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Molecular analysis of genetic stability in *in vitro* regenerated plants of broccoli (*Brassica oleracea* L. var. *italica*)

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Genetic integrity of tissue culture-raised plantlets of broccoli cv. Solan green head from India was assessed using RAPD. First, highly efficient, reliable and high-frequency shoot regeneration was achieved in leaf (62.96%) and petiole (91.11%) explants on MS medium containing 4.5 mg/l BAP + 0.019 mg/l NAA, and 4.0 mg/l BAP and 0.5 mg/l NAA respectively. Maximum rooting ability (93.99%) with healthy and vigorous roots was observed on MS medium containing 0.20 mg/l NAA. The regenerated plantlets with well-developed shoot and root system were acclimatized successfully. For genetic stability studies, a total of 66 amplicons were amplified using 15 informative primers with a high degree of monomorphism (88.45%) across the mother plant and 20 randomly selected *in vitro* regenerated plantlets.

Keywords: Broccoli, genetic fidelity, leaf and petiole explants, plant regeneration, RAPD–PCR.

BROCCOLI is an important vegetable crop of the cabbage family Brassicaceae (formerly Cruciferae) with chromosome number $2n = 18$. It is nutritionally rich with medicinal property and classified as the *italica* cultivar group of the species *Brassica oleracea.* It is high in vitamins C and A, soluble fibre and contains the medicinally important anticancerous compound sulphoraphane with potential application in the pharmaceutical industry[1,2]. Plant tissue culture is an important aspect of plant biotechnology because genetic manipulation is now necessary to harness its potential to overcome crop yield losses due to biotic and abiotic stresses. So establishment of a highly efficient, reliable and stable plant regeneration system without the risk of genetic instability is a major step in genetic improvement. Scaling-up of any micropropagation protocol is severely hindered due to incidence of somaclonal variations[3]. The occurrence of somaclonal variations is a potential drawback when the propagation of elite plant is intended[4], where assessment of the tissue culture-raised variations using clonal fidelity is required to maintain the advantages of the desired elite genotypes such as superior growth, resistance to abiotic and biotic stresses and other horticultural and agronomically important

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traits. Several techniques have been developed to assess the genetic purity of the tissue culture-raised plants such as morphological, physiological and cytological studies, isozymes[5], field assessment and molecular studies[6]. Several DNA markers have been successfully employed to assess the genomic stability in regenerated plants, including those with no obvious phenotypic alterations[7].

In this communication, we report the development of a reproducible high-frequency plant regeneration protocol of broccoli (*Brassica oleracea* L. var. *italica* cv. Solan green head) using leaf and petiole explants. Broccoli cv. 'Solan green head' was developed at the Department of Vegetable Science, Dr Y. S. Parmar University of Horticulture and Forestry, Solan, India and is used in our breeding programme because of its good yield potential and early maturity. The ability of this regeneration protocol to deliver genetically uniform and stable regenerants was evaluated by RAPD markers. Presently, this regeneration protocol is being used in our laboratory for the development of transgenic broccoli (*Brassica oleracea* L. var. *italica* cv. Solan green head) with insect resistance gene (*cryIAa*) through *Agrobacterium*-mediated gene transfer technique.

Certified, uniform and healthy seeds of broccoli were germinated in pots in the glasshouse in our Department. *In vivo*-grown seedlings (18–20 days old) were used as a source of explants. Young tender leaves and petiole explants were excised and surface-sterilized using 0.1% $HgCl_2$ for 1 min and 30 sec and washed thrice with distilled water. After treatment with $HgCl_2$, the explants were treated with 0.2% bavistin for 2 min and 30 sec and again washed thrice with sterilized distilled water in order to remove traces of sterilants. The explants were cut into pieces of 0.5–1.0 cm size and cultured on Murashige and Skoog (MS) basal medium[8] supplemented with various concentrations and combinations of benzylaminopurine (BAP) and napthaleneacetic acid (NAA) for multiple-shoot induction. The pH of the medium was adjusted to 5.8 before adding agar agar to it. The medium was poured into culture vessels and sterilized at 1.08 kg/cm^2 for 15 min in an autoclave. All the aseptic manipulations were carried out in a laminar airflow chamber. After inoculation, all the cultures were kept in our culture room at 26 ± 2°C and 70–80% humidity under 16 h photoperiod with a light intensity of 40 m $mol/m^2/s$ provided by cool white fluorescent lamps.

Leaf explants were cut and their surface was gently tapped with scalpel blade to cause injury and the petiole explants were cut into small pieces. The explants were then cultured on MS basal medium supplemented with different combinations and concentrations of BAP and NAA (mg/l). Five flasks with five explants each were inoculated for every combination and kept in the culture room. Each experiment was repeated thrice and observations were recorded at weekly intervals. Explants were evaluated for percentage shoot regeneration and average number of shoots per explant. Shoot proliferation and elongation were obtained on the same best shoot regeneration medium. After shoot multiplication, the *in vitro* developed shoots were transferred to root regeneration medium containing different concentrations of NAA. After the regeneration of roots, the percentage of shoots forming roots was calculated.

The regenerated plantlets with well-developed shoot and root system were carefully taken out and washed gently under running tap water to remove traces of medium from the roots. The *in vitro*-regenerated plantlets were transferred to earthen pots containing a mixture of sand, soil, and farmyard manure (1 : 1 : 1 ratio). During initial days of acclimatization, high humidity (70–80%) was maintained by covering the pots with polythene bags. The percentage survival of the hardened plants was recorded after 4–5 weeks of transfer to the pots.

For RAPD analysis, 20 hardened plantlets were randomly selected and evaluated for genetic similarity with each other and with the mother plant. The modified CTAB (cetyltrimethylammonium bromide) method with some modifications[9] was used to extract genomic DNA from leaves of the mother plant and *in vitro*-regenerated clones. PCR amplification of genomic DNA of the mother plant and *in vitro*-regenerated plantlets of broccoli was carried out using 21 random decamer oligonucleotide primers (Sigma Alderich, USA) in BIORAD MJ Mini DNA amplification system. The PCR reaction mixture of 25 μl contained 14.20 μl autoclaved double-distilled water, 2.50 μl *Taq* DNA polymerase buffer, 2 μl random primer, 2 μl dNTPs mixture, 0.3 μl *Taq* DNA polymerase and 4 μl genomic DNA. PCR was carried out in a thermocycler with a total of 40 cycles. Each cycle consisted of denaturation at 94°C for 1 min, annealing at 36°C for 1 min, and extension at 72°C for 2 min. All the PCR samples were given 5 min pre-PCR amplification at 94°C and 10 min post-amplification at 72°C. The amplification products were resolved by electrophoresis on a 1.2% agarose gel, stained with ethidium bromide, photographed in Alpha-imager gel documentation system and the images were saved for analysis. The size of the amplicon was determined from a 100–10,000 bp DNA ladder. Random primers, each consisting of 10 nucleotides, were obtained and used for RAPD–PCR studies. Out of 21 primers, only 15 gave amplification and clearly identifiable amplicon. These were used for genetic fidelity studies.

Data were analysed for different parameters of plant regeneration and genetic fidelity studies. Plant regeneration studies based on mean values per treatment were made using analysis of variance for completely randomize design[10]. Data analysis of genetic fidelity studies was carried out only for those primers which gave scorable patterns for the clones under study. After amplification, total scorable bands were calculated. The number of monomorphic and polymorphic bands, percentage of monomorphism and size range of amplicon in base pairs

Table 1. Effect of various concentrations and combinations of BAP and NAA (in MS basal medium) on shoot regeneration from leaf explants of broccoli (*Brassica oleracea* L. var. *italica*)

Medium composition	Average number of shoots regenerated per explant	Per cent shoot regeneration
MS medium + 3.0 mg/l BAP + 0.5 mg/l NAA	1.063	44.44 (41.80)
MS medium + 3.5 mg/l BAP + 0.5 mg/l NAA	0.930	37.22 (37.58)
MS medium + 4.0 mg/l BAP + 0.5 mg/l NAA	1.490	62.96 (52.52)*
MS medium + 4.5 mg/l BAP + 0.5 mg/l NAA	0.997	57.78 (49.48)
MS medium + 5.0 mg/l BAP + 0.5 mg/l NAA	1.353	53.33 (46.92)
MS medium + 5.5 mg/l BAP + 0.5 mg/l NAA	2.483*	62.22 (52.13)
$CD_{0.05}$	0.317	9.060 (5.287)
SE ±	0.145	4.158 (2.426)

Values in parenthesis are arc sine transformed. *Denotes highly significant values.
BAP, Benzyl amino purine; NAA, Naphthaleneacetic acid; MS, Murashige and Skoog.

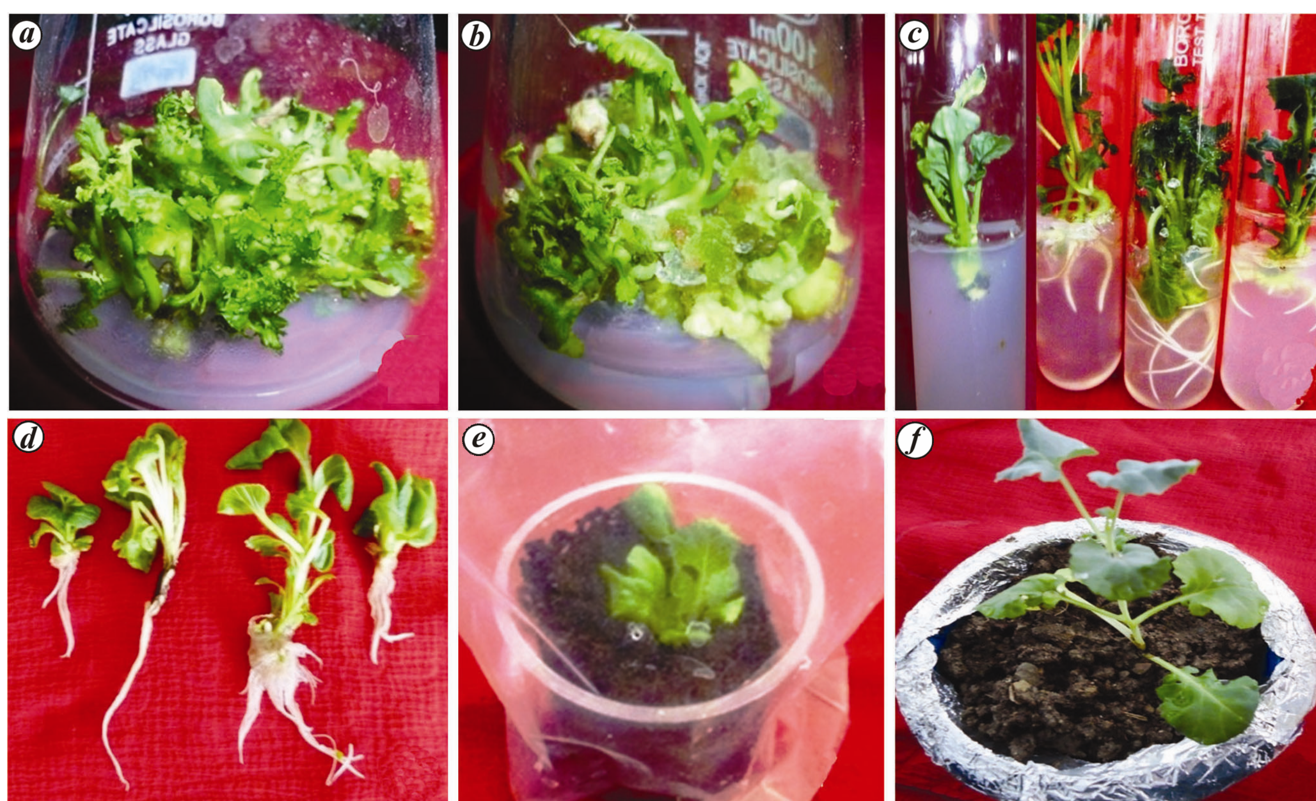

Figure 1. *In vitro* plant regeneration studies in broccoli (*Brassica oleracea* L. var. *italica* cv. Solan green head). ***a***, Shoot regeneration from leaf explants after 32 days in culture on MS medium supplemented with 4.0 mg/l BAP and 0.5 mg/l NAA. ***b***, Shoot regeneration from petiole explants after 28 days in culture on MS medium supplemented with 4.5 mg/l BAP and 0.019 mg/l NAA. ***c***, Root regeneration in *in vitro* regenerated shoots after 18 days in culture on MS medium supplemented with 0.20 mg/l NAA. ***d***, Development of complete plantlet. ***e***, Hardening of *in vitro* regenerated plantlet. ***f***, Successful acclimatization of plant in the pot.

were also calculated separately to assess the genetic fidelity of tissue culture-raised plants.

The requirement of various plant growth regulators for inducing shoot-bud differentiation varies from organ to organ and from plant to plant. It depends on the intrinsic status of these growth-promoting substances within the plant species. In our experiments, we used young, tender, completely green leaf and petiole explants from *in vivo*-grown seedlings for efficient shoot regeneration. The explants began to expand after one week of culturing and callus proliferation occurred after two weeks of culturing for both explant types. After 6–7 weeks, significant differences were observed among the treatments for percentage of explants forming shoots. Multiple shoot induction was achieved using different concentrations and combinations of BAP and NAA for both explant types. In the case of leaf explants, maximum shoot regeneration frequency, i.e. 62.96% and 62.22% was obtained on MS basal medium supplemented with 4.0 mg/l BAP and 0.5 mg/l NAA, and 5.5 mg/l BAP and 0.5 mg/l NAA

Table 2. Effect of various concentrations and combinations of BAP and NAA (in MS basal medium) on shoot regeneration from petiole explants of broccoli (*B. oleracea* L. var. *italica*)

Medium composition	Average number of shoots regenerated per explant	Per cent shoot regeneration
MS medium + 2.0 mg/l BAP + 0.019 mg/l NAA	1.013	51.11 (45.64)
MS medium + 2.5 mg/l BAP + 0.019 mg/l NAA	1.177	46.66 (43.09)
MS medium + 3.0 mg/l BAP + 0.019 mg/l NAA	1.620	60.37 (51.02)
MS medium + 3.5 mg/l BAP + 0.019 mg/l NAA	2.053	69.63 (56.61)
MS medium + 4.0 mg/l BAP + 0.019 mg/l NAA	2.573	83.33 (65.97)
MS medium + 4.5 mg/l BAP + 0.019 mg/l NAA	3.617*	91.11 (72.88)*
$CD_{0.05}$	0.356	7.809 (5.248)
SE±	0.163	3.584 (2.408)

Values in parenthesis are arc sine transformed. *Denotes highly significant values.

Table 3. Effect of various concentrations of NAA (in MS half strength basal medium) on per cent root regeneration from *in vitro* developed shoots of broccoli (*B. oleracea* L. var. *italica*)

Medium composition	Per cent root regeneration
MS (half strength) medium + 0.05 mg/l NAA	78.15(8.837)
MS (half strength) medium + 0.10 mg/l NAA	81.11(9.006)
MS (half strength) medium + 0.20 mg/l NAA	93.99(9.693)*
$CD_{0.05}$	8.429
SE ±	3.444

Values in parenthesis are arc sine transformed.
*Denotes highly significant values.

respectively (Table 1 and Figure 1 *a*). For the petiole explants, the highest shoot regeneration (91.11%), with highest mean number of shoots (3.61) per explant was recorded on MS medium supplemented with 4.5 mg/l BAP and 0.019 mg/l NAA (Table 2 and Figure 1 *b*). The regenerated shoots obtained from both the explants were separated and subcultured on the same best shoot regeneration medium. Shoot multiplication and elongation took place on the same medium.

During the present study, BAP and NAA used in MS medium for shoot regeneration studies were quite effective in multiple shoot induction in leaf and petiole explants compared to previous studies[11,12]. High-frequency shoot regeneration has been reported by different researchers using BAP and NAA in leaf, petiole, cotyledon and hypocotyl explants of broccoli[11,12], tomato[13] and cabbage[14]. Between these two explants, petiole (91.11%) was found better for shoot regeneration and multiplication compared to leaf (62.96%).

In vitro-developed elongated shoots (about 2–3 cm long) obtained from leaf and petiole explants were excised and cultured on MS medium containing different concentrations of NAA. Healthy and vigorous roots were formed after 10–14 days of inoculation. Maximum root regeneration response of 93.99% was obtained on MS medium supplemented with 0.20 mg/l NAA and well-developed complete plantlets were observed after 20–22 days (Table 3, Figure 1 *c* and *d*). It has been reported in a previous study[15] that high concentration of NAA is more effective for root regeneration compared to high concentration of IAA or IBA. It has also been reported that medium supplemented with 0.2 mg/l IBA is most suitable for root regeneration with maximum root regeneration response[14]. The regenerated plantlets of broccoli were successfully acclimatized in pots containing mixture of soil and organic manure 1 : 1 with 75% survival frequency and were morphologically uniform.

During the present study, the genetic fidelity of tissue culture-raised plantlets of broccoli was assessed using RAPD. The capacity to analyse the genomes (nuclear and organellar) with the number of molecular DNA marker represents one of the most recent developments in molecular biology. Genetic molecular markers are considered to be reliable in monitoring variability in the DNA sequence of regenerated plantlets. There are reports available which have applied the RAPD technique to detect somaclonal variation[16–18] and to identify micropropagated plants and cultivar[19–21]. Genetic fidelity of *in vitro*-raised plantlets of broccoli was studied using 21 random decamer primers. Among these, only 15 had shown scorable banding patterns and yielded 66 amplicons with an average of 2.96 bands per primer. Of the 66 amplicons, 56 showed monomorphism and 10 were observed to be polymorphic resulting in 88.45% monomorphism among all the *in vitro*-regenerated plantlets and mother plant studied. The number of bands for each primer varied from 1 to 7 and the size of the amplification product ranged from 124 to 4823 bp (Table 4 and Figure 2). The RAPD fragments obtained after amplification of genomic DNA from 20 randomly selected *in vitro*-raised plantlets and from the mother plant of broccoli were scored for presence as 1 (band present) and absence as 0 (band absent) for each plantlet. The results show that the regeneration protocol has not induced any gross genetic changes and also the RAPD approach suitable for the detection of variants.

Some variations have been observed among the *in vitro*-raised plantlets and the mother plant of broccoli that

Table 4. Total number of amplified fragments and number of monomorphic and polymorphic fragments generated by RAPD-PCR using 15 informative random decamer oligonucleotide primers.

Primer	Sequence (5′ → 3′)	Size range of amplified bands in base pairs	Total number of amplified bands	Total number of monomorphic bands	Total number of polymorphic bands	Percentage monomorphism
OPB-04	GGACTGGAGT	206 to 1387	4	4	–	100
OPB-05	TGCGCCCTTC	181 to 1250	5	3	2	60
OPB-10	CTGCTGGGAC	523 to 921	3	2	1	66.66
OPB-11	GTAGACCCGT	189 to 3552	7	5	2	71.40
OPB-12	CCTTGACGCA	655 to 885	2	2	–	100
OPB-14	TCCGCTCTGG	828 to 4823	6	6	–	100
OPB-16	TTTGCCCGGA	320 to 3676	7	7	–	100
OPC-05	GATGACCGCC	635 to 1000	3	3	–	100
OPC-06	GAACGGACTC	124 to 542	3	3	–	100
OPC-07	GTCCCGACGA	1268	1	1	–	100
OPC-09	CTCACCGTCC	192 to 2283	7	2	5	28.57
OPC-10	TGTCTGGGTG	761 to 1870	3	3	–	100
OPC-18	TGAGTGGGTG	328 to 1165	5	5	–	100
OPC-19	GTTGCCAGCC	151 to 1871	7	7	–	100
OPC-20	ACTTCGCCAC	390 to 853	3	3	–	100
Total			66	56	10	88.45

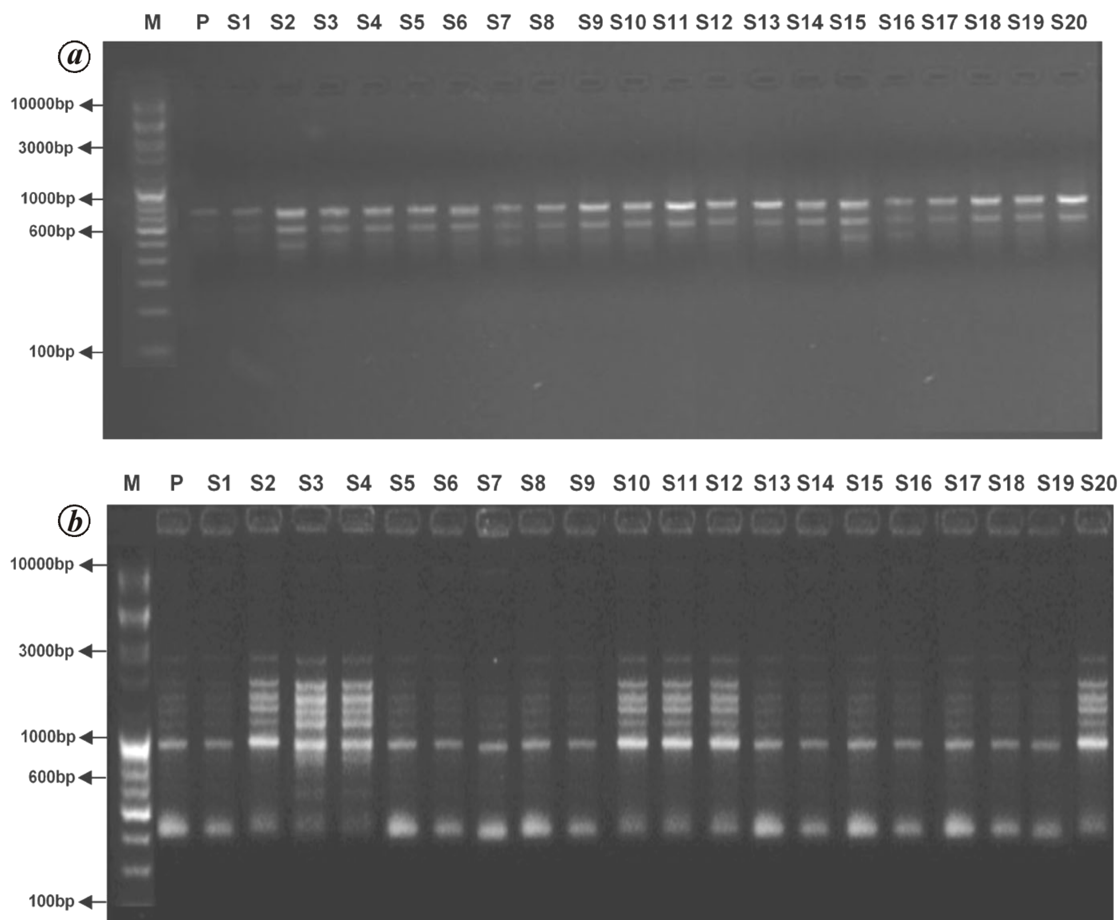

Figure 2. Genetic fidelity studies in *in vitro* regenerated plantlets of broccoli (*B. oleracea* L. var. *italica*) using RAPD markers. ***a***, RAPD profile generated by amplification of DNA of mother plant (P) along with 20 *in vitro* raised plantlets of broccoli with primer OPB-10. ***b***, RAPD profile generated by amplification of DNA of mother plant (P) along with 20 *in vitro* regenerated plantlets of broccoli with primer OPB-16. M, Marker/ladder (100–10,000 bp), P, Mother plant of broccoli. S_1–S_{20}, Twenty randomly selected *in vitro* regenerated plantlets of broccoli.

might arise during the cell division or differentiation under *in vitro* conditions[22]. The proposed mechanism is DNA methylation that may in part explain the changes that occur under *in vitro* conditions[23–25]. Qin *et al.*[26] have analysed the genetic stability in tissue culture-raised plantlets of broccoli using RAPD–PCR. A total of 62 arbitrary decamer primers were screened and a very low percentage of polymorphism was reported; the similarity ranged from 98% to 100%. Whereas in the present study 10–12% polymorphism in tissue culture-raised plantlets and mother plant of broccoli has been reported. In conclusion, analysis of genetic stability of *in vitro* regenerants using RAPD markers is an effective and rapid technique for assessing the molecular stability of and ensures the true-to-type nature of plants so obtained. It is also the utmost demand for carrying out genetic manipulations at cellular level.

A high frequency plant regeneration protocol has been developed from leaf and petiole explants of broccoli. Using RAPD for genetic fidelity, we conclude that a high-efficiency plant regeneration system for broccoli can be developed without risk of genetic instability. This protocol can be favourably exploited for genetic transformation studies of broccoli for improvement of agronomically important traits like aphid resistance, disease resistance, herbicide tolerance, salinity and drought tolerance, quality improvement, etc.

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