



Characterization of bolting behaviour in garlic (*Allium sativum*) using SNP

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ABSTRACT

The present research was conducted to evaluate the bolting (incomplete bolters and non-bolters) behaviour in garlic (*Allium sativum* L.) accessions collected from different geographical areas. A total of 96 genotypes collected from different sources were used for genotype sequencing to identify SNP but only 73 genotypes among them showed good amplification. A primer (AsLFY) was designed by using reference of already designed gene specific primer (gALFY-2) by Rotem *et al.* (2007). After amplification, sequencing of these 73 genotypes was done; SNP (CCGTCG) was at 301 bp from initiation codon, i.e. G for non-bolters genotypes and A for incomplete bolter genotypes. Thus, the identified SNP would help in detecting incomplete bolter and non-bolter genotypes at seedling stage which can be useful for garlic breeding programmes.

Key words: Garlic, Incomplete bolters, Non-bolters, SNP

Garlic (*Allium sativum* L.) reproduces exclusively by underground cloves and rarely by vegetative topsets or bulbils in the inflorescence and is mostly sterile. Although male fertility in wild garlic has been observed in nature and genetic linkage maps from S1 (family of plants produced by self-pollination) are already available (Ipek *et al.* 2005, Zewdie *et al.* 2005). Breeding programs depend on the genetic variability and identification of traits of interest, which require a rigorous characterization of accessions within germplasm collections. Significant levels of genetic variability were detected in garlic germplasm by morphological traits, isozymes, and molecular markers (Ma *et al.* 2009). Garlic genotypes are categorized as non-bolting, semi-bolting, and bolting types (Takagi 1990, Kamenetsky and Rabinowitch 2001). Genetic diversity studies using isozymes, RAPD, and AFLP markers were able to distinguish bolting, incomplete-bolting and non-bolting garlic clones from each other by clustering them into separate groups. These studies also demonstrated that there was significant genetic variation within and among groups of bolting garlic clones (Al-Zahim *et al.* 1999). These studies did not identify a particular molecular marker associated with bolting, but an easy-to-evaluate simple marker differentiating bolting garlic clones from

non-bolting or incomplete-bolting ones could be useful to categorize clones in garlic germplasm collections, or to identify quickly garlic clones with a potential for flowering and seed production.

Recently, SSR markers have been reported in specific *Allium* species, such as garlic, bunching onion and bulb onion, and have been used for genetic analysis within species (Song *et al.* 2004, Ma *et al.* 2009). Besides, SNP technique provides locus specific markers which can be used in gene mapping, detection of mutation at molecular level and identification of disease causing genes. In garlic, SNPs have not been extensively used so far. For the garlic genome, however, a limited number of SSR markers and SNPs have been developed and only few are available to generate high density genetic maps in a crop with such a large genome size (Cunha *et al.* 2012, Ipek *et al.* 2012, Khar *et al.* 2012). To improve genetic maps and genetic diversity analysis, more SSR markers and SNPs need to be developed for the garlic genome. Keeping all above points in view, the present study aimed to evaluate the bolting behaviour in garlic accessions collected from different geographical areas.

MATERIALS AND METHODS

The present study for identification of bolting behaviour in garlic was carried out at the Vegetable Research Farm, Department of Vegetable Science and Molecular Biology Laboratory, School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana, during 2013. Total 96 accessions were used which represent the collection from different geographical regions. For molecular studies, primer was designed using Primer 3 software

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(), for the formerly described 682-bp fragment of the gene (Rotem *et al.* 2007, GeneBank AY563104 <http://www.ncbi.nlm.nih.gov/nuccore/48476628>). The primer was designed from gene gaLFY-2 with forward primer (5'-TCGACGACATGATGACCACT-3') and reverse primer (5'-TCTTGGGCTTGTTGATGTAGC-3') having amplicon 675 bp. After detecting the position of SNP, gene specific primers were designed by using Primer3 software (<http://frodo.wi.mit.edu/primer3>) for the formerly again described flowering gene 682-bp fragment of the gene (Rotem *et al.* 2007, GenBank AY563104 <http://www.ncbi.nlm.nih.gov/nuccore/48476628>). Then again primer was designed from gene AsLFY with forward primer (5'-GCAGTCGCTCGTCCTAATT-3') and reverse primer (5'-CAATCGCCTGAACCTGAAC-3') having amplicon size 400 bp. Genomic DNA of all the 96 cultivars/accessions was isolated using the modified CTAB (Cetyltrimethyl ammonium bromide) method of Saghai-Marooof *et al.* (1984). Quantity and quality of DNA samples were determined by gel electrophoresis. *In vitro* amplification using polymerase chain reaction (PCR) was performed in a 96-well microtitre plate in an eppendorf master cycler in a 20 µl reaction volume (Saiki *et al.* 1988). Amplification was performed using temperature profiles as initial denaturation at 94°C for 2 min, denaturation at 94°C for 30 s, annealing at 55°C for 45 s, extension at 72°C for 60 s, final extension at 72°C for 10 min. Steps 2-4 were repeated 35 times. Annealing temperature varied according to the primer used. The agarose block containing the amplified DNA band was excised from the gel with a clean, sharp scalpel blade and transferred to a 1.5 ml micro centrifuge tube. The excised DNA fragments were purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Inc.) as per manufacturer's protocol. The information on nucleotide sequence of the targeted insert DNA was generated using forward and reverse primer with Big Dye Terminator v3.1 (ABI Sequencer 3730xL). Chromatograms were analyzed for sequences manually. The sequences were then extracted from these using CHROMAS Lite 2.1.1 (<http://technelysium.com.au/>). Further, sequences were trimmed to remove poor quality reads at both ends. Reads were assembled using DNA Baser v.4.16.0 (<http://www.dnabaser.com/>) to generate contigs. Multiple sequence alignment, using the gene sequences obtained for all genotypes was carried out using Clustal X 2.1. Based on the alignment, SNPs were detected. The detected SNPs were then manually curated by analyzing and comparing chromatogram files with Clustal X alignment files. Only those parts of reads for which Q-value equaled

or exceeded 20 were considered for finding SNPs. The bases which had Q-value below 20 were not considered to be SNPs.

RESULTS AND DISCUSSION

For commercial cultivation point of view, bolting varieties are not preferred as there is diversion of nutrients from bulbs to topsets, and scape and flowers which reduced the bulb size and ultimately the yield. Moreover, non-bolting garlic varieties have longer shelf life than bolting types (Kamenetsky R 2007). Out of 96 genotypes, 70 were observed to be incomplete bolters and 26 were non-bolter.

With the help of ABI Sequencer 3730xL a total 71 genotypes of garlic were sequenced for that region. These genotypes were already identified in field by taking phenotypic data and their bolting behaviour was confirmed by sequencing these genotypes. As we already gave the sequence of garlic genome described in Fig 1 from that sequence we were able to distinguish the genotypes from the SNP position whether the genotype is incomplete bolter or non-bolter. The sequence is same for both incomplete bolter and non-bolter genotypes but the only difference among these genotypes is the SNP position as incomplete bolting genotypes having A in the sequence (CCGTCA) and non-bolting genotypes having G in the sequence (CCGTG). The SNP lies at 301 bp from initiation codon. The sequencing was applied to 71 genotypes and after these was aligned with sequence alignment technique (Jalview software). The sequences of all 71 genotypes were compared with the reference sequence of garlic. As a result, 71 sequences of different length and the SNP were found among these 71 genotypes at 315bp position in the sequences so obtained. Therefore, the SNP differentiate

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TTGAGCTCGACGACATGATGACCACTCTCTCCACCTCTCCGCTGGGACGTCTCTGTC
GGTGAACCGTATGGCATCAAGGCCGCCGTGAGAGCCGAACGCCGCCGTCTCGACCACC
CTTCTCTCGACGATGGACGGCGCAGATTCTCCGACGTGCCCTCGATGCCGTTTCTCAA
GAAGGTACCGCAGTCGCTCGTCTAATTGACTCATTCACAACCCTTTCATAGATTTAAA
CCATGCAGGACTATCAGAAGAGCGAGCACACCACGAGAGTGAGGAGAGAAGTGATAAC
CGATAAAGGGAAAAGAACCGTCGCTGCAAGAAAAACCAAACAGAAAGGACAACA
TCAAGAAGAAGAAGAAGAAGGCACCCGCCGAGGTGACAGGTGAAGGAGAACGGGAA
ACCGATTTCGATTTCGGATTTCGGATTGGAAGCGAGTCCGGTACGGAACGGAGCGGCAA
AGGGAGCACCCGTTTCATAGTTACGGTGCCAGGCGAAGTGCGGAGAGCGAAGAAGAAT
GGGCTGGACTACCTGTTCCATCTGTATGAGCAGTGCCGTGAGTTTCTCGTTTCAGGTTCA
GGCGATTGCGAAAGAGCAAGGGGACAAGTGTCGACGAAGGTGACAAACCAGGTGTT
TAGGTATGCGAAGAAGGTGGGAGCGAGCTACATCAACAAGCCCAAGAA
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*The highlighted point is the position of SNP in the flowering gene sequence of garlic

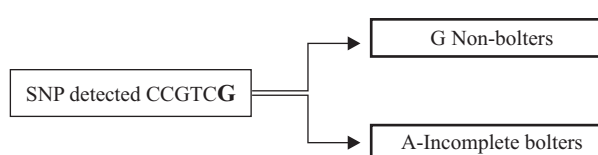


Fig 1 Flowering gene sequence of garlic.

the 71 genotypes into incomplete bolters and non-bolter genotypes by distinguishing each other and by showing the difference in A and G position. Out of total 71 genotypes, 14 genotypes showed non-bolter sequence and rest 57 genotypes showed incomplete bolter sequence. The data obtained from the sequencing technology of 71 genotypes of garlic correctly matched with the phenotypic data.

Length of approximately 600bp reads was obtained for most of accessions. In a few cases, the reads were limited to 300bp length. The sequences were extracted from Chromatograms using ChromasLite (Fig 2). Out of 71 accessions, sequences of 8 accessions were extracted from forward primer only, whereas in 63 accessions sequences were retrieved from reverse primer only. Reverse strand sequences were reverse complemented to bring all the sequences on same strand. For example ACTGCTAG would read as CTAGCAT on reverse complementation. Reverse complementation of reverse strands is necessary because sequence obtained starts from end at which primer binds, thus without this step, alignment and further analysis would not be possible. Then both forward and reverse strand sequences were aligned with reference in single alignment file using Clustalx2.0.11. It was observed that sequences reads obtained aligned perfectly with reference. After alignment the SNP position of reference and genotypes were compared and difference between incomplete bolter and non-bolter genotypes were observed.

The data presented in the above Table 1 depicts that the genotypes taken for molecular study with their phenotypic data and their position of SNP at 301 bp from initiation codon. A total of 71 genotypes which were used for sequencing and they were from different geographical regions out of which genotypes, viz. F-III-SF, AVTG-2, G-50, GRS-1328, BGSD-1228, PG-12, F-II-SF, GRS-1332, PG-17, PG-31, NRCRG-I, CG-114, PG-I, PG-24, 5323, 5366, 7281, AC-50, JNDG-213, W6-35675, BGSD-1217, W6-35695, 5477, 5303, F-IX-G, AVTG-7, F-I-R, 7274, PI-540356, GRS-1345, PI-540319, 5476, BGSD-1237, IETG-I, W6-29777, CGSD-1252, CGSD-1263, CGSD-1265, PG-19 (Pink), GRS-1349, PI-540367, PI-164521, W6-35696, PI-493124, AVTG-I, W6-17074, G-189, IETG-2, PG-18, F-VI-SF, 7107, PG-35, PG-32, BG-117, F-VII-G, F-VI-N, F-II-R, F-V-F, ASRG-1107 showed incomplete bolting as well as SNP (CCGTCA) was present in the sequence at 315bp position as well. The genotypes, viz. AVTG-6, F-IV-R, F-XIV-R, PG-30, PG-20, JNDG-219, PG-33, PG-26 (Pink), W6-12836, F-XV-C, BG-108, GRS-1337, F-X-CK, AG-102 showed non-bolting and the SNP (CCGTCG) was found when compared to non-bolting reference sequence.

The genotypes collected for the study they all have different geographical region, i.e. they were all collected from different sources and they might have different ancestors, their center of origin is different. A total of seventy-one genotypes were collected, they all were from different sources. Out of seventy-one genotypes, twenty-five genotypes, viz. PG-30, PG-20, PG-33, PG-26 (Pink), F-IV-R, F-XIV-R, F-XV-C, F-X-CK, F-III-SF, PG-12,

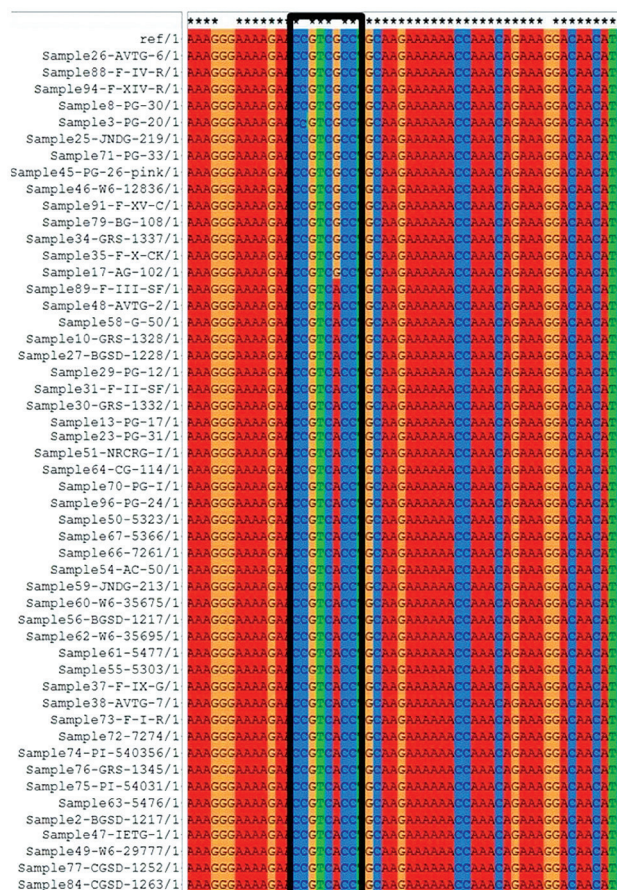


Fig 2 Demarcated area showing the difference of SNP (G/A).

F-II-SF, PG-17, PG-31, PG-I, PG-24, F-IX-G, PG-19 (Pink), PG-18, F-VI-SF, PG-35, PG-32, F-VIII-G, F-VI-N, F-II-R, F-V-F were collected from Punjab. All the twenty-five genotypes, although they were collected from Punjab and had same source but the bolting behaviour of these genotypes was different as seventeen genotypes, viz. F-III-SF, PG-12, F-II-SF, PG-17, PG-31, PG-I, PG-24, F-IX-G, PG-19 (Pink), PG-18, F-VI-SF, PG-35, PG-32, F-VIII-G, F-VI-N, F-II-R, F-V-F were incomplete bolter genotypes and eight genotypes, viz. PG-30, PG-20, PG-33, PG-26 (Pink), F-IV-R, F-XIV-R, F-XV-C, F-X-CK, were non-bolter genotypes, this showed that seventeen flowering genotypes which were from same source they might have same ancestor and other eight non-flowering genotypes having same source belongs to same ancestor. Twelve genotypes, viz. ASRG-1107, GRS-1349, CGSD-1252, CGSD-1263, CGSD-1265, GRS-1345, BGSD-1217, CG-114, GRS-1332, BGSD-1228, GRS-1328, GRS-1337 they were from AINRPOG out of these genotypes eleven genotypes, viz. ASRG-1107, GRS-1349, CGSD-1252, CGSD-1263, CGSD-1265, GRS-1345, BGSD-1217, CG-114, GRS-1332, BGSD-1228, GRS-1328 were incomplete bolter types and might have same ancestor and one non-flowering genotype, GRS-1337 out of them were non-flowering, having same origin showed that it might have some different ancestor. Nine genotypes, viz. 7107, PI-540367, 5476, 5477, 7261, 5366, 5323, W6-29777, W6-12836 collected from USA.

Table 1 List of genotypes with their sequences at position of SNP and data obtained in the field

Genotype	Bolting behaviour	Source	Position of SNPs at 315 bp
AVTG-6	Non-bolter	IIVR, Varanasi	AAAAGAACCGTC G CCTGCAA
F-IV-R	Non-bolter	Punjab	AAAAGAACCGTC G CCTGCAA
F-XIV-R	Non-bolter	Punjab	AAAAGAACCGTC G CCTGCAA
PG-30	Non-bolter	Punjab	AAAAGAACCGTC G CCTGCAA
PG-20	Non-bolter	Punjab	AAAAGAACCGTC G CCTGCAA
JNDG-219	Non-bolter	Gujarat	AAAAGAACCGTC G CCTGCAA
PG-33	Non-bolter	Punjab	AAAAGAACCGTC G CCTGCAA
PG-26 (Pink)	Non-bolter	Punjab	AAAAGAACCGTC G CCTGCAA
W6-12836	Non-bolter	U.S.A	AAAAGAACCGTC G CCTGCAA
F-XV-C	Non-bolter	Punjab	AAAAGAACCGTC G CCTGCAA
BG-108	Non-bolter	IIVR, Varanasi	AAAAGAACCGTC G CCTGCAA
GRS-1337	Non-bolter	AINRPOG	AAAAGAACCGTC G CCTGCAA
F-X-CK	Non-bolter	Punjab	AAAAGAACCGTC G CCTGCAA
AG-102	Non-bolter	Punjab	AAAAGAACCGTC G CCTGCAA
F-III-SF	Incomplete bolter	Punjab	AAAAGAACCGTC A CCTGCAA
AVTG-2	Incomplete bolter	IIVR, Varanasi	AAAAGAACCGTC A CCTGCAA
G-50	Incomplete bolter	Haryana	AAAAGAACCGTC A CCTGCAA
GRS-1328	Incomplete bolter	AINRPOG	AAAAGAACCGTC A CCTGCAA
BGSD-1228	Incomplete bolter	AINRPOG	AAAAGAACCGTC A CCTGCAA
PG-12	Incomplete bolter	Punjab	AAAAGAACCGTC A CCTGCAA
F-II-SF	Incomplete bolter	Punjab	AAAAGAACCGTC A CCTGCAA
GRS-1332	Incomplete bolter	AINRPOG	AAAAGAACCGTC A CCTGCAA
PG-17	Incomplete bolter	Punjab	AAAAGAACCGTC A CCTGCAA
PG-31	Incomplete bolter	Punjab	AAAAGAACCGTC A CCTGCAA
NRCRG-I	Incomplete bolter	Maharashtra	AAAAGAACCGTC A CCTGCAA
CG-114	Incomplete bolter	AINRPOG	AAAAGAACCGTC A CCTGCAA
PG-1	Incomplete bolter	Punjab	AAAAGAACCGTC A CCTGCAA
PG-24	Incomplete bolter	Punjab	AAAAGAACCGTC A CCTGCAA
5323	Incomplete bolter	U.S.A	AAAAGAACCGTC A CCTGCAA
5366	Incomplete bolter	U.S.A	AAAAGAACCGTC A CCTGCAA
7261	Incomplete bolter	U.S.A	AAAAGAACCGTC A CCTGCAA
AC-50	Incomplete bolter	Maharashtra	AAAAGAACCGTC A CCTGCAA
JNDG-213	Incomplete bolter	Gujarat	AAAAGAACCGTC A CCTGCAA
W6-35675	Incomplete bolter	Uzbekistan	AAAAGAACCGTC A CCTGCAA
BGSD-1217	Incomplete bolter	AINRPOG	AAAAGAACCGTC A CCTGCAA
W6-35695	Incomplete bolter	New Zealand	AAAAGAACCGTC A CCTGCAA
5477	Incomplete bolter	U.S.A	AAAAGAACCGTC A CCTGCAA
F-IX-G	Incomplete bolter	Punjab	AAAAGAACCGTC A CCTGCAA
AVTG-7	Incomplete bolter	IIVR, Varanasi	AAAAGAACCGTC A CCTGCAA
F-I-R	Incomplete bolter	Punjab	AAAAGAACCGTC A CCTGCAA
PI-540356	Incomplete bolter	Georgia	AAAAGAACCGTC A CCTGCAA
GRS-1345	Incomplete bolter	AINRPOG	AAAAGAACCGTC A CCTGCAA
PI-540319	Incomplete bolter	Poland	AAAAGAACCGTC A CCTGCAA
5476	Incomplete bolter	U.S.A	AAAAGAACCGTC A CCTGCAA
BGSD-1237	Incomplete bolter	AINRPOG	AAAAGAACCGTC A CCTGCAA

Cond.

Table 1 (Concluded)

Genotype	Bolting behaviour	Source	Position of SNPs at 315 bp
IETG-I	Incomplete bolter	IIVR, Varanasi	AAAAGAACCGTC A CCTGCAA
W6-29777	Incomplete bolter	U.S.A	AAAAGAACCGTC A CCTGCAA
CGSD-1252	Incomplete bolter	AINRPOG	AAAAGAACCGTC A CCTGCAA
CGSD-1263	Incomplete bolter	AINRPOG	AAAAGAACCGTC A CCTGCAA
CGSD-1265	Incomplete bolter	AINRPOG	AAAAGAACCGTC A CCTGCAA
PG-19 (Pink)	Incomplete bolter	Punjab	AAAAGAACCGTC A CCTGCAA
GRS-1349	Incomplete bolter	AINRPOG	AAAAGAACCGTC A CCTGCAA
PI-540367	Incomplete bolter	USA	AAAAGAACCGTC A CCTGCAA
PI-164521	Incomplete bolter	USA	AAAAGAACCGTC A CCTGCAA
W6-35696	Incomplete bolter	Bulgaria	AAAAGAACCGTC A CCTGCAA
PI-493124	Incomplete bolter	Poland	AAAAGAACCGTC A CCTGCAA
AVTG-I	Incomplete bolter	IIVR, Varanasi	AAAAGAACCGTC A CCTGCAA
W6-17074	Incomplete bolter	Jordan	AAAAGAACCGTC A CCTGCAA
G-189	Incomplete bolter	Maharashtra	AAAAGAACCGTC A CCTGCAA
IETG-2	Incomplete bolter	IIVR, Varanasi	AAAAGAACCGTC A CCTGCAA
PG-18	Incomplete bolter	Punjab	AAAAGAACCGTC A CCTGCAA
F-VI-SF	Incomplete bolter	Punjab	AAAAGAACCGTC A CCTGCAA
7107	Incomplete bolter	USA	AAAAGAACCGTC A CCTGCAA
PG-35	Incomplete bolter	Punjab	AAAAGAACCGTC A CCTGCAA
PG-32	Incomplete bolter	Punjab	AAAAGAACCGTC A CCTGCAA
BG-117	Incomplete bolter	IIVR, Varanasi	AAAAGAACCGTC A CCTGCAA
F-VIII-G	Incomplete bolter	Punjab	AAAAGAACCGTC A CCTGCAA
F-VI-N	Incomplete bolter	Punjab	AAAAGAACCGTC A CCTGCAA
F-II-R	Incomplete bolter	Punjab	AAAAGAACCGTC A CCTGCAA
F-V-F	Incomplete bolter	Punjab	AAAAGAACCGTC A CCTGCAA
ASRG-1107	Incomplete bolter	AINRPOG	AAAAGAACCGTC A CCTGCAA

Non-bolter: AAAAGAACCGTC**G**CCTGCAA; Incomplete bolter: AAAAGAACCGTC**A**CCTGCAA

All genotypes from source U.S.A were of incomplete bolter types except one genotype, i.e. non-bolter type showing that eight genotypes of incomplete bolter types were from same origin and they also had some common ancestor and one non-bolter type had some different ancestor even it was from same source. Eight genotypes, viz. AVTG-6, BG-108, AVTG-2, AVTG-7, IETG-I, AVTG-I, IETG-2, BG-117 they were collected from IIVR, Varanasi. Two genotypes AVTG-6 and BG-108 were non-bolter and might have same ancestor and rest six genotypes, viz. AVTG-2, AVTG-7, IETG-I, AVTG-I, IETG-2, BG-117 they were incomplete bolters and they might have same pedigree. Maharashtra collection includes AC-50, NRCRG-1, G-189 and all the three genotypes were of incomplete bolter type they were from same source showing that they were having same ancestor. G-50 genotype collected from Haryana was incomplete bolter type. W6-35675 was from Uzbekistan source and of incomplete bolter type. Poland also includes two genotypes, PI-540319, PI-493124 that were incomplete bolter type showing same ancestor. Gujarat collection also had two genotypes, JNDG-213, JNDG-219, but out

of these two genotypes JNDG-213 was incomplete bolter and JNDG-219 was non-bolter type having same origin but might have different ancestor. New Zealand, Georgia, Bulgaria, Jordan and Uzbekistan includes genotypes W6-35695, PI-540356, W6-35696, and W6-17074, W6-35675 respectively these genotypes had different origin but they were of incomplete bolter types so they might have same ancestor. Out of total seventy-one genotypes fifty-seven genotypes were of incomplete bolter types and fourteen out of seventy three were of non-bolter types. Although the incomplete bolter genotypes they all were from different sources but having same character of incomplete bolting and non-bolter genotypes were also from different sources having same character of non-bolting.

Garlic is mainly propagated asexually and its flower is nearly, or completely, sterile. A flowering gene in garlic was already been detected by not has been utilized for further studies. Therefore, the present study was conducted to identify the SNP in flowering gene of garlic which can detect the non-bolters and incomplete bolters in early stage of plant. The sequencing of all 73 genotypes was done

and SNP for non-bolters and incomplete bolter garlic was detected by using a newly designed primer (AsLFY). This SNP (CCGTCG) lied at 301 bp from initiation codon i.e. G for non-bolters genotypes and A for incomplete bolter genotypes. Thus, this SNP will help in detecting incomplete bolter and non-bolter genotypes of garlic at seedling stage which can be useful for garlic breeding programmes.

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