Journal of Medicinal Plants Studies

www.PlantsJournal.com

ISSN 2320-3862
JMPS 2014; 2(4): 1-4

Received: 03-06-2014
Accepted: 11-06-2014

Vimarsha H. S.
Business Development Executive, Liveon Biolabs.

Sowjanya M.S
Junior Research Fellow, Biotech Dept. UASB, GKVK

Rajmohan K
Faculty, College of Agriculture, Vellayani. Trivandrum, Kerala.

Soni K. B.
Faculty, College of Agriculture, Vellayani. Trivandrum, Kerala.

Swapna Alex
Faculty, College of Agriculture, Vellayani. Trivandrum, Kerala.

Correspondence:
Vimarsha H. S.
Business Development Executive, Liveon Biolabs.

Attfl-1 gene homolog in spike branching black pepper type (*Piper nigrum* L.) and local variety karuimunda

Vimarsha H. S., Sowjanya M.S, Rajmohan K., Soni K. B., Swapna Alex

ABSTRACT
Spike branching in black pepper is very rare phenomenon. Profuse spike branching is observed in an uncharacterized black pepper type from idukki district of Kerala. Single spike had three to four times more berry yield, compared to improved varieties such as Panniyur-1. This type needed to be characterized at morphological and molecular levels to know its parental descendence and possible involvement of gene regulation responsible for spike branching. As an initial approach, molecular analysis was done to find out the presence of *tfl-1* homolog which has been reported to be involved in inflorescence branching in modal plants.

Keywords: Spike branching, Black pepper, flowering, Inflorescence architecture, *tfl-1*, Floral meristem, Berry yield, Karimunda. Peppertekkan, Inflorescence branching.

1. Introduction

The genus Piper has records of the highest diversity, compared to the other genera of Piperaceae family. The International Plant Name Index (www.ipni.org) has recorded 6704 Piper species [1]. Species diversity and varietal diversity are considerable in black pepper [2]. It is believed that ecosystem diversity does not contribute much to its biodiversity [3, 4]. India is considered as the primary center of diversity for black pepper, which is indigenous to the rain forests of south-western states [5]. Considerable variation exists regarding its morphology, yield and quantitative traits. With respect to the spike characteristics, variability has been observed in spike length, floral composition, floral arrangement, fruit number and size.

In black pepper, flowers are borne in the axils of ovate, fleshy bracts in long pendant spikes, which are single in nature and appear opposite to the leaves on the plagiotropic branches. The sessile, white, small flowers on a spike may vary in number from 25 to 100, arranged in 4 to 5 rows [6]. The apical buds of the plagiotropic branches transform into inflorescence. The apical meristem of the inflorescence grows in length before any organs are formed

Normally, black pepper species have unbranched spikes. Spike branching is very rare. The varieties 'Aimpiriyan' and 'Kathirinmelkkathir' show a tendency for spike branching. However, the branches are rudimentary and bear only very few berries. Contrary to this, recently, a black pepper type showing profuse spike branching has been reported from a farmer's field in Idukki district [4]. The proliferating spikes are of indeterminate growth with pronounced bracts and bear up to 30 branches with about 300 berries altogether. This is about four times the reported yield of berries from spikes of the highest yielding varieties, Panniyur-1, Panniyur-3 and Panniyur-5.

Spike branching in black pepper is of great economic significance as it is a quantitative trait. Identification and characterization of genes involved in spike branching is important if they are to be transferred. Traditional crop improvement programs can be adopted for transferring the trait to superior varieties.

Inflorescence branching has been observed in several plant species [7]. The involvement of specific genes have been reported. In Arabidopsis, genes *tfl-1, lfy, cal, ful, agl24, ap1, ft, ag* and *ufo* have been reported to be involved in determining inflorescence architecture [8], of which, the genes *tfl-1* and ft have been delineated to have prominent roles.

In this context, the present study was taken up as an initial step towards identifying and characterizing the genes involved in spike branching in black pepper, which is a prerequisite for biotechnological interventions in crop improvement. Specific objective of the study was to examine the presence of *tfl-1* homolog in seven non-spike branching cultivars and spike

branching type collected from same farm for analysing marker-linked trait and genetic variability associated with *tfl-1* allele.

2 Materials and Methods

2.1 Sample Collection

Plant samples of black pepper showing spike branching (Pepper Tekkan – as named by the farmer) were collected from an innovative farmer, Thomas.T.T. of Kanjiar village, near Kakkatikada, Kattappanataluk, Idukki district (Figure 2). Plant samples of seven varieties and cultivars from the same farm viz., Karimunda, KumbakalKodi, Arayan Mundi, Vella Mundi, NarayaKodi, Panniyur 2 and Panniyur 4 were also collected. Semi mature leaves were collected and transported to laboratory in ice boxes.

2.2 DNA Extraction

Modified C-TAB method [9] developed by Kalisz Lab (http://www.pitt.edu/Kaliszlab/Protocols/DNAExtraction.doc) was used for genomic DNA isolation of all eight black pepper samples. For five milliliter of extraction buffer (2% w/v C-TAB; 3 M NaCl; 100 mMTris-HCl of pH 8, 0.5M EDTA) 200 mg (4% w/v) of Poly Vinyl Pyrrolidone (PVP) was added prior to DNA extraction. The PVP was dissolved completely by warming the buffer, using a water bath. Thereafter, 25 µl (0.5% v/v) of β-mercaptoethanol was added to the extraction buffer, mixed well and kept warm in water bath.

Next, the deep frozen leaves were washed and dried using sterile blotting papers. One gram of leaf sample was chopped and transferred to a dry, sterile mortar. Chopped material was ground well to a fine powder using liquid nitrogen. Warm extraction buffer was added immediately and the mixture was homogenized. The extract was then subjected to incubation at 55 to 60 °C for sixty minutes with intermittent shaking. The tubes were then brought back to room temperature. The mixture was centrifuged at 10,000 rpm for 8 minutes at 4 °C and the supernatant was collected. For each 500µl of supernatant, 250 µl of phenol: chloroform: isoamyl alcohol (25:24:1) was added and slowly mixed by gentle inversions for 2-3 minutes and centrifuged at 7000 rpm for 7 minutes. Then, the supernatant was extracted twice with equal volume of chloroform: isoamyl alcohol (24:1) and centrifuged at 7000 rpm.

The DNA from the supernatant was then precipitated with chilled 100 per cent ethanol and stored overnight at -200C. The precipitate was then centrifuged at 14,000 rpm and washed twice with 70 per cent ethanol and the pellet was air dried for 15 minutes. Thereafter, the pellet was slowly dissolved in 60µl of TE buffer (10 mMTris-HCl (pH 8.0), 1mM EDTA) and stored at -200C (Rotek deep freezer). Spectrophotometric analysis of the extracted DNA samples was made for determining quality and quantity of DNA.

2.3 Primer Designing

i) Primers *tfl-1*-F1 & *tfl-1*-R1

Forward and reverse primers were designed using Primer 3.0 software for the fourth exonic sequence which is the longest and conserved. 1172 bp DNA sequence (c1024640-1025811) of the gene *tfl-1* of locus (NC_003076.4) from chromosome 5 of the model plant Arabidopsis thaliana was used for the purpose. Factors were set to achieve the priming position in fourth exonic sequence (833 bp -1172 bp).

ii) Primers *tfl-1*-F2 &*tfl-1*-R2

First primer pair was specifically made on exonic sequence without consideration of intronic gaps. However, Primers (*tfl-1*-F2 &*tfl-1*-R2) were designed using the conserved sequences obtained using the multiple sequence alignment program (Clustal W) for the homologous sequences for the gene *tfl-1*. GI: 30680238 (Arabidopsis thaliana), GI: 187761640 (*Malus domestica*), GI: 149250805 (*Piceaabies*), GI: 82791228 (*Aquilegia Formosa*), GI: 83628279 (*Populustrichocarpa*) sequences were used to find conserved sequence. Position of left primer was fixed and reverse primer was designed using Primer 3.0 tool. (Table 1)

Table 1: List of primers made for *Attfl-1* gene.

Sl. No	Gene identity	Primer notation	Tm (°C)	GC %	Primer Length (nt)	Sequence of Primer
1	*tfl-1* Arabidopsis. Exonic region 4	*tfl-1*-F1	54.0	55.5	18	5'CAGGCAGAAGCAAACG3'
		tfl-1-R1	56.2	55.5	18	5'ACTAGCGTTTGCGTGCAG3'
2	Multiple Sequence Alignment (gi: 30680238,187761640, 149250805,82791228, 83628279)	*tfl-1-F2*	50.5	45.0	20	5'CACTTTGGTGATGATAGACC3'
		tfl-1-R2	47.8	40.0	20	5'GATCTCTCGAAGGGATATTA3'

2.4 PCR using the designed primers

The Genomic DNA of black pepper was amplified using specific primers designed for the gene *tfl-1*. A 20µl of reaction mixture was prepared in 0.2 ml flat capped eppendorf PCR tubes with the reaction mixture constituents as mentioned below. PCR was done using Eppendorf Mastercycler and PTC-100 thermo cyclers. The reaction mixture is depicted in Table 2.

Thermo cycler program was set for 44 PCR cycles with initial denaturation at 94 °C for 4 minutes followed by repeated cycles of denaturation at 94 °C for 1minute, annealing at 51 °C for 1 minute and extension at 72 °C for 2 minutes. Final extension was done for 4 minutes at 72 °C. Varied annealing temperatures were screened. Control was set without DNA to distinguish the target product from non-target products and primer dimers.

Table 2: Reaction mixture ingredients for PCR

Component	Volume (µ1)
Genomic DNA sample (20 ng/µl)	2.0
10X assay buffer A	3.0
Taq DNA Polymerase (3U/µ1)	0.5
dNTP mix (10mM)	2.0
10 pM Reverse Primer	1.0
Sterile distilled water	10.5
Total Volume	20.5

3. Results and Discussion

3.1 General morphology of plant

The height of plant varied from 3 to 5 meters. Plagiotropic branches were protruding out prominently from main shoot axis. Shoot tip colour was light green. Lateral branches were semi erect and the average branch length was more than 30 cm, consisting of 7 to 12 nodes per lateral branch.

3.2 Leaf characters

Leaf length was medium ranging from 11 to 15 cm and average leaf width was 7 cm. Leaf petiole length varied from 1 to 2.5 cm. Leaf lamina was ovate elliptic in shape with the widest axis at the midpoint of the leaf. Leaf base shape was acute, with margin straight to convex, forming a terminal angle of 40 to 50 degrees. Leaf margin was smooth without any indentations or incisions on margins.

3.3 Spike characters

Young spikes were greenish yellow in colour and did not show any branching, but almost all matured spikes were profusely branching. Branching spikes had peculiar protruding bracts, compared to normal ones (Figure 3). Peduncle length was 1.3 to 2.1 cm. Wide variation in spike length was also observed, ranging from 9.4 cm to 18.6 cm. Although main spikes were branching irregularly, they showed complete indeterminate growth status. Seed setting was also irregular and loose on branched spikes and number of berries per spike varied from 60 to 240. More than 4 spikes per lateral branches were observed. Number of spike per plant varied from 87 to 162. Berry shape was round and bold.

Fig 2: Farmer T. T. Thomas with Spike branching black pepper Type

Fig 3: Prominent bracts in spike branching black pepper type

3.4 Anatomical studies of the spike

The spikes with pronounced bracts were subjected to anatomical studies. The cross section of the branching spike showed the formation of inflorescence primordia at the base of floral meristem (Figure 4). Longitudinal sections of young spikes observed under 40x magnification (Nikon SMZ 10A) showed the variation at the floral junction.

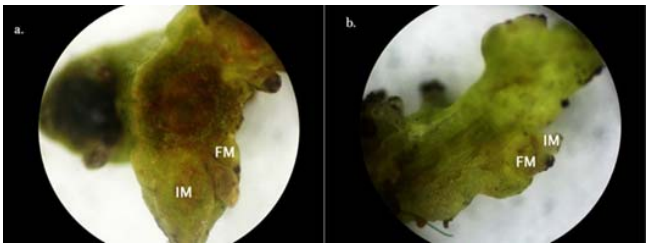

Fig 4: The variation in inflorescence primordial at floral junction.

3.5 PCR analysis with *tfl-1* gene specific primers

Polymerase Chain Reaction was carried out using the primers designed for the genes *tfl-1*. Genomic DNA of spike branching pepper type and seven non-spike branching cultivars were used as template DNA. PCR carried out using the primers *tfl-1*-F1 and *tfl-1*-R1 yielded a specific amplicon equivalent to 700 bp DNA ladder. The amplified DNA fragment was separated on 1.4 per cent agarose gel. The amplification was prominent at 51.5 °C and the product was reproducible in Karimunda, Spike branching pepper type, and Vellamundi cultivar/varieties (Figure 5). However, *tfl-1*-F2 and *tfl-1*-R2 primers designed from the conserved regions obtained from multiple sequence alignment of various species did not amplify any genomic regions.

Presence of sequence homology for the gene *tfl-1* (*tfl-1* F1 & R1) indicated the possible involvement of *tfl-1* gene, which had been reported to be associated with inflorescence branching in Arabidopsis thaliana [10] in the spike branching trait of the black pepper type. This result is significant, as five out of the eight cultivars tested did not give any positive response for the primer pair designed based on *tfl-1* gene. However, two non-spike branching varieties including Karimunda and Vellamundi have also shown amplification for the *tfl-1* primer pair.

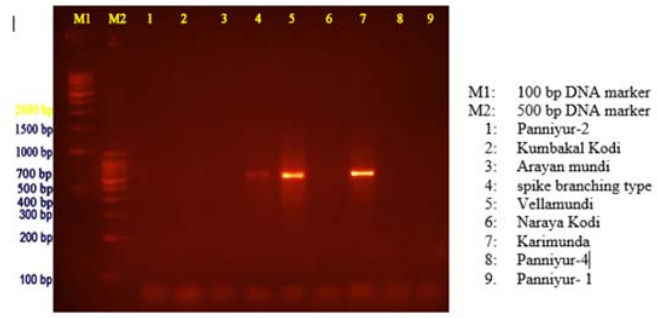

Fig 5: DNA amplification profile for primer pair *tfl-1* F1 & R1.

Amplifications for *tfl-1* specific primers in the three cultivars need to be analysed in detail for verification of the presence of full length gene. Presence of the sequence similarity for *tfl-1* specific primers in non-spike branching cultivars is interesting. This necessitates detailed analysis for the presence of full length gene, as well as investigations on gene expression. *tfl-1* is a well-known regulator of gene expression. It belongs to the MADS box transcription regulator [11]. The expression level of the gene rather than its presence, may be critical for the spike branching trait.

It would be worthwhile to analyze for the presence and expression of *tfl-1* gene in the cultivars Aimpiriyan and Kathirinmelkathir, showing rudimentary spike branching. Such an analysis as well as sequencing of the amplicons might help to confirm that the amplification was related to spike branching trait. Such studies can improve and aid the genomic information on least studied black pepper species.

4. Acknowledgment

We like to acknowledge research funding provided for the current work. Heartfelt thanks to department of plant biotechnology, COA, Vellayani and Kerala Agricultural University for the grants and support provided. We also like to thank scientist of IISR, Calicut. We like to acknowledge Mr. Thomas T. T. Ummachan [12] of Kanjiyar village, katappana. Idukki. Kerala for providing us the new spike branching variant

5. References

1. Jaramillo MA, Manos PS. Phylogeny and patterns of floral diversity in the genus Piper (Piperaceae). American J botany 2001; 88(4):706-716.
2. Hooker JD. The Flora of British India, Todays and Tomorrows. Printers and Publishers, New Delhi, 1886, 331-333.
3. Ibrahim KK, Pillai VS, Sasikumar S. Genotypic and phenotypic correlations among yield and its components in black pepper (*Piper nigrum* L.). Agric Res J Kerala 1985; 23:150-154.
4. Sasikumar B, Saji KV, George JK. Spike proliferation in black pepper (Piper nigrum L.) A new variation. Journal of the Pepper Industry 2006; 3(1):11-13.
5. Anandaraj M, Devasahayam S, Iswar BA, Zachariah TJ, Sannthosh J, Eapan B *et al*., Black pepper (Exiension Pamplet). Indian institute of Spices Research. Calicut, 2005, 20.
6. Parthasarathy VA, Sasikumar B, Nair RR, George JK. Black pepper-Botany and Horticulture. Hort. Review 2007; 33:55,
7. Brown PJ, Klein PE, Bortiri E, Acharya CB, Rooney WL, Kresovich S. Inheritance of inflorescence architecture in Sorghum. Theor Appl Genet 2006; 113:931-942.
8. Espinosa-Soto C, Padilla-Longoria P, Alvarez-Buylla ER. A gene regulatory network model for cell-fate determination during Arabidopsis thaliana flower development that is robust and recovers experimental gene expression profiles. The Plant Cell 2004; 16:2923–2939.
9. Doyle JJ, Doyle JL. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 1987; 19:11-15
10. Bradley D, Ratcliffe OJ, Vincint C, Carpenter R, Coen ES. Inflorescence commitment and architecture in Arabidopsis. Science 1997; 275:80-83
11. De-Folter S, Immink RGH, Kieffer M, Parenicova L, Henz SR, Weigel D *et al*., Comprehensive interaction map of the Arabidopsis MADS box transcription factors. Plant Cell 2005; 17:1424-1433.
12. Thomas TT, ICAR Innovation Awardee, and the founder of Spike Branching Black pepper variant. http://www.icar.org.in/en/node/2285.

.