical approval This manuscript does not contain studies performed by any of the authors involving human or animals.

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