

Molecular screening of Indian garlic genotypes (*Allium sativum* L.) for bolting using DNA based *Bltm* markers

Ashwini Prashant Benke*, Abhilash Nair, Ram Krishna, Anandhan S., Vijay Mahajan and Major Singh

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Abstract

In the present study, we screened 32 Indian garlic (*Allium sativum*) germplasm to identify bolting genotype using three mitochondrial DNA based molecular markers, P1, P2, and P3. In two different combinations P1+P2 and P1+P3. 1.4 kb amplicon was obtained with P1+P2 bolt marker in all the 32 screened accessions which showed the inherent ability of bolt with accessions and further needs to impose to intense winter. Further, the consistency of result was confirmed with P1+P3 mitochondrial DNA based marker which produces the amplicon of 3.7kb size, on the basis of non-synchronization in molecular profiling, the whole population has been classified in two groups, i.e. bolting and not bolting based on presence or absence of both or one of them. Group-1 includes 22 (656, 662, 663, 664, 665, 667, 668, 669, 675, GG-1, GG-2, G-41, G-355, AC-50, AC-183, AC-316, SG-1, CG-1, Phule Baswant, Bhima Purple, Bhima Omkar, and AKG-2) accessions having the amplicons of both P1+P2 and P1+P3 markers 1.4 and 3.7 kb respectively which indicates chimeric gene arrangement. Group-2 consists of 10 accessions showing 1.4 kb amplicon with P1+P2 marker, including bolting genotypes (one complete, G-5 and nine, G-3, 654, 671, Ranibennur Local, Gadag Local, GG-4, G-282, AC-378, Godavari) hence it can be concluded that Indian garlic genotypes do not need chimeric gene arrangement for bolting. This is the first step towards the identification of short day bolted garlic in India.

Key words: Garlic, *Allium sativum*, molecular marker, screening, bolting.

Introduction

Garlic (*Allium sativum* L.) is the second most important crop after onion belongs to the family Amaryllidaceae. Garlic is a perennial crop cultivated from ancient times,

contains high nutritional, nutraceutical and medicinal value. Garlic foodstuffs are widespread around the world because of its antibacterial, antifungal, anti-cardiovascular, antidiabetic and anticancer properties (Liu et al. 2007; Li et al. 2016; Ried, 2016; Varshney and Budoff, 2016; Fan et al. 2017). Being sexually sterile, garlic follows clonal propagation worldwide (Shemesh et al. 2015). Presently, plant tissue culture techniques are being used for garlic improvements like directed mutagenesis, genetic engineering and polyploidy breeding (Park et al. 2002; Fan et al. 2017). The tissue culture techniques are the most effective virus eradicating and rapid propagating tool for garlic but it has limitations and requires a wide range of improvement. The clove carries the extreme load of viruses and seed born diseases which not only reduces its yield potential but also deteriorates the quality of seed material (Taþkýn et al. 2013; Gimenez et al. 2016). Genetic improvement through breeding techniques also could not applicable due to non-flowering ability. Due to the sexual sterility in garlic, possibilities of its improvement are very less for higher yields, better quality, and resistance to pests and disease. Therefore, the induction of flowering in garlic becomes a prerequisite to overcoming the above-cited limitations. Further restoration of the sexual reproduction in garlic is anticipated to exchange different inheritable traits via classical breeding. Additionally, garlic propagation via seed will remarkably change the scenario of the garlic seed industry. This seed multiplication will reduce the cost of virus elimination, damage caused by propagules transmitted diseases and pest, cost of storage and loss of propagules during storage. Therefore many workers attempted to restore fertility in garlic (Konvicka, 1984; Etoh et al. 1988) and suggested the presence of vegetative top sets is the major cause of sterility. Among all the varying attributes of garlic, the bolting habit has been the primary characteristic used to classify the garlic clones. On the basis of bolting ability garlic clones are divided into three classes, Complete bolting, incomplete bolting, and non-bolting. In complete bolting, plants many

ICAR-Directorate of Onion and Garlic Research,
Rajgurunagar-410505, Pune, Maharashtra

*Corresponding author; Emails: ashwini.ashwini@gmail.com,
ashwini.benke@icar.gov.in

flowers and top sets produced on a long thick flower stalk, while incomplete bolting includes plants producing a short thin flower stalk having a few large topsets, and mostly flowers formation are unusual, and non-bolting plants normally do not form flower stalk and produce cloves within incomplete scape. The degeneration of tapetum leads this sterility (Novak 1972); which is induced by disease pathogens like mycoplasma, rickettsia, viruses (Konvicka 1973); deletions of chromosomal regions (Ethoh 1985); competition between floral and vegetative buds (topsets) for nutrients during inflorescence development (Koul and Gohil 1970). However, fertility restoration in bolting garlic was achieved by the constant elimination of developing topsets (Cheng 1982; Etoh 1983; Pooler and Simon 1994; Jenderek 1998). Therefore bolted garlic will be the primary requirement to stimulate flowering in garlic along with environmental manipulations like photoperiod, nutrient and growth hormones. Ipek et al. (2007) identified a DNA based bolt marker having a close association with bolting in garlic. On the basis of this present experiment planned to screen the Indian garlic genotypes for bolting trait and its association with morphological behavior. This will bring the possibilities of identification of genotypes with stimulated flowering.

Materials and Methods

Indian garlic accession: A total of 32 accessions were chosen for the study from different agro-climatic zones of India (Table 1). Accessions were selected on the basis of their three years phenotypic behavior for bolting in normal atmospheric conditions of respective locations. All the thirty-two genotype showing phenotypically diverse characters were grown in half kg plastic pots containing a mixture of sand and soil in a 1:1 ratio under glasshouse condition.

DNA extraction and molecular characterization of bolting genotype: For the DNA isolation, tender leaves were collected from three weeks old plants and the DNA was extracted from 200 mg leaf samples by using modified cetyltrimethyl ammonium bromide (CTAB) method (Fütterer et al. 1995). The quality of DNA was checked electrophoretically on 0.8% agarose gel and the concentration measured using a nano spectrophotometer (Eppendorf Mini-Fluorometer) at 260/280 nano meter. The characterization of bolting genotype done by earlier reported bolting specific molecular marker by Ipek et al. (2007), P₁, P₂ and P₃ with their respective sequences (5' AAGGAGCATCACGTTGGCTTTG 3', 5' CAGCAGCCAGGT GCGAAGC3' and 5' GGGAAAGGGTAGAAGAATGGG3'). The two

combinations of bolting markers P₁+P₂ and P₁+P₃ made and PCR amplification was carried out in a volume of 15 iL reaction mixture contained 1 iL template DNA, 1.5 iL 10× PCR buffer, 0.35 iL of dNTPs (25 mM), 0.3 iL MgCl₂ (1.5 mM), 1.2 iL random primer (10 pM), 0.25 iL Taq-DNA polymerase, and 10.4 iL sterile Milli-Q water. The PCR was performed in a thermal cycler (DNA Engine Dyad® ALD1234; Biorad, USA) and the PCR machine was programmed as initial denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 45 seconds and extension at 72°C for 1 minute and final extension at 72°C for 7 minutes. The amplicons were analyzed by electrophoresis on 1% agarose gels in TAE buffer with a 1 kb DNA ladder (Gene Ruler Ladder, Thermo Fisher, Mumbai, India) and photographed using a gel documentation system (AlphaImager™ 3400, USA).

Results and Discussion

Garlic (*Allium sativum* L.) cultivars are mainly sterile and propagated vegetatively. In India among whole garlic diversity, accessions from the northern part of the country have the ability to develop scape, however, two accessions from Central Institute of Temperate Horticulture (CITH) Srinagar are reported as being able to develop sterile florets. In Indian subtropical parts like Maharashtra, Karnataka and Madhya Pradesh few lines consistently develop scape, especially during *Kharif* season. The variation in lowering with respect to the environment, location and season reported by Kamenetsky et al. (2004) and proposed induction need for flowering in garlic bolting lines. In India especially for a short day, garlic flowering induction is the utmost for a breakthrough in improvement. Ipek et al. (2007) developed a mitochondrial gene-based *Bltm* marker which differentiates bolting lines from non-bolting and incomplete bolting lines. In the present report mitochondrial DNA based bolt markers were used to screen 32 garlic accessions diverse in their geographic origin (Table 1). Amplification of bolt marker (1.4 kb) was observed in all the accessions has the ability to bolt (Fig. 1A). However when we correlate the bolting behavior of these accessions at their native condition, G-3 and G-5 only form bulbils or florets respectively (Fig. 2A & 2B) at long day garlic center CITH, Srinagar (34.083656, 74.797371) and Gadag local, G-654, G-671NE, G-282, G-44 shows axis scape induction at Rajgurunagar, Pune location (18.851743, 73.881408) and further axial bulbil formation (Gadag Local, G-671NE) at Maharashtra and Karnataka region (Fig. 2C) this shows that other might have inherent capacity and needs to be triggered by imposing suitable environment

or this could be due to low expression of associated genes due to environmental interaction. These results were confirmed by amplifying another reliable mitochondrial DNA based markers (3.7kb, Fig. 1B) which depict bolting behavior along with the chimeric arrangement of the gene. Amplification of this marker observed in only twenty-two accessions (Fig. 1 and Table 2) illustrates the chimeric gene arrangement. The other ten genotypes have shown amplification of only 1.4kb indicating the absence of chimeric gene arrangement. Here, out of these ten accessions, seven ecotypes (G-3, G-5, Gadag local, G-282, G-671, G-654, and GG-4) are showing scape induction at their native locations and bulbil formation in G-3, G-5, Gadag local genotypes. Therefore, this can be concluded that Indian garlic genotypes do not have chimeric gene arrangement for bolting or scape induction. Ipek *et al.* (2007) identified that in garlic 3.7kb region is a mitochondrial part where 4.8 kb chloroplast DNA is inserted into the mitochondrial genome. In fact, the mitochondrial genome is more sensitive to genetic aberrations like insertion or deletion than the plastid genome. Further, they also explained that the sequence of the gene of the chimeric region is variable than other crops viz *Arabidopsis*, rice and wheat. In addition to this, they also described the occurrence of nucleotide changes due to mutation in the chimeric region which leads to non-functionality of the plastid gene sequence. In our study primer P1+P2 combination amplified 1.4kb

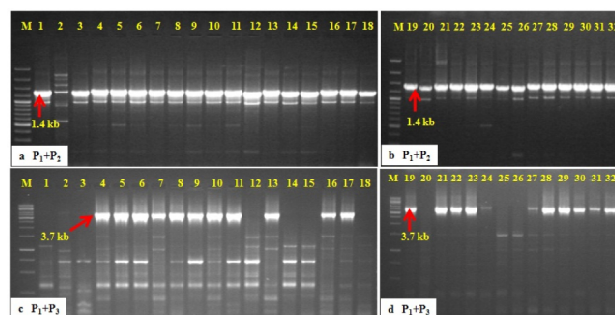


Figure 1: Banding pattern of primer combinations P1-P2 and P1-P3, the 1.4kb band is amplified in all 32 accessions with P1-P2 and 3.7kb band is amplified in 22 accessions with P1-P3 combination. The 3.7kb band lacking accessions show bolting.

Fig 1: (a) 1.4 kb amplicons; M-ladder, GeneRular™ DNA ladder Mix; 1, G-3; 2, G-5; 3, 654; 4, 656; 5, 662; 6, 663; 7, 664; 8, 665; 9, 667; 10, 668; 11, 669; 12, 671; 13, 675; 14, Ranibennur local; 15, Gadag local; 16, GG-1; 17, GG-2; 18, GG-4; (b) 1.4 kb amplicons; M-ladder, GeneRular™ DNA ladder Mix 19, G-41; 20, G-282; 21, G-355; 22, AC-50; 23, AC-183; 24, AC-316; 25, AC-378; 26, Godavari; 27, SG-1; 28, CG-1; 29, Phule Baswant; 30, Bhima Purple; 31, Bhima Omkar; 32, AKG-2) (c) 3.7 kb amplicons; M-ladder, GeneRular™ 1kb DNA ladder; 1, G-3; 2, G-5; 3, 654; 4, 656; 5, 662; 6, 663; 7, 664; 8, 665; 9, 667; 10, 668; 11, 669; 12, 671; 13, 675; 14, Ranibennur local; 15, Gadag local; 16, GG-1; 17, GG-2; 18, GG-4; (d) M-ladder, GeneRular™ 1kb DNA ladder; 19, G-41; 20, G-282; 21, G-355; 22, AC-50; 23, AC-183; 24, AC-316; 25, AC-378; 26, Godavari; 27, SG-1; 28, CG-1; 29, Phule Baswant; 30, Bhima Purple; 31, Bhima Omkar; 32, AKG-2)

Table 1: Geographic origin and bolting status of garlic genotypes at India

| Sr. no. | Genotype | Origin/ Source | Status | Sr. no. | Genotype | Origin/ Source | Status |
|---------|-------------------|----------------|--------------------|---------|---------------|-----------------|-------------|
| 1 | G-3* | J & K | Complete bolting | 17 | GG-2 | Gujarat | Non-bolting |
| 2 | G-5* | J & K | Complete bolting | 18 | GG-4 | Gujarat | Non-bolting |
| 3 | 654 | Sikkim | Non-bolting | 19 | G-41 | NHRDF, Nashik | Non-bolting |
| 4 | 656 | Sikkim | Non-bolting | 20 | G-282 | NHRDF, Nashik | Non-bolting |
| 5 | 662 | Sikkim | Non-bolting | 21 | G-355 | NHRDF, Nashik | Non-bolting |
| 6 | 663 | Sikkim | Non-bolting | 22 | AC-50 | ICAR-DOGR, Pune | Non-bolting |
| 7 | 664 | Sikkim | Non-bolting | 23 | AC-183 | ICAR-DOGR, Pune | Non-bolting |
| 8 | 665 | Sikkim | Non-bolting | 24 | AC-316 | ICAR-DOGR, Pune | Non-bolting |
| 9 | 667 | Sikkim | Non-bolting | 25 | AC-378 | ICAR-DOGR, Pune | Non-bolting |
| 10 | 668 | Sikkim | Non-bolting | 26 | Godavari | Maharashtra | Non-bolting |
| 11 | 669 | Sikkim | Non-bolting | 27 | SG-1 | ICAR-DOGR, Pune | Non-bolting |
| 12 | 671 | Sikkim | Non-bolting | 28 | CG-1 | ICARDOGR, Pune | Non-bolting |
| 13 | 675 | Sikkim | Non-bolting | 29 | Phule Baswant | Maharashtra | Non-bolting |
| 14 | Rannibennur Local | Karnataka | Non-bolting | 30 | Bhima Purple | ICAR-DOGR, Pune | Non-bolting |
| 15 | Gadag Local | Karnataka | Incomplete bolting | 31 | Bhima Omkar | ICAR-DOGR, Pune | Non-bolting |
| 16 | GG-1 | Gujarat | Non-bolting | 32 | AKG-2 | Gujrata | Non-bolting |

*Genotypes showing the formation of bulbils along with sterile flowers at CITH, Srinagar, India

Table 2: Grouping of accessions on the basis of amplification of DNA based *Bltm* marker

| Group | Amplicon size | No. of accessions giving amplification | Bolting/ Flower | Name of accessions |
|-------|---------------------|----------------------------------------|-----------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1 | Both 1.4 and 3.7 kb | 22 | Absent | 656, 662, 663, 664, 665, 667, 668, 669, 675, GG-1, GG-2, G-41, G-355, AC-50, AC-183, AC-316, SG-1, CG-1, Phule Baswant, Bhima Purple, Bhima Omkar and AKG-2. |
| 2 | 1.4kb | 10 | Present | G-3, G-5, 654, 671, Ranibennur Local, Gadag Local, GG-4, G-282, AC-378, Godavari |

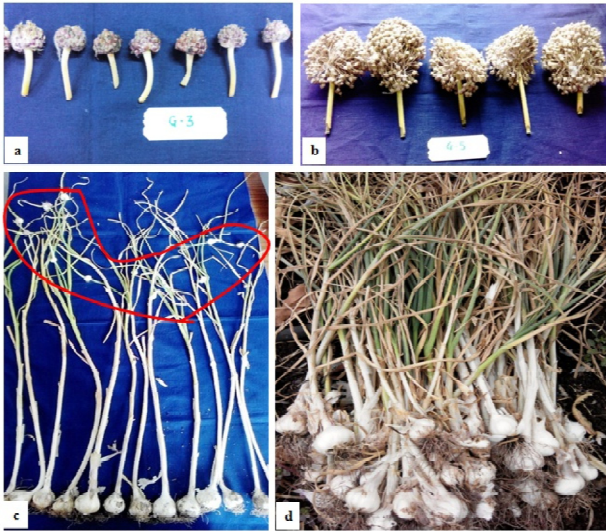


Figure 2: Field performance of various garlic varieties: (a and b) completely bolted long day garlic varieties G-3 and G-5; (c) incomplete bolted short day garlic variety Gadag Local and (d) bolting free short day garlic variety.

region which possesses the *psbA* gene derived from the *cox 3* gene and primer P1+P3 amplified 3.7kb region which contains protein-coding gene *rp123*, *rps19*, *rp12* and *ycf2* and one tRNA gene *trnH-GUG* (Celiński et al., 2017). Hence in the case of garlic, the plastid genome diversity focusing genomic region is playing a major role in flowering behavior (Ipek et al. 2007). Earlier the alteration in flowering behavior by environmental manipulation was achieved by Kamenetsky et al. (2004). The field performance and flowering incidence comparison can be predicted using *Bltm* marker, which will be helpful for determining the usefulness of this marker over diverse locations. As Indian complete bolted line only possesses 1.4 kb region it shows that there is nearly 1.4kb from chloroplast further incomplete and non-bolted garlic lines show amplification of both 1.4 and 3.7 kb region which explains diverse fragment insertion of plastid genome among Indian garlic population. These findings also revealed that bolting and non-bolting are not directly related to this marker in Indian conditions. Generally, the plastid genome is proven to be useful in phylogenetic relationships between species (Yang J. et al 2017) and the same can be explored to find an evolutionary pattern of Indian garlic with special focus to degeneration of its fertility. The present reports is showing the linkage of bolting with mitochondrial and plastid genome and provide the future site to study the molecular evolutionary history of Indian garlic in connection to sterility.

Conclusion

Out of 32 screened genotypes, bolting and scape induction behavior at native conditions of ten genotypes

correlated with *Bltm* marker (P1+P2) amplification of 1.4 kb. These genotypes showed the potential for flowering and further use in breeding purposes through environmental manipulation like photoperiod, temperature, storage period and growth hormones. Hence it is interesting to note that the bolting behavior of Indian garlic genotypes does not link with chimeric gene arrangement. Further, this can be validated by assessing plastid genome diversity among them. This will generate information on evolutions that occurred in the plastid genome of short day garlic genotypes. The present report is showing the link of bolting with association mitochondrial, and plastid genome and provides a future site to study the molecular evolutionary history of Indian garlic in connection to sterility.

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सारांश

वर्तमान अध्ययन हेतु भारतीय लहसुन के 32 जननद्रव्यों में तीन माइटोकॉन्ड्रियल डी.एन.ए. आधारित आण्विक मार्कर, पी.-1, पी.-2 और पी.-3 का उपयोग करते हुए बोल्टिंग करने वाले प्रभेदों की पहचान करने के लिए जांच किया गया। दो अलग-अलग संयोज में पी.-1 + पी.-2 और पी.-1 + पी.-3, 1.4 किलोबेस के एम्प्लिकॉन को सभी 32 जननद्रव्यों जांच किये गये प्रविष्टियों में पी.-1 + पी.-2 पुष्प चिन्हकों द्वारा प्राप्त किया गया, जिनमें प्रविष्टियों के साथ पुष्प की अंतर्निहित क्षमता और तीव्र सर्दियों को सहन करने की आवश्यकता थी। इसके अलावा परिणाम की स्थिरता की पुष्टि हेतु पी.-1 + पी.-3, माइटोकॉन्ड्रियल डी.एन.ए. आधारित चिन्हक के साथ की थी जो 3.7 किलोबेस लम्बाई के एम्प्लिकॉन का उत्पादन करते हैं, आण्विक प्रोफाइलिंग में गैर तुल्यकालन के आधार (दोनों में से एक की उपस्थिति या अनुपस्थिति के आधार) पर पूरी आबादी को दो समूहों अर्थात् पुष्पनीय और अपुष्पनीय में वर्गीकृत किया गया है, समूह-1 में 22 (656, 662, 663, 664, 665, 667, 668, 669, 675, जी.जी.-1, जी.जी.-2, जी.-41, जी.-355, ए.सी.-50, ए.सी.-183, ए.सी.-316, एस.जी.-1, सी.जी.-1, फुले बसवंत, भीमा पर्पल, भीम आंकार और ए.के.जी.-2) सम्मिलित है, दोनों पी.-1 + पी.-2 और पी.-1 + पी.-3 चिन्हक 1.4 और 3.7 किलोबेस दोनों लम्बाई के एम्पलीकॉन वाले अभिगम है जो कि संकर (काइमेरिक) जीन व्यवसायी को इंगित करता है। समूह-2 में पी.-1 + पी.-2 चिन्हक के साथ 1.4 किलोबेस लम्बाई के एम्प्लिकॉन दिखाने वाले 10 प्रविष्टियाँ पायी गयी जिसमें पुष्पीय प्रभेदों (जी.-5 पूर्णरूपेण और नौ, जी.-3, 654, 671 रानीबेनूर स्थानीय, गडग स्थानीय, जी.जी.-4, जी.-282, ए.सी.-378 और गोदावरी आंशिक रूप से) सम्मिलित इससे निष्कर्ष निकाल जा सकता है कि भारतीय लहसुन की प्रभेदों

को पुष्पन के लिए संकर जीन की व्यवस्था की आवश्यकता नहीं है। यह अध्ययन भारत में अल्प दिवसीय लहसुन में पुष्पन के पहचान की दिशा में पहला प्रयास है।

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