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Quantum leap in the light of molecular elucidation of garlic genome

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Review

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ABSTRACT

Garlic, a popular vegetable cum condiment is known widely for its health benefits, pharmacological properties and in curing several pathological conditions. This compelling horticultural bulb crop is propagated asexually from individual bulbils or cloves. It is an obligate apomict that lost its fertility and blooming potential long ago and probable reason for evolution from fertility to sterility to greater contiguity of human selection to asexual propagules as they are used in culinary as and when required. The crop is likely to be sterile owing to nutritional competition between topsets, pollen degeneration, chromosomal deletion, irregular chromosomal pairing and abnormal meiosis during gametogenesis and thus curbing genetic variation is needed utmost for its improvement. With asexual reproduction, molecular studies are challenging due to its expected and complex genome. Alongside classical molecular markers like RAPDs, AFLPs, SRAPs, SSRs, and isozymes; recent high-throughput genotyping-by-sequencing (GBS) approaches like DArTseq has allowed characterization, mapping, whole-genome profiling, DNA fingerprinting among others in garlic. However, in recent years, bio-technological tools, genetic transformation via biolistic or *Agrobacterium tumefaciens*, polyploidization or chromosomal doubling have emerged as a potent breeding tool in enabling the improvement of vegetatively propagated plants such as garlic. In recent times biological responses of garlic and its compounds have been studied using epigenomics, proteomics and transcriptomics by researchers in preclinical studies instigating the biological effects of garlic and such gene expression revealed many early mechanistic events which may clinically underlie important health benefits pertaining to garlic intake. This review thus encompasses efforts achieved till the present date towards the elucidation of garlic genome with regard to molecular, biotechnological analysis and gene expression in terms of *in vitro* and *in vivo* studies.

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Introduction

Garlic, botanically *Allium sativum* L., and one of the popular vegetable cum condiments is consumed and cultivated worldwide for a considerable period. Vavilov (1) and Kazakova (2) suggested Central Asia was suggested to be its primary centre of origin which unfurled to other parts of the world through colonization and trade. Theorgano-sulphur compounds in garlic exhibit antioxidant, antibacterial, antidiabetic, immunomodulatory, lipid-lowering and antithrombotic capabilities (3,4). Besides, this monocotyledonous bulbous vegetable cum condiment is also well known for its beneficial effects in curing pathological conditions like hyperlipidemia (5), cardiovascular disorders and arteriosclerosis (6). It is an obligate apomict that lost its fertility and blooming potential millennia ago.The probable reason fore volution from fertility to sterility might be due to the greater propinquity of human selection to asexual propagules as they are used in culinary as and when required (7,8). The crop is, thus, likely to be sterile. Sterility is attributed to nutritional competition between top-sets, pollen degeneration, chromosomal deletion, irregular chromosomal pairing and abnormal meiosis during gametogenesis(9). Consequently, vegetative propagation using cloves, bulbils or top-sets are the sole propagation system used (10). Such an asexual mode of propagation curbs genetic variation which is needed utmost for genetic improvement in garlic but can be achieved possibly by somaclonal variations, critical selection, spontaneous and induced mutations of available clones.Such propagules especially cloves need to be reproduced every year since they possess storability issues which adds an extra cost and hassle tothe preservation of large germplasm collection. This peculiarity in garlic reproduction can out-turn low genome diversity, since through clonal propagation meiosis is not involved (11). This popular bulb crop is a diploid species (2n=2x=16), but its nuclear genome is reported to be dramatically complex

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having an estimated haploid (1C) size of 15.9 gigabase pairs (Gbp) along with a large ratio of repetitive sequences (91.3%). Molecular markers have played an important role in the evaluation of germplasm for the last 10 years but reports on the such aspect are less in garlic due to large genome size complicating molecular analysis. The reason for such a complex nuclear genome might have aroused from multiple copies of genes and other duplications, involving tandem repeats and non-coding sequences (12,13). Although it is a challenging task to develop full sequencing of the garlic genome using next-generation assembly accompanying transcriptome assembly may be utilized for the generation of functional genomic data effectively (14).In recent years, biotechnological tools, polyploidization or chromosomal doubling have emerged as a potent breeding tool in enabling the improvement of vegetatively propagated plants such as garlic (15). Nevertheless, there is a need to drive techniques in order to smash barriers pertaining to vegetative propagation and low bulb yield through the annexation of ingenious breeding techniques to boost garlic improvement. Thus this review encompasses efforts achieved towards the elucidation of the garlic genome with regard to molecular, biotechnological analysis and gene expression in terms of *in vitro* and *in vivo* studies are discussed here.

Chemical composition and medicinal traits

Water (65%), carbohydrates (25-30%) especially fructose polymers constitute major components, whereas, lipids, proteins, minerals, saponins and fibres comprise minor constituents of garlic. The crop is an armoury of more than 33 sulphur-containing active substances among which allicin (thio-2-propene-1-sulfinic acid S-allyl ester) (16-21) is the major one (22). This allicin possesses antibiotic, antimicrobial, antioxidant, anticoagulation, antimycotic, antiparasitic, anti-aging, antiviral, antitumoral, antihypersensitive, heavy metal detoxification, lipid-lowering capacity, immunity modulator and enhancer (23,24). Besides, garlic also produces sulphur producing compounds such as alliin [(+)-S-(2-propenyl)-L-cysteine sulfoxide] and γ- glutamylcysteines which confabulates its biological activity, odour and flavour (25). Alliin alone can reckon 1.4% of bulb's fresh weight (26). In addition, Alliin is known for its antibiotic effects on microorganisms especially *Staphylococcus epidermis*, *Salmonella typhi*, *Helicobacter pylori* (bacterium causing stomach cancer). The activity of allinase enzyme is reported to be 10 times higher in bulbs than in leaves involving hydrolysis of sulphur compounds (27).The crop is profoundly produced as a cash crop to earn coinage from foreign countries like the USA, Europe and the Middle East, fetching encouraging returns and achieving food security goals. Garlic can be processed in granules, chips, flakes and powder. Besides dehydrated and garlic extracts are widely accepted for home usage and industrial purpose for producing repellents, pharmaceutical drugs and explosives.

Cytology, Biosystematics and Diversity

Cytologically, garlic aka *Allium sativum* L. is a diploid species with its haploid chromosome number n=8. Other species related to garlic are tetraploid, 2n=32, whereas, *A. sativum* var. *scordoprasum* is triploid, (3n=24). On biosystematics point of view, *Allium sativum* L. belongs to the genus *Allium* and section *Porrum* and the family *Alliaceae*. The classification of garlic is still ambiguous, based mainly on bolting and non-bolting habit. Bolting species includes *Allium sativum* var. *ophioscorodon* and *Allium sativum* var. *scorodoprasum* and the non-bolting species is *Allium sativum* var. *sativum* (garlic). In another classification, *Allium sativum* var. *vulgare* Kuz. and *Allium sativum* var. *sagitatum* Kuz. is classified as non-bolting and bolting species respectively. The subspecies are again divided into ecotypes. The bolting subspecies include three ecotypes, viz., Caucasian, Asian and Seaside, whereas, the non-bolting subspecies include East Mediterranean, South Russian and Continental(28).

Garlic plant exhibits wide variations especially in shape, size and bulb color, flavour, maturity date, size and number of cloves, peeling ability, pungency, flavor, number of flowers in an inflorescence, bolting capacity, sizes and number of topsets (29,30). Such a variety of phenotypic expressions certainly validates the fact that there lies a strong interaction between environments(31).On the basis of the ability to produce flower stems, some garlic producers distinguished varieties as soft-neck and hard-neck (32). Nevertheless, the terms bolters and non-bolters are more accurate from the physiological point of view. On the basis of inflorescence development and scape elongation garlic can be classified as (i) *Complete bolters*-which produces thick and long flower stalk along with many flowers and topsets, (ii) *Incomplete bolters*-which produces short and thin flower stalk bearing only a few topsets which usually forms no flowers, (iii) *Nonbolters*- possesses an incomplete scape that doesnot form flower stalk or produce cloves (33). Such observed traits might be altered by a wide range of environmental conditions, but the mechanisms involved in their regulation are still unknown. Worldwide, garlic cultivars based on physiological and morphological phenotypes were classified into several horticultural groups, demonstrating wide diversity in the crop itself. The "*Purple Stripe*" group (which includes bolting hard neck cultivars) is considered genetically contiguous to the origin of garlic. Other groups vary in bulb structure and bolting ability which include Marbled Purple Stripe, Silver skin, Glazed Purple Stripe, Porcelain, Creole, Rocambole, Asiatic, Artichoke and Turban types (30). But behavioural patterns of these cultivars alter due to the effect of environmental variations and thus phenotypic expression of the same variety changes dramatically under different

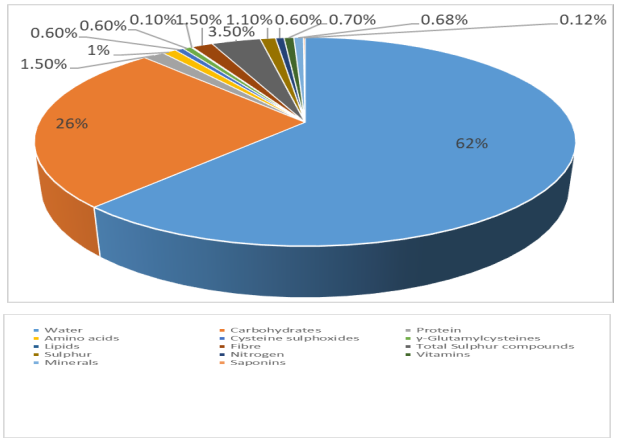

Figure 1. Chemical composition of garlic bulb (27).

climatic situations. Volk et al.(34) reported duplications of 41-64% of garlic accessions while conducting AFLP analysis in 211 genotypes collected from National Plant Germplasm System (NPGS) in the USA. Thus, the precise distinction between different groups and cultivars needs the application of further molecular tools(35).

Insubstantial genetic variability

Garlic has been identified as an obligatory apomict, owing to which the crop stands limited in terms of genetic variation. This led to difficulty in inculcating conventional breeding methods for its improvement especially breeding for higher yield, better quality and disease resistance, owing to the vegetative mode of propagation in garlic (36). Presently, most of the genetic improvement has been achieved through clonal selection for the characters earliness, clove number and size (37). However, for improving traits like obtaining true garlic seeds and fertility, selections for enhanced inflorescence traits are done which might result in achieving genetic stocks having reduced or no bulb-lets having complete pollen fertility in the future (37). To further add, the selection and induction of molecular markers with aid of marker-assisted selection have widened opportunities for genetic and molecular studies for breeding garlic (38). In fact, utilization and validation of these markers on germplasms available especially on long day regions can assists effectively in the development of male fertile plants in the development of true seed parents/lines. Such parents/lines upon inbreeding can serve either as the base material for further selections or as genetic contributors in F_1 hybrids. Shemesh et al(39) and Ipek et al(40) reported significant genetic variation *via* true seeds for important plant and bulb traits among garlic lines. Such results certainly validate an emerging success in achieving genetic variation by virtue of sexual reproduction either by exploring genetic diversity or *via* normal selection. Furthermore, the application of breeding tools is much higher in long-day garlic in contrast to short-day garlic cultivars as they are more naturally pre-disposed to sexual reproduction.

Genomic size

Among vegetable crops, garlic's genomic size is very large approaching almost that of onion. Its genomic size is 32.7 pg DNA per 2C nucleus having low GC content along with a large number of repetitive sequences. Such large genomic size and duplication are the major setbacks in instigating biotechnological tools especially molecular markers, genomic libraries, genetic fingerprinting, tagging and mapping of genes. Development of a genetic map requires genetic variability to arise straight from sexual reproduction, but garlic is inherently apomictic which limits genetic variation thereby compromising locating genes of interest. Thus employing MAS in garlic remained largely constrained for a considerable period of time. In last decade, fortunately, the commencement of laboratory-based sexual reproduction in garlic paved the way for the construction of few genetic maps, but, the density of such markers including RAPDs, AFLPs, SSRs and isozymes reported to be low. Nevertheless, few gene-specific markers had been mapped for allinase, Chalcone synthase (CHS), male sterility and Somatostatin (SST) (41), such genetic maps unveiled duplication in germplasms, genetic diversity and the nature of the garlic genome.

Global uses of RAPDs, AFLPs, SRAPs, SSRs and isozymes to study genetic diversity and population structure

Predominantly, garlic is a sterile species, while its ancestor *Allium longicuspis* has been disputed as a separate species. Nonetheless, the existence of great genetic variation in terms of physiological and morphological features involving varyingdegrees of flowering and bolting led to the postulation of three botanical varieties. A collection of 110 garlic clones was investigated for an interspecific classification using morphological and isozyme methods by Pooler and Simon (42). They detected 17 different enzyme groups, among which 13 isozyme systems were tested; due to lack of variability or inconsistency in staining, only four were useful. Besides floral characteristics were found to be correlated well with isozyme data, whereas, traits related to bulbs or geographical origin exhibited low predictive value for the genetic relationship of the accessions studied by them. 300 garlic clones with isozymes and 48 of thesewere tested with RAPDs by Maaβ and Klaas(43) to compare two marker systems). Accessions/clones taken under study by them were suitable for judging the genetic relationship between cultivated clones having primitive features and a feral accession of *Allium longicuspis*. They tested 12 isozyme systems and identified 22 loci, out of which 10 were reported to be polymorphic with 16 defined isozyme groups. Expectedly, 125 RAPD fetched detailed distinction but both the markers substantially paved a good delimitation of varieties, especially *ophioscorodon* and *sativum*. The third variety *pekinense* was not found to be discernable enough; neither by marker from *longicuspis*-type plants nor an accession conditioned as *longicuspis*-separated from more partially fertile garlic based on molecular markers. Ipek et al (44) reported that in garlic clones AFLP is a useful technique for gauging genetic relationships and found out ten groups in 9 diverse garlic collections.Al-zahim et al(45) investigated a similar range of accessions but their results depicted differences in some important aspects. They structured twenty-seven garlic cultivars having 63 polymorphic bands generated from 26 primers. Their study inferred that 11 accessions were assigned to variety *sativum*, another 11 to variety *ophioscorodon*, and the remaining five were assigned to *A. longicuspis*. However, these workers have found substantial genetic differences within *ophioscorodon* and also found interspersal, especially with *longicuspis* accessions. Such findings exhibited contrast genetic homogeneity of the *ophioscorodon* (80 accessions were investigated by isozymes and seven among them were investigated by RAPDs), being clearly distinct from *longicuspis*-type accessions genetically as reported by Maaβ and Klaas (43). Differences in such results might have stemmed from the difference in morphological classification of the material used in prior molecular studies; rather than misapplication of RAPD markers in either case as comparable number of primers and markers per taxon were used in both laboratories. A collection of 20 Australian garlic accessions along with five RAPD markers were investigated by Bradley et al(46) resulting in 65 marker bands. The approach befitted to grouping major

Australian cultivars with respect to place of origin, early and late types and bolting behaviour. Al-Zahim et al (45) found difficulties in distinguishing *A. ophioscorodon* from *A. longicuspis* based on exerted anthers. Ipek et al (47) drew an interesting comparison among RAPD, AFLP and isozymes. The researchers evaluated three *A. longicuspis* clones and 45 garlic clones and compared AFLP results with that of RAPD and isozymes and observed a total of 183 polymorphic fragments from three AFLP primer combinations generations. Similarities between the clusters were low (>0.30) and with AFLP analysis they also observed similarity (>0.95) within the clones studied. In addition, 16 clones represented only six different banding patterns sharing 100% polymorphic RAPDs and AFLPs and were expected to be duplicates. Volk and colleagues (34) performed an AFLP analysis on 211 accessions involving both *sativum* and *longicuspis* taken from USDA NPGS and other commercial sources. They applied several statistical tools to assess how clonal lineages of these accessions are differentiated genetically and how those patterns of differentiations correspond to phenotypic classification to recognize. The analysis inferred that there lies a fine line between soft-neck and hard-neck garlic types and showed that soft-neck garlic revealed less genetic diversity than hard-neck garlic types. They further suggested that phenotypic categories correlated well with the data pertaining to genetic distance. This distinctive study winded up the fact that AFLP is useful and permits discerning duplicates for epitomizing the portion genotyped from NPGS garlic collection for cryoconservation by presuming that genetically unique accessions, as well as simple accession, should have priority over duplicate samples. Mota et al (48) assembled 12 Brazilian cultivars to study genetic diversity among them and categorized them as 'half noble' and 'noble' by amplifying 279 fragments using 80 primers. The 'half noble' and 'noble' cultivars presented 54.2% and 57.1% of similarity respectively. Panthee et al (49) laid an investigation on 179 garlic accessions collected from Nepal using morphological traits which were eventually grouped into three clusters and concluded the presence of duplicates on the collection. Rosales-Longo and Molina-Monterroso (50) contemplated based on AFLP studies that genetic variability in cultivated garlic populations was low in Guatemala.Buso et al(51) analysed the diversity using 206 RAPD markers for 17 mostly-grown Brazilian garlic cultivars in a separate study. By bootstrap analysis, it was exhibited that number of markers was efficient and sufficient enough to receive a coefficient of variation of 10%. Besides, similarity ranged in between 16 and 98% and cluster analysis revealed that a genetic similarity, in general, correlates well with morphological traits of the cultivars and variation in the production cycle. The study further emphasizes the suitability of RAPD for genetic diversity analysis revealing the existence of significant genetic variation among genotypes taken under study. Paredes et al (52) observed low genetic diversity among Chilean garlic accessions. The analysis revealed a total of 398 bands generated from 68 accessions with 40 RAPD primers and they realized that there was no association between patterns that emerged out by primers employed, morphological characters and geographical origin of clones.Zhao et al (53) on AMOVA analysis in garlic accessions revealed that 84.4% of variation was due to within-population differences and the remaining 15.6% of the variance was due to divergences between groups. Lampasona et al (54) conducted AFLP based diversity study in garlic accessions and reported the existence of several duplicates. Jo et al (55) analysed genetic diversity using SSR markers in 120 garlic accessions collected from five different countries and inferred that geographical regions correlate with genetic diversity. Shaaf et al (56) studied and collected 31 different accessions from 31 different regions of Iran using ISSR and RAPD markers and observed that those 31 accessions were assigned into two clusters having no admixed classes. Cunha et al (57) studied 130 accessions collected from Brazil and other countries. Their study indicated the presence of duplicates in the collection and all garlic entries were assigned into two groups. In addition, a core set with 17 genotypes representing their entire collection was also selected by them.Singh et al (58) and Raja et al (59) observed high morphological diversity in garlic accessions for clove rind color, bolting habit, bulb size, clove weight and number using SSR markers. Polyzos et al (60) studied genetic diversity in garlic genotypes based on morphological traits and observed that these traits are correlated with environmental conditions such as fertilizing regimes, soil properties, cultivation practices and genetic compositions. Their findings might explain the reasons behind the drawback of morphological-based traits genetic diversity analysis.Mokate et al (61) used 16 garlic genotypes to study genetic diversity using molecular markers *viz*. RAPD, ISSR and SSR and realized that reliability of one particular marker doesnot fulfill in the identification of genotypes. Benke et al (62) used SRAP markers for characterizing 625 garlic accessions with thirty primer combinations that showed polymorphism ranging from 33.33 to 100% and with an overall polymorphism of 80.59%. In addition primer combinations generated an average of 5.1 bands per primer and the number of primers ranged between 2 to 9 possessing 100 to 1500 base pairs between them. All of the above studies provide good opportunitiesfor developing and identifying DNA markers from the genome-wide level in the garlic genome.

DArTseq in genetic diversity and genotyping-by-sequencing (GBS) analysis in garlic genome

For genetic analysis and crop improvement the role of molecular markers as a tool has gained importance over the years (63). The use of such molecular markers has indeed become common in important crops and species in terms of polymorphism assessment and crop-breeding studies (64). Wide-scale identification of molecular markers especially Single Nucleotide Polymorphism (SNP) on transcriptome and genome stands in for interesting approaches (65). In garlic, classical molecular markers used to assess genetic diversity and polymorphisms were described by Ovesna et al (66) and Ipek et al (67). Other molecular marker includes Random Amplified Polymorphic DNA (RAPD) (43), Amplified Fragment Length Polymorphism (AFLP) (68), Simple Sequence Repeat (SSR) (69) and Insertions-deletions (InDel) (70). Still, genetic diversity analyses in the species arequite disputable (71). Nevertheless, recent technological advancements, fortunately, have overcome previous limitations. Such developments include Second-generation-sequencing (SGS) and

third-generation sequencing (TGS) approaches. Third-generation sequencing cryptically known as next-generation sequencing (NGS) was reviewed by Dorado et al(72). Henceforth, a very high-throughput GBS aka genotyping-by-sequencing technology known as DArTseq has been developed which combines Diversity array technology (DArT) with TGS/SGS (73,74). This advanced technology allows identifying SNP as well. DArT markers are actually polymorphic segments of DNA found mainly at specific genomic sites being detected by hybridisation after complexity reduction. Such markers might exhibit codominant or dominant inheritance (75). To analyse DNA polymorphisms, DArT markers escapade DNA-microarray manifesto, requiring no prior knowledge of DNA sequence. Applications of such markers include genetic mapping, germplasm characterization, profiling whole-genome, and DNA fingerprinting among others (76). By selecting the most appropriate complexity-reduction method (includes both sizes of representation of assays and snippet of genome selected), DArT can be optimized for each application and organism. Application of the DArT marker is thus hugely relevant for garlic, which possesses a large and much-anticipated complex genome. Besides, DArTseq technology has established itself well as a "Proof-of-Concept" in analysing huge garlic-germplasm banks. Egea et al (77) used DArTseq in evaluating genetic diversity and structure of 417 garlic samples (out of which 408 were collected from the garlic-germplasm bank by them). The results of their study revealed that there exists general consistency between geographic origins, accessions taken under study and groupings acquired from known/expected garlic specification and individuals could be divided into three main groups (I, II and III). Furthermore, they observed statistical probability of belonging to a group was high and the individual exhibited a similar association pattern in hierarchical cluster analysis. In addition, samples maintained their patterns from C1 to C5 (with respect to previously known information). Their findings thus finally acknowledged the fact DArTseq markers proved to be the fruitful, compelling and congruous genotyping approach to gauge genetic structure and diversity. To further add on, DArTseq is the "cost-effective genotyping tool" for creating and maintaining germplasm banks, generating genetic patterns and high-quality whole-genome profiles, and genetic dissection of important traits with exponentially increased resolution commensurate to previous methodologies (78).

First genetic linkages among expressed regions of the garlic genome

Commercial garlic production is mediated mostly through asexual propagation and sterility in it is mostly attributed to several factors including anther degeneration, competition for nutrients between flowers and topsets, chromosomal deletion and irregular chromosomal pairing. Garlic improvement has been limited to sports or clonal selection of spontaneous mutants with traits involving cloves per bulb, bulb color, and non-flowering phenotype (79). Moreover, the discovery of male-fertile garlic accessions in Central Asia escalated research on sexual reproduction (80). Nevertheless, garlic genetics is at a nascent stage, with one of the largest genomes (1.59×10^{10} base pairs) among all vegetable crops (81). Other than molecular markers, recent advances in garlic genetics involvethe development of a Bacterial Artificial Chromosome (BAC) library of garlic, suitable for studying cytogenetics at the molecular level and transformation by particle bombardment (82). Genetic linkage maps are mighty embellishments for understanding marker-assisted breeding, the genetic basis of complex traits, and map-based cloning of important traits (83,84). In addition, genetic improvement of garlic would be enhanced by constructing a genetic linkage map which would not only allow the identification of genes controlling economically important traits but also aid in marker-assisted selection. Zewdie et al (27) developed the first genetic linkages among expressed regions of the garlic genome to bring clarity to the genetic basis of male fertility. They were the first group of researchers in the public research sector to develop the S_1 family of garlic by self-pollinating a single plant where on average one SNP occurs in every 210 bases in garlic. The prevalence of SNP transitions (63%) over transversions (37%) of their study agreed well with the findings of Kuhl et al (85) in *Allium cepa*. Their study unravelled segregation for molecular markers and morphological traits unveiling that the parental plant taken under study was heterozygous for loci conditioning those traits. Major factors contributing to segregation warp might be due to the loss of progeny lines due to the linkage up with deleterious alleles. As because garlic has been known to asexually propagated for centuries which is likely to be the fact that mutation has delivered deleterious allelesthatare maintained in the heterozygous state. They also observed 18 markers to get segregated as the absence vs. presence of amplicons, in fact, insertion-deletions events or mutations especially at the priming sites might result in no amplifications from ¼ of the S_1 plants.In addition, their study laid down the identification and mapping of major locus *Mf* affecting male fertility in garlic, ideal for garlic improvement and its seed production. Moreover, identifying molecular markers linked close to *Mf* locus will permit eventually MAB in male-fertile garlic thereby allowing the breeding of superior garlic cultivars.

Genomic insights into diversification and evolutionary history of bulb traits

Maps based on comprehensive genomic variation are a dynamic tool for the analysis of genetic diversity and have been used widely for exploring the scores and evolutionary history of crops (86-88). Although garlic is diploid species, its nuclear genome is dramatically complex along with a huge ratio of repetitive sequences (91.3%). Nevertheless, the recently sequenced garlic genome addressed such challenges with five advanced sequencing methodologies leading to an assembly of a high degree of wholesomeness (89). By genotype-by-sequencing (GBS) technology, Li et al (90) sequenced 230 garlic accessions to infer population structure and thereby studied evolutionary history by resequencing 84 accessions from three main garlic types. The said research group identified 120, 857, 927 SNPs and 8,551, 841 indels in the garlic genome unfolding resources for breeding and genetic studies in garlic. In fact, dissecting the genetic architecture of traits like clove number can be achieved using these variations as a basic tool.They used five taxonomic groups of garlic which include the *longicuspis* group (considered as

basalgroup), *pekinense* group (probably a subgroup of *longicuspis*), Mediterranean *sativum* group, subtropical group and *ophioscorodon* group. Their study revealed four garlic groups were based on genomic variations among which two groups were existed in CG1, CG2 and China. In addition, the subtropical group was hypothesized to originate independently from *longicuspis* group, whereas, *pekinense* group was seen to diverge from *longicuspis* group ~1500 years ago (43). Genomic evidences of their findings further unravelled that both CG1 and CG2 diverged from its ancestral group preceding domestication of the crop by human as sterility results in reproductive isolation and are independently domesticated. Further findings also revealed that OG accessions had a narrow genetic relationship with that of the accessions of outgroup species indicating OG accessions had more genetic information of garlic wild progenitor in their genome than that of the genome of accessions from the other three groups. The study also revealed a small selective genome shared in CG1 and CG2 and among three approaches used in detecting the selective signal of the genome, only the XP-CLR approach identified a genome of 1.6 Mb which underwent common selection in CG1 and CG2 (91). Thus, few extremely ancient selectionswere observed commonly between CG1 and CG2 validating further independent domestication. Their study also unfolded large clove size in CG1 accessions obtained from natural selection for the purpose of adaptation to cold environment, but well performance of cloves CG2 accessions was obtained from domesticated selection conducted by human thereby explaining deep insight on evolutionary history in garlic genome.

Genome-wide identification of WRKY transcription factors (TFs) in response to heat and salt stress

During growth and development plant experiences a wide range of abiotic stresses like heat, drought, salinity, and low and high temperature. Several transcription factors (TFs) are engaged in such stress responses which regulate plant's response to stress by specifically binding and recognizing to *cis*-acting elements in the promoter regions,especially of downstream genes(92). By regulating RNA transcription and expression, TFs can regulate plant's growth and development.WRKY TFs, which comprise one of the largest families of TFs in plants, control various processes involving stress responses, physiological functions, growth and development.WRKY proteins comprise a domain with 60 amino acids and the regulatory role (transcriptional) is conciliated through highly scrimped WRKYGQK motifs (towards the end of N-terminus), binds specifically to W-box. The W-box is nothing but the shortest sequence (C/TTGACT/C sequence) known to bind DNA, where TGAC is known as 'core-conserved region'. Preceding studies showed that WRKY TFs were involved in the different physiological phenomena and several abiotic and biotic stress responses, plant seed development, epidermal hair development and leaf senescence. The WRKY proteins can be categorized into three groups depending on a number of WRKY domains and symbolic features to their zinc-finger-like motifs. In *A. thaliana*, the classification of WRKY TFs family identified to be in three major groups *i.e.*, G1-G3, where in G2 five subgroups are reported and in G3 group all members are reported to be responding well towards a wide spectrum of biotic stresses (93). Identification of WRKY gene family members emphasized a push towards the scientific community since the release of the garlic genome. In addition, very few studies were reported pertaining to WRKY proteins in *A. sativum* L. Yang et al (94) took an effort to identify and characterize WRKY family members from the garlic genome. They identified as many as 78 *AsWRKYs* TFs. Based on garlic transcriptome, genome and amino acid sequence outside the WRKY domain, the identified WRKY TFs were grouped into groups G2 a, G2 b, G2 c, G2 d, and G2 e. Their study revealed that among all the subgroups studied, the subgroup G2 c possessed highest number of proteins and the result was found consistent with other species as well (95, 96). Such TFs and their interacting proteins frisk a vital role in plant growth processes (97). The study of such proteins interacting with WRKY TFs can fetch high insight into the mode of action of WRKY members and its regulation. Assays upon RT-qPCR disentangled expression of nine WRKY TFs in response to abiotic stress.The same research group further constructed an interaction network between *A. thaliana* and garlic WRKY TFs and observed 40 garlic genes to have been involved in the interaction network suggesting WRKY TFs involvement in the multiple biological phenomena. In addition, the relative expression level of four *AsWRKY* genes was up-regulated rather especially under high saline and temperature stress conditions. Functional analysis of *AsWRKY* genes in garlic and the key function of WRKY TFs in response to plant stress suggests that such specific WRKY TFs could be regulated or over-expressed with the aid of genetic engineering to improve the existing ones or their response to multiple stress factors.

Biotechnological upsurge in garlic genome

Biotechnology in agriculture is an amalgamation of scientific approaches to improve animals, plants including microorganisms. Biotechnology enables breeders to devise improvements in the crop which is not possible through conventional/traditional breeding. Tools such as micropropagation, meristem culture, somaclonal variation and genetic transformation furnished ease in garlic breeding (98).

Micropropagation

Micropropagation for garlic production started in 1970. The tissue culture approach has proved to be more commanding over clove propagation as the former requires only tissue fragments or cells to generate a large number of plants. Such tissue culture technique is mediated through two morphogenetic processes (1) organogenesis-depicting generation of organs *i.e*., shoots and roots; (2) somatic embryogenesis-depicting formation of structures possessing similarity in morphology as compared to that of the zygotic embryo. Both direct and indirect processes are involved before the callus phase. In garlic, the morphogenetic ability decreases as the callus continues to grow older and on this account regeneration that does not include the previous callus phase is highly accepted. Nevertheless, several protocols pertaining to micropropagation have been set up using different types of explant; but the majority of protocols were developed

Table1. An overview of in vitro propagation techniques used in garlic.

Cultivars	Explant	Plant growth Regulator	Morphogenic pathway	Efficiency	References
Aben	Root tip of micropropagated plant	C: 2,4-D/Kin:4.5/4.6µM R: BA: 4.4 µM	Organogenic callus	170 S/g C in 4months	(99)
Fc	Immature inflorescence bulbils	S induct: NAA: 5.4µM	Bulbil development	33 to 46 bulbils induced per inflorescence	(100)
Malepur	Clove's basal tissue	Direct emb: 2,4-D/kin:1/0.5 mg/l	Direct somatic embryogenesis	60% explant gave 20-25 embryos/ explant	(101)
Rouge Reunion	Root sections, young leaves, bases of leaf (2 mm)	C:2,4-D/NAA/IAA/ Kin:0.5/0.2/0.2/0.1 mg/l R:2,4-D/Kin:0.1/0.5 mg/l C:2,4-D/IAA:1.5/1 mg/l R: Kin: 6 mg/l	Somatic embryogenesis Somatic embryogenesis	37 embryos per 150 mg of C 70% embryogenic C, 6.8P/C	(102)
Danyang	Shoots of micropropagated plant	S prolif: 2-Ip:0.5 mg/l B: NAA: 0.1 mg/l	Proliferation of shoot in liquid medium	15 shoots/explant in 3 weeks	(103)
Morado, Morasol, Messidrome	1 cm shoot	C:Picloram-iP: 20.7/0.5µM R: Kin: 1 mg/l	Organogenic callus	C: 34% explant. R: 47% C	(104)
Printanor, Morasol, Morado, Messidrome	Young leaves	Liq Med.: 2,4-D/BAP :0.3/0.1 R: 2,4-D/Kin: 0.1/0.5 mg/l	Cell suspension culture	10^{11} embryos annually from 1 clove	(105)

following organogenetic pathways.

Organogenesis

Meristem culture in micropropagation is a technique for getting virus-free plants. Mohamed-Yassen et al (106) developed a novel regeneration protocol by sectioning and dissecting shoots developed from cloves of the cultivar *Extra Select Sets* and were cultivated on a medium of NAA (0.1µM) and BA (8µM) producing eight more shoots in five weeks in comparison to ones kept intact. Haque et al (107) cultivated root tips in a medium of BA (10µM) and NAA (1µM) achieving up to 380 shoots from a single clove having no intermediate callus phase. Robledo-Paz et al (108) regenerated 169 plants per gram of callus after transferring the calli to a medium with 4.4µM BA having the ability to form microbulbs. Khan et al (109) regenerated adventitious shoots from root tips of two garlic varieties; they further observed that when a combination of kinetin (23.8µM) and 2,4-D (6.8µM) was used frequency of callus formation was high. *In vitro* garlic micropropagation was observed by Myers and Simon, (110) by developing roots from adventitious shoots when cultivated in a medium comprising of 4-amino-3,5,6-trichloro-picolinic acid (picloram) (1.4µM) and BA (13.3µM) enabling regeneration of 5.4 shoots per explant. Haque et al(111)devised a protocol for bulb formation and plant regeneration from the root and shoot meristems of the *Bangladesh Local* cultivar, where the meristems were cultivated without growth regulators or a combination of NAA (1-5µM) and BA (1-10µM) on MS medium. None of these combinations proved to be more highly responsive than their absence. On the other side, Luciani et al (112) experimented with different explants in micropropagation of 069 varieties, cultivated in BDS medium with BA,picloram and 2,4-D where meristems and basal plates resulted in the highest shoot regeneration. They further realized that on using a combination of 4.43µM BA and 0.25µM 2,4-D, 100% of explants were able to produce calli differentiated both into shoots and embryos. Wu et al(113) conducted a study on hyperhydric garlic shoots regenerated *in vitro* based on biochemical and ultra-structural traits. They observed the organelles especially chloroplasts and mitochondria were compresseda gainst the cell wall with a significant decrease in protein content. Besides, H_2O_2 and O_2 generation rates increased by 63.9% and 45.3%, respectively. Their study also reported an increase in oxidative stress-related enzymes especially ascorbate peroxidase, superoxide dismutase, catalase, lipoxygenase and peroxidase concluding the fact that the hyperhydric condition of tissues has a close bearing on oxidative stress.

Somatic embryogenesis

For the first time in 1977, embryoids (formation of structure) were reported. In the presence of IAA (10µM) and kinetin (20µM), differentiated calli wereobtained from

bulb leaf discs and stem tips. Al-Zahim et al (114) observed this response again when floral receptacles and basal plates were cultivated on a medium comprising BA (10µM) and NAA (1µM). In a different work established cultures of the variety *Chonan*in suspension obtained from calli on MS medium of 10µM kinetin, 5µM 2,4-D and 5µM picloram. Laterwards, Sata et al (101) used basal sections of clove to obtain somatic embryos from the cultivar *Malepur* grown on white medium supplemented with 2.3µM kinetin and 4.5µM 2,4-D upon which 20 to 25 embryos are formed from each explant. When placed in a higher concentration of kinetin and 2,4-D, masses of hyperhydric tissues are produced. Following the trend, Fereol et al (102) developed plants and somatic embryos of variety *Rouge de la Réunion* by cultivating calli produced from root tips on a B5 modified medium augmented with kinetin (2.3µM) and 2,4-D (0.4µM). Fereol et al(105) developed a protocol for embryo regeneration by using young leaf sections of clove from the variety *Morasol* through suspension cultures. Calli (embryogenic) were obtained when explants were grown on B5 medium along with 0.47µM kinetin and 4.5µM 2,4-D. By using the same culture condition induction of suspension culture (embryogenic) of four garlic cultivars named *Printanor*, *Rouge de la Réunion*, *Messidrome* and *Morasol*. After two months of culture, 90% of calli differentiated into embryos, especially at the globular stage. 50% out of the total regenerated embryos developed into plants were established successfully in the greenhouse. Such culture's histological analysis unfolded the fact that somatic embryos had basically unicellular origin (115).

Meristem culture

The technique is widely known for the production of virus-free clones. Such elimination through meristem culture is based on the fact that meristematic tissues are free of virus and thus regenerated plants from it will also be virus free. To execute this process, explants of 5mm (max.) are recommended, but their size may sometimes limit theirs*in vitro* establishment. Nevertheless, meristem culture has been a driving force to produce virus-free plants in various parts of the world. Ma et al(116) used stems and scape tips *Red Six Cloves* variety. This allowed the formation of adventitious shoots when cultured on a medium comprising kinetin (2.3µM) and NAA (2.6µM). Eventually, plants turned into garlic mosaic virus (GMV) free 65 days after the onset of the culture. Alternative protocols for generating virus-free plants from roots, bulbils and inflorescence meristems were also executed by some researchers. These plant parts are reported to be available in high numbers than that apical meristems. Following the trend, Verbeek et al (117) cultured meristems taken from bulbils and cloves (0.15-1.00mm) 71% of which plants were regenerated, 38% of explants through meristem culture and thermotherapy OYDV in plants of *Ptujksi-spomladanski* cultivar was eliminated in Sloveni. 0.3-0.6 mm of meristems were cultivated first on B5 medium along with 1µM BA and 1µM IAA and then were transferred into multiplication medium comprising of 5µM 2iP and 5µM jasmonic acid. Meristems that had undergone thermotherapy exhibited a lower number of shoots (1.0-2.2) than non-treated plants (9.3), in addition, 90 to 100% of plants were found OYDV free (118). Through meristem culture and chemotherapy, Sidaros et al(119) took attempt to produce plants from three cultivars (*Chinese*, *Italian* and *Balady*). They observed that meristems of 3mm fetched 100% of virus-free plants when cultivated on MS medium containing 50mg L^{-1}Virazole. In a different trial the meristem culture, chemotherapy and thermotherapy were combined to obtain plants free potyvirus. Thermotherapy exhibited negative results on ELISA at 3°C forone week, followed by 36°C for two weeks and 38°C for three weeks. From the clove of these plants, embryos were removed and cultivated in 205µM ribavirin. Regenerated meristem (0.1-0.5mm) plants exhibited negative results by ELISA. The results proved to be a fact that thermotherapy had a negative impact on plant survival rate as compared to chemotherapy and meristem culture. On the basis of virus elimination, thermotherapy proved to be more efficient (60.0 to 70.9%) as compared to meristem culture (64.0%) and chemotherapy (10.7%)(120).

Somaclonal variants

A somaclonal variation is known to be one of the tissue culture techniques in creating crop variation. Somaclonal variation as variation (phenotypic) observed in *in vitro* regenerated plants with respect to the original plant. Somaclonal variation at the genetic level can be brought about by various changes in DNA includes; (a) Gene mutation, (b) Somatic recombination (c) Polyploidy (d) Aneuploidy (e) transposons movement (f) genetic conversion (g) Gene expression modification by amplification, methylation, reactivation or inactivation (h) chromosomal rearrangements). Badria and Ali (121) regenerated somaclonal variants from calli obtained from root meristems cultured on MS medium comprised of IAA, 2,4-D and kinetin. The regenerated somaclonal variants developed bulbs without undergoing division in the first generation alongside exhibited normal phenotypes in the next generations. They observed cytogenetical activities where somaclonal varieties exhibited the same chromosome number to that of plants (original). Upon quantification of allicin production, some somaclones exhibited allicin content thrice (14.5mg g^{-1}) to that of plants taken under control (3.8mg g^{-1}). The researchers suggested that such a technique would be helpful in alleviating allicin content in garlic.El-Aref (122) regenerated plants from roots and leaves of cultivar *Balady* cultured first on BDS medium along with kinetin (9.5µM) and 2,4-D (4.5µM) for initiation of callus and then in presence of 5.5µM NAA and 9µM BA for plant regeneration. In addition, isozyme analysis exhibited that out of 29 regenerated plants, 9 plants were different from the original plant in respect to the enzymes malate dehydrogenase, phosphatase acid, esterase and alcohol dehydrogenase. Among all these enzymes acid phosphatase and Esterase exhibited a higher degree of polymorphism than that malate dehydrogenase and alcohol dehydrogenase. In another study, Mukhopadhyay et al(123) observed chromosome stability in plants from the callus generated from the cultivar *Rossete* and found that plants thatwere generated on solid MS medium with 2,4-D (9µM) and kinetin (0.93µM) and thereby sub-cultured in the liquid medium along with kinetin and NAA revealing chromosome stability. In addition, ones that were grown on initiation solid medium possessed hyper- or hypo-diploid cells accompanying diploid ones.

Genetic transformation

Although a wide spectrum of methods isavailable to introduce DNA into plant cells, the majority of which has been developed directly with aid of the bacterium *Agrobacterium tumefaciens* or indirectly via biolistic as a vehicle. To introduce DNA directly into plant cells a series of chemical, physical and electrical methods (e.g. biolistic and electroporation) have been generated. From the successful transformation of monocotyledonous crops such as maize and soybean using the biolistic method, turned out to be one of the most used systems for gene transfer. The mechanism comprises bombarding target cells with tungsten or DNA-coated gold microparticles accelerated through a high-speeded gene gun, thereby allowing the crossing of cell walls. Various gene guns exist, the PDS1000 helium which is designed by Dupont is one most widely used gene gun for monocotyledonous plants. Inspite of having a wide range of genetic transformation protocols, the technology has not yet been applied toevery species. Quite a number of species in the genus *Allium* especially garlic few reports are available todate in this regard.

Genetic transformation via*Agrobacterium tumefaciens*

Using *Agrobacterium tumefaciens* as a vehicle, Kondo et al (124) were the first research group to establish a transformation protocol in garlic. Firstly, they infected the morphogenetic calli using the strain EHA101 carrying the plasmid pIG12, which contained *nptII*, *hph* and *uidA* genes subdued by promoter CaMV35S. On using this protocol it was possible to regenerate 15 plants (transgenic) from 1000 inoculated calli which were grown on selective culture medium for about five months. Zheng et al(125) developed a transformation system that enabled the introduction of genes for insect resistance apart from imparting plant resistance to herbicides or antibiotics. Calli of three European cultivars were inoculated using the AGLO strain carrying four different plasmids containing *hpt*, *gusA*, *HO4* and *cry1Ca* genes from *Bacillus thuringiensis*, conferring resistance to insect *Spodoptera exigua*. They observed the highest transformation frequency (1.47%) with the cultivar *Printanor* and pPB34 plasmid. In addition, out of regenerated plants, the ones that are integrated with *cry1Ca* gene resumed good growth and bulb formation under greenhouse conditions. Such transgenic plants also exhibited *Spodoptera exigua* resistance when bioassays were carried out *in vitro*. Later, some of the immature embryos were inoculated by

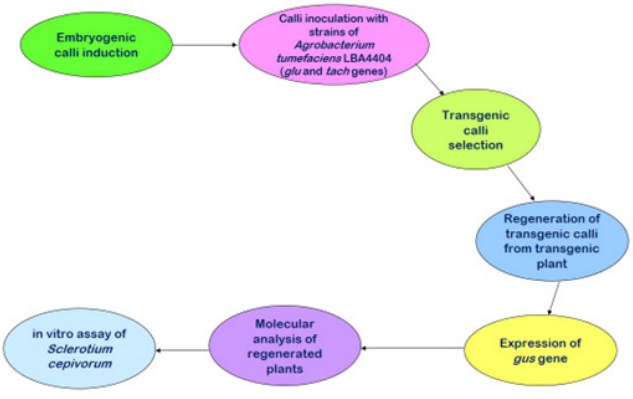

Figure 2. *Agrobacterium tumifaciens* mediated genetic transformation protocol in garlic using tobacco chitinase and glucanase genes.

Eady et al (126) using the strain LBA4404 carrying the vector pBIN *m-gfp-ER* and the gene *gfp* (which encodes the green fluorescent protein) and gene *nptII*. Only two transgenic plants (0.06%) out of 3200 infected embryos were regenerated. The transitory expression of the reporter gene *gusA* was studied by them in two garlic cultivars after infecting them with two plasmids carrying *Agrobacterium tumefaciens*. Plasmid pTOK233 (4.1%) was reported to induce a lower transformation frequency than Plasmid pCAMBIA 1301 which induced higher frequency (7.4%). Resistance-governing genes are yet not commercially used for fighting phytopathogens. Robledo-Paz et al (99) in an attempt to incorporate glucanase and chitinase genes in garlic to confer *Sclerotium cepivorum* resistance. The experiment inferred that the regenerated transgenic plants did not show total resistance to the fungus with a delay in the speed of infection.

via biolistic

Garlic transformation protocol was reported for the first time in 1998. On the callus, immature bulbs, cloves and leaf tissue of the cultivar *Morado de Cuenca* Barandiaran et al (127)were bombarded with four constructs (pAHC25, pCW101, pActl-D and pDE4). Out of all these vectors taken under experiment by them, the one carrying reporter uidA gene (gusA) (coding for β-glucuronidase) under control of the promoter 35S [from cauliflower mosaic virus (CaMV35S)] along with terminator of the NOS (Nopaline Synthase gene) allowing the expression of *uidA* gene in 76.7% of bulbs, 43.3% of leaf explants, 23.3% of clove tissue and 13% of calli. The *uidA* gene's transitory expression could only be detected after the tissues were treated with a nuclease inhibitor (aurintricarboxylic acid). Still, the goal of achieving transgenic plants through this protocol remained unaccomplished. In a similar pattern, Myers and Simon (110) used cell suspensions of *RAL27* clone and bombarded it with kanamycin conferring resistance genes *i.e*., *gusA* and *nptII*. These two genes were under the control of NOS and CaMV35S promoters. Shoots were regenerated after 16-18 weeks on a culture medium. Nevertheless, the incorporation of *nptII* and *gus* genes into garlic transgenic plants was affirmed by PCR assays. Ferrer et al (128) later used biolistics to introduce the selection gene *bar* and the reporting gene *uidA* which actually codes for N-acetyl-transpherase, into embryogenic calli, basal plate discs and leaf tissues of cultivar *Moraluz.* The *bar* and *uidA* genes were under the control of maize ubiquitin (Ubi) and CaMV35S promoters, respectively. It was also observed that expression of *uidA* genes was found maximum in leaves and calli. In another experiment, Sawehel (82) executed a transformation system using calli obtained from immature cloves of *Giza 3* cultivar. Such calli were bombarded with pBI22.23 plasmid containing *hpt* gene (which codes hygromycin phosphotranspherase enzyme conferring resistance to hygromycin) along with reporting gene *gusA*. To inhibit activity of endogenous nucleases, calli had been previously treated with aurintricarboxilic acid. After histochemical analysis and southern blot analysis, the protocol proved to aid the transfer required, stable integration of transgenes and its expression in the DNA of the garlic genome. At the around same time, Park et al (129) after bombarding calli of *Danyang* cultivar using

pC1301-ALS plasmid containing *hpt*, *als* (coding for acetolactate synthase) and *gus* genes under the promoter CaMV35S, received some transgenic plants resistant to herbicide chlorsulfuron. Out of 1900 calli, only 12 regenerated plants exhibited resistance to chlorsulphuron (3mg L^{-1}) and eventually reached maturity and formed bulbs. The entire process of expression and integration of transgenes into the garlic genome was confirmed on Northern, Southern blot assays and PCR. Robledo-Paz et al (99), using embryogenic calli derived from root tips of *GT96-1* cultivar established a transformation protocol. Calli were thereafter bombarded with pWRG1515 plasmid possessing *gusA* and *hpt* genes, both of which were under the control of CaMV35S promoter and 3' region of the *nos* gene. Transgenic calli (putative) were identified after culturing them on a medium hygromycin (20mg L^{-1}) after four months, which later developed into plants. The transgenic nature of regenerated plants was confirmed using GUS (histochemical) and southern blot (molecular) analysis. In addition, the results revealed a transformation efficiency of 2.2 clones per fresh weight gram of callus bombarded.

Chromosomal doubling

In the breeding program, autopolyploidization (mediated by whole genome duplication) is considered as a mutational force in the evolutionary process (130). In addition, it serves as a powerful tool to broaden the germplasm base (131). Although comprehensive alterations in terms of morphological, physiological, agronomic, histological and genomic levels have been inducing artificially through polyploidy in the large number of plant species the consequences and efficacies of the such protocol are still in ambiguity (132,133,134). *In vitro* techniques among several polyploidy, induction techniques can simultaneously increase the efficiency of polyploidy induction and bring down mixoploid formation from complex influence external or internal factors which guarantees the multiplication of mutants throughout the year (135,136). Nevertheless, uniformity in environmental factors such as light and temperature might result in meristem cell division along with a simultaneous increase in tetraploid and a decrease in mixoploid progeny (137).

For genetic manipulation or polyploidy induction, an efficient and stable regeneration system is a prerequisite (138). Nowadays, nodal segments, axillary buds, callus, leaf explants and shoot tips are most commonly used explants for *in vitro* polyploidy induction(139).Major problems in mutation breeding of *in vitro* asexually propagated vegetable plants are somatic elimination (diplontic selection) and chimera formation of mutated sectors after mutagenic treatment.Thus, best-suggested method for *in vitro* polyploidization is by direct adventitious shoot regeneration without meristem tissue or shoot bud, as young meristems are more flexible to induction of polyploidy as it provides more permeability to antimicrobial chemicals (135). To establish a polyploidization system, garlic inflorescence as explant with active meristematic status was explored by high-frequency direct organogenesis, amenable mainly to the regeneration of autopolyploid shoots and in subsequent ploidy alterations (140,141). The use of antimitotic agents is sometimes critical in the induction of polyploidy. Colchicine among different antimitotic agents has been the most commonly used chemical as per the criteria (142). But it has some side effects such as chromosomal rearrangements or losses, abnormal growth, sterility and gene mutation. The reasons behind such side effects are its toxic effects on mammals and the negative impact environment which happens due to the high affinity of colchicine towards microtubules of animal cells. But colchicine is reported to bind poorly to plant tubulins and for that, it is used widely in high concentrations. For these multiple limitations, herbicides inhibiting mitosis having more affinity to plant tubulin dimer have gained attraction as its ideal alternative (143). Reports suggested that these herbicides outperformed to that of colchicine in terms of polyploidy induction with higher efficiency which includes agricultural crops, and forage (144), vegetables (145), ornamentals (146) and fruits (147). But colchicine can still be used as optimal mutational agent. Reports suggested that colchicine can induce tetraploidy at the rate of 21.8% as compared to oryzalin (4.3%) (140). Nevertheless, the exposure time and concentration of specific antimitotic agents are the most vital factors. Excessively high concentrations are toxic while too low doses might be ineffective and sometimes reduced viability may even be lethal. In addition, high exposure times and concentration could result in higher ploidy levels than required (148). To date, the investigation has confirmed that lower concentrations are steered up by a longer duration of exposure and vice versa. But one cannot affirm the fact that one antimitotic agent is the most significant and successfuleven for certain species, as it is significantly affected by the explant of donor plants and genotypes(139).

Hormonal, morphological and functional metabolite variations on chromosome doubling in garlic genome

Genome doubling is an event of a single macro mutation having many phenotypic consequences (150). Superiority in terms of genetic adaptability, morphological changes and environmental stresses along with the giga effect as bigger organs were preeminent in autopolyploids as compared to diploids (151). However, polyploidy does not always reveal higher enlargement and/or quality (152).It exemplifies phenotypic stochastic changes which are initiated by ploidy and are species-dependent(139). Autotetraploid/autopolyploid garlic was reported to be accompanied by wider, thicker, shorter leaves and developmentally delayed roots accompanying difficulty to acclimatize in a greenhouse environment (153, 154, 155, 156, 157).

As compared to the diploid counterpart, dwarfing is reported to be a more noticeable phenotype in tetraploid garlic. Following genome doubling, extreme dwarfness after tetraploidization was reported in several other crops especially cabbage (158), Chinese jujube (159) and apple (160). Moreover, polyploidization alters plant phenology, morphology and physiology not only in one generation but also in a few other generations (161). In addition, individuals may experience "genomic shock" following polyploidization which fetches disruption between cytoplasmic and nuclear components inhibiting meiosis and mitosis (162,163). Furthermore, escalation in cell volume after polyploidization might slow down metabolic activity and reduce the rate of cell division, consequently resulting in slow rather low growth rates

(164,165).Recent studies have revealed that ploidy levels for organ development and dwarfism were regulated by a complex interaction of different phytohormones (166).In addition, mutational defects in biosynthesis or signalling of plant hormones can result in dwarfism (167), whereas, deficiency of brassinosteroids (BRs) and active GA was also reported in the dwarf phenotype of tetraploid apple, Arabidopsis and rice(168).Convincingly, studies have further revealed that deficiency of active GA results in dwarf and semi-dwarf rice and reduces plant height (169). Such impaired GA biosynthesis is observed in dwarf banana phenotypes (170). Besides, lower accumulation of GA can suppress additional cell division and lower the size of the division zone inhibiting thereby leaf growth and contributing to semi-dwarf phenotype in maize (171), also defective mutants in BR synthesizing genes also reduce plant height in rice and barley (172). This might be attributed to the inhibition of transcription factors, genes, or enzymes related to BR synthesis and signal transaction pathways affecting cell expansion or elongation (173). Genome multiplication bestows enhancement of secondary and functional metabolites which has got significant commercial value for medicinal and industrial importance (141). Studies have also revealed that the dwarfing tetraploid plantlets showed higher levels of allicin, DADS, and DATS than their diploid counterparts, suggesting the potentiality as a breeding method in garlic for abundant production of pharmaceuticals(140).

Gene expression analysis and its use in *in vitro* and *in vivo* studies

Biological responses to garlic and its compounds can be studied by metabolomics (changes in small molecule metabolites), epigenomics (DNA methylation and histone modification), proteomics (effects on activities and concentration of protein) and transcriptomics (patterns of gene expression) (174). In garlic, here, the measurement of gene expression refers measurement of mRNA at the transcript level. This expression is measured either by targeting a small number of genes of interest or by determining a broad array of genes. It has been frequently used by researchers in preclinical studies instigating the biological effects of garlic. The changes in mRNA correspond to the determination of protein changes. It is of great importance as it sometimes accompanies the measurement of mRNA. In addition, means of other elucidating biological mechanisms are present but those are not restricted only to garlic-induced and garlic-derived metabolites, measurement pertaining to enzyme activity, activation of the transcription factor, morphologic changes at the cellular level (*i.e*, changes occurring apoptosis), redox status (175-180). Gene expression thus can reveal many early mechanistic events which may clinically underlie important health benefits pertaining to garlic intake

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Cell studies

In an investigation, Zhou et al(181) used molecular modes of action lipid-lowering effects of semi-quantitative RT-PCR, oligonucleotide microarrays and lipid-lowering effects of DATS applied to HepG2 cellscomprising 452 genes related to hyperlipidemia and atherosclerosis. By DATS treatment, the hepatocyte nuclear factor 4a (*HNF4A*) and the mRNA of the peroxisome proliferator-activated receptor α (PPARA) were elevated. On the other hand, the mRNA of the cytochrome 450 of family 7 and subfamily A, member 1 (*CYP7A1*) was lowered which suggests the involvement of these genes in the lipid-lowering effects of DATS. Horev-Azariaet al(182) in their study used RT-PCR and oligonucleotide arrays and treated allicin to human umbilical vein endothelial cells to try-out the hypothesis that allicin elevates the expression of cellular reduced glutathione (GSH) and oxidative-stress–related genes. They observed that out of 22,277 genes on the microarray chip, 100 genes were down-regulated by at least one-half and 116 genes were up-regulated by at least 2-fold. Besides, among different modified oxidative stress-related genes, solute carrier family 7 (*SLC7A11*), glutamate-cysteine ligase modifier (*GCLM*) and thioredoxin reductases 1 and 2 (*TXNRD1* and *TXNRD2*, respectively) were elevated by 2.1 to 3.2 fold to that of heme oxygenase 1 (*HMOX1*) by 11-fold. The study thus revealed that when *HepG2* cells were treated with DATS (diallyl trisulphide), DADS (diallyl disulphide) and diallyl disulphide (DAS), *HMOX1* was upregulated. In response to allicin treatment, glutathione concentration gets increased. In addition, *SLC7A11* (solute carrier family 7) andglutamate-cysteine ligase modifier (GCLM), the rate-limiting enzyme of glutathione synthase, contribute to the maintenance of GSH (reduced glutathione) concentrations. Besides, *TXNRD1* and *TXNRD2*were reported to reduce thioredoxin and thus help in protecting against oxidative stress. Taking the entire context together, the upregulation of oxidative stress-related genes might result in increased glutathione concentrations and decreased cellular damage by reactive oxygen species.

Nevertheless, on the basis of the biological phenomenon being investigateda relatively small number of genes have been targeted. Zenget al(183) reported that in *LO2* cells *HMOX1*(heme oxygenase 1) was the only gene to have protective effects of DADS against ethanol-induced oxidative stress. This happens as *HMOX1* can mediate hepatoprotection induced by DADS. When subjected to ethanol, DADS increases *HMOX1* protein and *HMOX1* mRNA in *LO2* cells. In addition, DADS can lower ethanol-provoked cytotoxicity by increasing concentrations of GSH and suppresses concentrations of malondialdehyde, aspartate transaminase and lactate dehydrogenase activities. Thus, DADS by upregulating *HMOX1* proved to be a protectant against ethanol-provoked liver injury. Lee et al(184) selected a single gene to investigate the effects of DAS on inflammatory joint disease which is modelled by an *in vitro* system using chondrocytes (primary), primary synovial cells and HIG-82 cells. The results revealed that when pro-inflammatory monosodium was co-incubated with DAS and united in *HIG-82* cells, expression of *PTGS2* (prostaglandin-endoperoxide synthase 2) got inhibited. Thus, by means of *PTGS2* suppression, DAS exerted anti-inflammatory effects. Kim et al(185) reported the ability of *S*-allylcysteine to inhibit the expression of nitric oxide synthase 2 (*Nos2*) in murine macrophage (*RAW264.7*) cells. Large quantities of NO are produced on activation of *Nos2* and its overexpression is correlated with dreadful diseases like atherosclerosis, rheumatoid arthritis and ulcerative colitis. *S*-Allylcysteine, the chief organosulfur component in AGE, has the potentiality to modify genes related to cardiovascular genes. Malekpour-Dehkordiet al(186) tested *S*-Allylcysteine to determine whether it

can modulate the expression of ATP-binding cassette transporter A1 (*ABCA1*)in human THP macrophages. *ABCA1* is associated with elevated HDL-concentrations and is a key factor of apolipoprotein-mediated cholesterol efflux. By treatment with *S*-allylcysteine (as compared to control), both *ABCA1* protein and mRNA were increased. This indicated that cholesterol efflux is stimulated by *S*-allylcysteine via *ABCA1*and thereby slows down the process of artherosclerosis. In a study, DADS,DAS and DATS were tested using primary rat hepatocytes to induce expression of *Gstp1* (glutathione S-transferase pi 1), which is a reliable marker for testing inhibitors of carcinogenesis. At 200 mmol/L, DATS and DADS elevated protein expression and *Gstp1* mRNA expression by 6.5- and 5.1-fold. In addition, the expression of *Gstp1*was correlated to a number of sulphur atoms i.e., DATS > DADS > DAS. In a study pertaining to apoptosis, garlic polysulfides can induce apoptosisin several cancer cell lines but do not affect normal cell lines. Such mode of action of apoptosis is mediated through the generation of reactive oxygen species and regulation of apoptotic B cell CLL/lymphoma 2 (*BCL2*) protein family(187). In another study by Chu et al (188), targeted the influence of garlic on the mechanism of cell death and treated *HepG2* and *Hep3B* cells with allicin. Apoptosis-inducing factor, apoptosis-related cysteine peptidase (*CASP3*) and mitochondrion-associated 1 (*AIFM 1*) were measured by them in their study after treating the cells with allicin. With allicin treatment, the expressions of these genes got significantly increased. As these genes were found active apoptotic pathways, the results were solid enough to prove that cell death induced by allicin was more apoptotic than autophagic.

Animal studies

Just as *in vitro* studies, gene expression has also been an important part of *in vivo* animal studies keeping the same goal pertaining to the possible health benefits of garlic. For reducing the risk of cardiovascular disease, the use of garlic has been an arena where gene expression has contributed to the understanding of protective mechanisms possible behind this. Mohammadi et al(189) measured the gene expressions of ATP binding cassette subfamily G member 8 (*Abcg8*), Niemann-Pick C1–like 1 (*Npc1l10*), ATP binding cassette subfamily G member 8 (*Abcg8*) and *Abca1* in murine duodenal tissue. Their motto was whether garlic could modulate cholesterol efflux mechanisms of the intestine to control hypercholesterolemia. *Abca1* has a potent role in the formation of HDL-cholesterol with a concomitant decrease in *Abcg5* and *Abcg8* cholesterol efflux transporters. On the other side, expression ofcholesterol transporter, Npc1l1 mRNA having a role in enterocytic cholesterol uptake was found to be low in jejuna of mice when fed with garlic as compared to the mice fed with the same diet without garlic. Ou et al(190) explored the influence of garlic oil on cardiac dysfunction (diabetes-related) and gene expression (cardiac) in type 1 diabetic rats when induced with streptozotocin. The rats were thereby fed 0, 10, 50, or 100 mg of garlic oil/kg body weight. It was observed that there was a shift in the expression of mRNA of myosin heavy-chain 6, cardiac muscle a (*Myh6*) to myosin heavy-chain 7, cardiac muscle b (*Myh7*) in diabetic rats, which is recognised as maladaptive response to cardiovascular stress. Such myosin drives contraction of cardiac muscle. They reported an imbalance of gene expression of *Myh6* and *Myh7* which was dose-dependent. This happened when garlic oil was fed to rat for 16 days which resulted in complete normalization of *Myh6* and *Myh7* ratio at a dose of 100 mg/kg. In addition, the co-occurrence of improvements induced by garlic-oil in cardiac α-actin, activities pertaining to apoptosis signalling and in superoxide dismutase 1 as well as amelioration in an imbalance of *Myh6* and *Myh7* ratio suggesting the protective nature of garlic-oil against diabetes-induced cardiomyopathy.There are several studies designed to judge the ability of garlic to inhibit cancer (experimentally induced) through the expression of genes related to xenobiotic metabolism. Bose et al(191) reported that on administering DADS to mice, benzo[a]pyrene induces protection against fore-stomach carcinogenesis, such chemoprotective activity has a correlation with the expression of phase II gene Gstp1. After DADS treatment, expression of Gstp1 was maximum between 6hr and 12 hr. In addition, by using DADS analogs, structure-activity studies were undertaken to assess the bioactivities of the disulphide chain and allyl groups of the DADS molecule. Thus these studies suffice the facts that disulfide chain and allyl groups were required both for high induction of Gstp1.Andorferet al(192) induced several other GSTs in the small intestine, colon, stomach and liver of mice which were administered with DADS or allicin.With 2 doses of allicin (2.2 mmol/mouse) or DADS (20 mmol/mouse) the expressions of proteins of glutathione S-transferase m 1 (*Gstm1*), glutathione S-transferase m 4 (*Gstm4*) and mRNA were induced. The results reported induction was more prominent in the small intestine and stomach than thecolon and liver. However, there were differences in gene expression with respect to tissue type. Infact, DADS induced the expression of Gsta5 and Gsta1 in the small intestine, liver, colon and stomach of the mouse. However, the research group reported that Gsta4 (glutathione S-transferase α 4) got increased in intestinal and stomach tissue. Contrary to an earlier study, DADS or allicin could not modify Gstp1 which might be due to a different dosing regimen. In addition, no effect was observed in the tissues of the brain and heart on the administration of DADS. Wu et al(193) studied xenobiotic metabolism in the liver of rats using garlic oil and its constituents using DADS, DAS and DATS. They used dosages of 20, 80, or 70 mg/kg of DAS, DADS, and DATS, respectively per kg of body weight. Northern blot analysis in this study was used to measure the level of mRNA. Whereas, Western blot and SDS-PAGE were used to measure proteins. It was observed that proteins and mRNA of cytochrome P450, family 2, subfamily B, polypeptide 1 (*Cyp2b1*), *Gstp1*, cytochrome P450, family 1, subfamily A, polypeptide 1 (*Cyp1a1*) and cytochrome P450, family 3, subfamily A, polypeptide 1 (*Cyp3a1*) were upsurged by garlic oil and its 3 diallyl sulphides, but cytochrome P450, family 2, subfamily E, polypeptide 1 (*Cyp2e1*) was suppressed. Furthermore, Hu et al(194) observed that the structure-activity relation of DADS, DAS and DATS for Gstp1 was found similar to that reported in primary rat hepatocytes when treated *in vitro*. Animal studies involving gene expression to judge possible ways of cancer protection by garlic do not only focus on xenobiotic metabolism. Balasenthil et al (195) studied tumour suppressor gene retinoic acid receptor (*Rarb*) in Syrianhamsters when subjected to DMBA-induced hamster buccal pouch carcinogenesis. This buccal

Table 2. Mechanisms of bioactivity induced by garlic and garlic-derived compounds in preclinical studies.

Studies taken in	Medicaments used	Outcomes	References
		Cell	
HepG2 and Hep3B	Allicin	Enhanced *AIFM1mRNA*, *CASP3* and *CASP3* protein	(188)
RAW264.7	S-Allylcysteine	Decreased *NOS2 mRNA*	(185)
Primary rat hepatocytes	DATS, DADS	Enhanced protein and *Gstp1 mRNA*	(196)
Primary synovial cells, HIG-82 and chondrocytes	DAS	Decreased protein and *PTGS2 mRNA*	(187)
HepG2	DATS, DADS, DAS	Enhanced *HMOX1* and *NQO1 mRNA*	(197)
THP-1 macrophages	S-Allylcysteine	Enhanced protein and *ABCA1 mRNA*	(186)
		Animal/tissue	
Rat liver	DAS, AMS	Enhanced protein levels and no effect on *Cyp1a2 mRNA* or *Cyp2e1*	(198)
Mouse liver/stomach	DADS	Enhanced Gstp1 mRNA	(191)
Rat liver	DATS, DADS, DAS and garlic oil	Decreased *Cyp2e1*, enhanced *Cyp3a1*, *Cyp1a1*, *Cyp2b1* and *Gstp1 mRNA*	(193)
Rat adipose tissue	Garlic extract	Enhanced*Ucp2 mRNA* and reduced *Srebf1*, *Pparg* and *Fabp4*	(199)
Mouse liver, small intestine, colon and stomach	DADS, Allicin	Enhanced *Gsta4*, *Gstm4*, *Gstm1*, *Gsta1*, protein and *Gsta5 mRNA*	(192)
Rat colonocytes	DADS	49 out of 588 genes modification related to detoxification, proliferation, signal transduction, cell cycle and transport	(200)
Buccal pouch of Syrian hamster	Garlic extract (acqueous)	Enhanced *Rarb mRNA* in DMBA-induced tumors	(195)

pouch has been used to use it as the model for oral cancer tumorigenesis. By altering DMBA treatment, one group acquired garlic extract (aqueous) at a dose of 250 mg/kg body weight for 14 weeks every other day but other groups received either of DMBA or garlic treatment or neither of the treatments. It was further observed that 100% of the hamsters when treated with only DMDA, well-differentiated squamous cell carcinoma developed. While on the other groups, no squamous cell carcinoma was developed, and only hyperplasia at a mild level was observed in only garlic and garlic + DMBA groups. The above experiment thus suffice the fact that expression of *Rarb* gene was restored to normal level validating the fact that *Rarb* gene has a potent role in chemoprevention using garlic extract.

Human studies

To the best of our knowledge, there are no human studies pertaining to mRNA gene expression in assessing the possible health benefits of garlic. The measurement of gene expression in humansdepending on the tissue of interest might carry a significant risk or next to impossible. Hajda et al(201) approved the challenges of assessing human tissue. Their objective was to determine how garlic extract can influence the drug metabolism of saquinavir, a drug (antiretroviral) in the treatment of HIV infection. After consuming saquinavir, midazolam was sedated to 10 volunteers and were subjected to gastroduodenoscopy for measuring P-glycoprotein (which is an ATP-dependent efflux pump) and CYP3A4 protein, especially in the excised duodenal intestinal mucosa. Subjects were then made to undergo a gastroduodenoscopy for the second time after ingesting garlic extract (600mg) twice for 21 days. The results revealed garlic extract-induced intestinal P-glycoprotein having no effect on CYP3A4 protein. But a significant degree of medical involvement is required to access the target tissue. Another difficulty is obtaining peripheral blood samples. But, blood's gene expression analysis may provide high insight into how garlic provokes the human immune system by identification of basophils, neutrophils, eosinophils, monocytes, T cells and B cells in response to the intake of garlic. In addition, such gene expression analysis of blood might serve as a gateway of indirect measures of biological activity throughout the body as blood cells connect and interact with cells of other organs thus the possibility of gene expression of blood cells might change in parallel to that of other cells. Liewet al(202) used expressed sequence tag and microarray hybridization approaches and observed that >80% of the blood transcriptome of humans was shared with that of tissue of the stomach, heart, brain, liver, lungs and spleen. Moreover, with the aid of RT-PCR measurement MYH7(typically associated with the heart) and INS (relatedto the pancreas) exhibited expression of genes in

blood thereby demonstrating expression of tissue-specific genes in blood.

Conclusion and future direction

At the global level, research in garlic is still underway and many issues pertaining to production and productivity needs to be resolved especially in the domestic and international market. Conservation of genetic diversity is of high importance for ensuring future breeding programs which might be the base for executing artificial selection. Huge germplasm collection which needs field trials and evolution of such trials in terms of yield and quality is high time taking to assess. In garlic, breeding has been hampered by the absence of appropriate methods to create genetic variability within the existing germplasms. The use of markers RAPDs, AFLPs, SRAPs, SSRs, isozymes and DArTseqh as proved to be a potent tool in complementing huge number of bioassays and are highly useful in breeding programs in garlic as it actually helps to track loci, genome regions and in the tracking of genes. In addition, genome duplication of unique or specific alleles present in particular germplasm/accession may contribute in genetic improvement in garlic. Furthermore, genome duplication not only multiplies hereditary materials but also mediates selection to act upon. Besides, in last couple of years both biolistic and *Agrobacterium* gene transfer systems havebeen widely developed in garlic. Nonetheless, in the coming future, a deeper and further understanding of the genetic make-up of reproductive traits of garlic essential for physiological, genetic knowledge and exploitation of the genetic potential of the crop is required. In addition, transcriptome and *omics* in garlic studies might actually help in the development of transferable and user-friendly co-dominant markers which would fetch efficient garlic breeding programs.

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Conflict interest

The authors declare no conflict of interest

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