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Greenhouse evaluation on the performance of heat tolerant transgenic broccoli and genetic diversity analysis using inter simple sequence repeat (ISSR) markers

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Abstract

Background: Broccoli, *Brassica oleracea* subsp. *italica* is one of the many valuable *Brassica* species which is still less cultured under *in vitro* condition. Heat tolerant transgenic and non-transgenic broccoli cv. Green Marvel plantlets with well-developed root system obtained through *in vitro* culture were transferred into disposable plastic pots containing sterilized potting mixture consisting of (peatgro^{TM}) + coconut dust (2:1) and maintained in a growth chamber.
Results: After one month, the hardened plantlets were transferred and maintained in a transgenic greenhouse. After four months of acclimatization in the transgenic greenhouse, the efficacy of HSP101 gene in increasing the heat tolerance of the transgenic broccoli was evaluated. Results showed that the transgenic plants could survive and performed normally, producing flower heads even at the highest tested temperature of 34ºC. Seven transgenic broccoli lines with different gene copy number of the *At*HSP101 gene as well as the control plant were assessed for genetic diversity using inter simple sequence repeat (ISSR) markers.
Conclusions: ISSR results showed polymorphism and phylogenetic relationship between the transgenic and non-transgenic (control) *Brassica oleracea* cv. Green Marvel.

Keywords: *At*HSP101 gene, *Brassica oleracea,* ISSR, transgenic broccoli.

INTRODUCTION

Acclimatization is the last stage in the regeneration of plants, which involves the transfer from heterotrophic to an autotrophic condition in the transgenic greenhouse. This stage is to prevent plantlets from stress and death prior to transfer under field condition. Generally, acclimatized plantlets could grow well on a mixture of coconut dust, peatgro^{TM} and vermiculite in the misting chamber (Hartmann et al. 2007). However, a substantial number of micropropagated plants do not survive the transfer from *in vitro* conditions to the greenhouse or field. The greenhouse and field have substantially lower relative humidity, higher light level and a septic environment that are stressful to the micropropagated plants compared to *in vitro* conditions (Hazarika, 2003). Pavlovic et al. (2010) reported 58% plantlet survival during acclimatization of *Brassica* vegetables. ISSR markers are very reproducible, abundant and polymorphic in the plant genomes (Bornet and Branchard, 2001; Bornet et al. 2002; Adams et al. 2003; Archak et al. 2003; Galván et al. 2003; Arolu et al. 2012) and their universality and easiness of development without the need to sequence data are most applicable in

plant breeding programs. This paper reports on the efficacy of HSP 101 gene in increasing the heat tolerance of transgenic lines of *Brassica oleracea* subsp. *italica* cv. Green Marvel in the greenhouse followed by evaluation on the genetic diversity of the transgenic and non-transgenic plants using ISSR markers.

MATERIALS AND METHODS

Four heat tolerant transgenic broccoli lines and non-transgenic plantlets about 2 cm in height, with primary and secondary roots were selected and washed with sterilized distilled water to remove the remaining agar. The plantlets were dipped briefly into 5% Benlate solution. Each plantlet was transplanted into disposable plastic pot (10 cm diameter and 10 cm height) containing sterilized potting mixture which consisted of (peatgro^{TM}) + coconut dust (2:1). The medium components had been previously autoclaved at 121ºC for 20 min. The potted plants were covered with similar plastic pots and placed at 25 ± 1ºC, under 16 hrs photoperiod with a light intensity of 30 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent tubes. They were watered using a spray two-three times a week. After one month, the plastic covers were removed completely and the transgenic plants were transplanted to bigger pots (30 cm diameter and 20 cm height) containing peatgro^{TM} + peatmoss + vermiculite (3:1:1). The transgenic and control plants were placed in the transgenic greenhouse under various temperature regimes (20ºC, 30ºC and 34ºC) for evaluation on the efficacy of HSP101 gene in increasing their heat tolerance. Non-transgenic plants were also placed under the different temperature regimes as controls. Parameters recorded were the percentage of plant survival and the morphological traits such as plant height (cm) attained, leaf colour, fruit size, growth habit and stem thickness. Data on percentage of plant survival were analyzed using the analysis of variance (ANOVA). Duncan New Multiple Range Test (DNMRT) at α = 5% was used for comparison between treatment means. The treatments were replicated three times and each replication per treatment contained four plantlets giving a total of 180 plantlets for the entire experiment.

For the analysis of genetic diversity 7 transgenic broccoli lines with different gene copy numbers and a control were used. DNA was extracted separately from leaves of 7 plants of each accession following a CTAB extraction protocol (Doyle and Doyle, 1990). Fourteen ISSR primers were selected which were UBC808, UBC809, UBC810, UBC811, UBC818, UBC826, UBC834, UBC835, UBC841, UBC842, UBC848, UBC849, UBC850 and UBC889, commercialized by UBCs [set no. 9, University of British Columbia (UBC), Vancouver, BC, Canada]. All reactions were carried out in a MyCycler^{TM}. Programmable Thermal Cycler (Bio-Rad Laboratories, Inc.) using the following Touch-down PCR program: initiation denaturation at 94ºC for 3 min followed by 10 cycles of 94ºC for 1 min, 60ºC for 1 min (decreases of 1ºC in each cycle) and 72ºC for 2 min; 30 cycles of 94ºC for 15 sec, the annealing temperature at 55ºC for 30 sec and 72ºC for 1 min. A final extension at 72ºC for 10 min was performed.

ISSR amplification products were analyzed by gel electrophoresis in 2% agarose in 1 x TBE buffer, stained with Midori Green DNA stain (1 µl/100 ml gel), and photographed under ultraviolet light. Fragment size was estimated by using a 100 base pairs (bp) molecular sized ladder (Fermentas, Germany). Amplified products were scored on the basis of the presence or absence of ISSR markers using UVIDOC software (version 99). Pairwise comparisons were calculated using the Jaccard's coefficient (Jaccard, 1901). The similarity values found were used to generate a phenogram via the unweighted pair group method analysis (UPGMA). The correlation between the similarity matrix and the cophenetic matrix for the clusters was computed. All the analyses were performed with NTSYSpc version 2.1 (Rohlf, 2002).

RESULTS AND DISCUSSION

Transgenic and control plantlets with well-developed root system (Figure 1a) were removed from the culture flasks and transferred to disposable plastic pots containing sterilized potting mixture consisting of peatgro^{TM} + coconut dust (2:1), (Figure 1b and 1c). After one month and once hardened the plantlets were transferred and maintained in the transgenic greenhouse. In the transgenic greenhouse the potting medium was checked daily to ensure adequate supply of moisture. In the first and second week of acclimatization water was sprayed thoroughly 2-3 times a week and then allowing it to drain out. Too much water may cause plant death. The composition of acclimatization medium (peatgro^{TM} + peatmoss + vermiculite; 3:1:1) used in this study was similar to studies on *Brassica* conducted by other

researchers (Ruiz and Blumwald, 2002; Qin et al. 2007). Vermiculite can hold and made available ammonium, potassium, calcium and magnesium to the growing plants and when combined with peatgro^{TM} promotes faster root growth and gives quick anchorage to young roots (Ruiz and Blumwald, 2002; Hartmann et al. 2007; Ravanfar et al. 2011). When the plants reached 5-10 cm in size they were fertilized with Welgro and transferred to different temperature regimes. After four months in the transgenic greenhouse, the efficacy of HSP101 gene in increasing the heat tolerance of the transgenic broccoli was evaluated based on the percentage of plant survival (Figure 1d) and the morphological traits. Data on the non- transgenic broccoli plants as controls were also recorded for comparison.

In Figure 2, no significant difference in terms of percentage of plant survival was observed between the transgenic lines and the control maintained at 20ºC. However at 30ºC, the percentage of plant survival significantly decreased in the control (16.67%) compared to the transgenic lines (above 93%). At 34ºC the percentage of plant survival in the control decreased to 6.67% followed by a decrease to 76.67% in transgenic line 4, both differing significantly from the other transgenic lines (Figure 2). The highest percentage of plant survival (100%) occurred in transgenic line 2 at all temperature regimes tested. The better survival of the transgenic lines even at 34ºC compared to the control plants is very likely due to the expression of the inserted *At*HSP101 gene in their genomes. Schirmer et al. (1994) strongly suggested that the high temperature inducible 101 kD6 HSP isolated from cDNA libraries of *Arabidopsis* (*At*HSP101) promotes survival at high temperature. The ability of *At*HSP101 to protect plant from severe heat stress has an important role in thermotolerance in higher plants.

At 20ºC all transgenic plants showed normal morphology with few changes compared with the non-transgenic plants (Table 1). The transgenic plants produced leaves which were darker green in colour compared to that of the control. The broccoli heads were more or less of equal size to that of the non-transgenic plants (Figure 3a and 2b). In addition, the transgenic plants were slightly shorter than the non-transgenic. The slight morphological differences of transgenic and non transgenic broccoli are probably due to position effect. Position effect is the effect on the expression of a gene when its location in a chromosome is changed and occasionally can cause gene silencing and DNA rearrangement. Similarly, Robson et al. (2004) reported that systemic expression of *IPT* gene in transgenic maize resulted in reduction of plant height, leaf number and greener leaves than the control. At 30 and 34ºC temperature regimes the percentage of plant survival significantly decreased in the controls (16.67%) and (6.67%), respectively. Also, at 30ºC and 34ºC the control plants have stunted growth and majority without flower heads. However, the morphological traits of the transgenic plants at 30 and 34ºC were normal and similar to those maintained at 20ºC (Table 2 and Table 3).

Table 1. Comparison of morphological traits between transgenic and non- transgenic broccoli after 5 months in the transgenic greenhouse at 20ºC.

Morphological trait	Transgenic	Non-transgenic
Growth habit	erect	erect
Leaf colour	dark green	green
Mean flower head size	9 cm	8.5 cm
Mean plant height	30 cm	33 cm
Mean stem thickness Seed colour (after six months)	2 cm yellowish	2 cm black

In the assessment of genetic diversity between the transgenic and non-transgenic broccoli plants, out of 14 ISSR primers evaluated 10 primers produced a total of 100 bands at an average of 10 bands per primer. Out of the 100 bands, 85 bands were polymorphic and 14 bands were monomorphic (Table 4). The overall percentage of polymorphism for the 10 primers across the transgenic and non-transgenic broccoli plants was 85. The polymorphic bands varied from 3 (UBC834) to 15 (UBC826) per primer (Figure 4). The amplified fragments ranged from 72 to 1590 bp in size. The UPGMA dendrogram of genetic distances between the transgenic and non-transgenic plants based on band polymorphisms generated by ISSR is shown in Figure 5. According to the dendrogram, the transgenic and non-transgenic broccoli plants were grouped into five major clusters at the coefficient level of 0.6. Jaccard's

genetic similarity co-efficient indicated a high level of genetic variation between the transgenic lines and the non-transgenic plants based on band polymorphisms generated by ISSR which ranged between 0.33 and 0.77 (Table 5). The highest similarity value of 0.77 was found between S_3 and S_4 transgenic lines.

ISSR markers can be used in population genetic studies of plant species as they effectively detect very low levels of genetic variation (Zietkiewicz et al. 1994). High reproducibility of ISSR may be due to the use of longer primers and higher annealing temperatures (Moreno et al. 1998) compared with those normally used in other DNA amplification-based techniques, such as the Random Amplification of Polymorphic DNA (RAPD). By using ISSR primers, we found a high level of genetic diversity for the transgenic and non- transgenic broccoli plants with 85% of bands being polymorphic. High genetic variation has been reported in other *Brassica* species (Burton et al. 2004; Fu et al. 2006; Huangfu et al. 2009).

According to Muminovic et al. (2005) the level and pattern of genetic diversity detected by ISSRs among radish varieties highly agreed with the analysis of morphological characters which is in agreement with the results obtained in this study on transgenic and non-transgenic broccoli. Morphological variations detected among the transgenic and non-transgenic broccoli were on plant height (cm), leaf colour and flower head size. The high genetic variability may be a consequence of different gene copy number present in the different transgenic lines, and possibly the occurrence of mutations during *in vitro* regeneration stage (Wang et al. 2012).

Table 2. Comparison of morphological traits between transgenic and non- transgenic broccoli after 5 months in the transgenic greenhouse at 30ºC.

Morphological trait	Transgenic	Non-transgenic
Growth habit	erect	erect
Leaf colour	dark green	green
Mean flower head size	9 cm	majority without flower heads
Mean plant height	30 cm	stunted growth (20 cm)
Mean stem thickness Seed colour (after six months)	2 cm yellowish	2 cm no seeds produced

Table 3. Comparison of morphological traits between transgenic and non- transgenic broccoli after 5 months in the transgenic greenhouse at 34ºC.

Morphological trait	Transgenic	Non-transgenic
Growth habit	erect	erect
Leaf colour	dark green	green
Mean flower head size	9 cm	majority without flower heads
Mean plant height	30 cm	stunted growth (20 cm)
Mean stem thickness Seed colour (after six months)	2 cm yellowish	2 cm no seeds produced

Table 4. ISSR primers; their sequence, the number and size range of bands produced among the transgenic broccoli lines and the non-transgenic plant.

ISSR Primer	Sequence (5'-3')	Total amplified fragment	Polymorphic band	Monomorphic band	Percentage of polymorphic band	Amplified fragment size
UBC808	5'-AGAGAGAGAGAGAGAGC-3'	10	8	2	80	225-730
UBC809	5'-AGA GAGAGAGAGAGAGG-3'	8	8	0	100	105-465
UBC810	5'-GAGAGAGAGAGAGAGAT-3'	9	9	0	100	110-970
UBC811	5'-GAGAGAGAGAGAGAGAC-3'	12	6	6	50	225-1170
UBC826	5'-ACACACACACACACACC-3'	17	15	2	88.24	317-1590
UBC835	5'-AGAGAGAGAGAGAGAGYC-3'	6	6	0	100	450-1440
UBC841	5'- GAGAGAGAGAGAGAGAYC-3'	11	11	0	100	255-1515
UBC848	5'-CAC ACA CAC ACA CAC ARG-3'	9	9	0	100	210-1525
UBC889	5'-DBDACACACACACACAC-3'	10	10	0	100	72-915
UBC834	5'-AGAGAGAGAGAGAGAGYT-3'	8	3	4	37.5	270-760
Total		100	85	14		

Table 5. Similarity coefficients between pairs of transgenic lines (S1-S7) and the non-transgenic (C) *Brassica oleracea* subsp. *italica* using ISSR molecular marker.

	S1	S2	S3	S4	S5	S6	S7
S2	0.62	1					
S3	0.45	0.58	1				
S4	0.46	0.60	0.77	1			
S5	0.45	0.44	0.53	0.56	1		
S6	0.33	0.38	0.60	0.60	0.42	1	
S7	0.37	0.52	0.55	0.6	0.50	0.60	1
C	0.44	0.39	0.51	0.52	0.51	0.38	0.40

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REFERENCES

ADAMS, R.P.; SCHWARZBACH, A.E. and PANDEY, R.N. (2003). The concordance of terpenoid, ISSR and RAPD markers, and ITS sequence data sets among genotypes: An example from *Juniperus*. *Biochemical Systematics and Ecology*, vol. 31, no. 4, p. 375-387. **[CrossRef]**

ARCHAK, S.; GAIKWAD, A.B. and GAUTAM, D. (2003). Comparative assessment of DNA fingerprinting techniques (RAPD, ISSR and AFLP) for genetic analysis of cashew (*Anacardium occidentale* L.) accessions of India. *Genome*, vol. 46, no. 3, p. 362-369.

AROLU, I.W.; RAFII, M.Y.; HANAFI, M.M.; MAHMUD, T.M.M. and LATIF, M.A. (2012). Molecular characterization of *Jatropha curcas* germplasm using inter simple sequence repeat (ISSR) markers in Peninsular Malaysia. *Australian Journal of Crop Science*, vol. 6, no. 12, p. 1666-1673.

BORNET, B. and BRANCHARD, M. (2001). Nonanchored inter simple sequence repeat (ISSR) markers: Reproducible and specific tools for genome fingerprinting. *Plant Molecular Biology Reporter*, vol. 19, no. 3, p. 209-215. **[CrossRef]**

BORNET, B.; GORAGUER, F.; JOLY, G. and BRANCHARD, M. (2002). Genetic diversity in European and Argentinean cultivated potatoes (*Solanum tuberosum* subsp. *tuberosum*) detected by inter-simple sequence repeats (ISSRs). *Genome*, vol. 45, no. 3, p. 481-484.

BURTON, W.A.; RIPLEY, V.L.; POTTS, D.A. and SALISBURY, P.A. (2004). Assessment of genetic diversity in selected breeding lines and cultivars of canola quality *Brassica juncea* and their implications for canola breeding. *Euphytica*, vol. 136, no. 2, p. 181-192. **[CrossRef]**

DOYLE, J.J. and DOYLE, J.L. (1990). Isolation of plant DNA from fresh tissue. *Focus*, vol. 12, p. 13-15.

FU, J.; ZHANG, M.F. and QI, X.H. (2006). Genetic diversity of traditional Chinese mustard crops *Brassica juncea* as revealed by phenotypic differences and RAPD markers. *Genetic Resources and Crop Evolution*, vol. 53, no. 7, p. 1513-1519. **[CrossRef]**

GALVÁN, M.Z.; BORNET, B. and BALATTI, P.A. (2003). Inter simple sequence repeat (ISSR) markers as a tool for the assessment of both genetic diversity and gene pool origin in common bean (*Phaseolus vulgaris* L.). *Euphytica*, vol. 132, no. 3, p. 297-301. **[CrossRef]**

HARTMANN, H.T.; DAVIS, F.T. and GENEVE, F.L. (2007). Plant Propagation. *Principles and Practices.* London: Prentice Hall, Inc. 784 p. ISBN 9788120331457.

HAZARIKA, B.N. (2003). Acclimatization of tissue-cultured plants. *Current Science*, vol. 85, no. 12, p. 1704-1712.

HUANGFU, C.H.; SONG, X.L. and QIANG, S. (2009). ISSR variation within and among wild *Brassica juncea* populations: Implication for herbicide resistance evolution. *Genetic Resources and Crop Evolution*, vol. 56, no. 7, p. 913-924. **[CrossRef]**

JACCARD, P. (1901). Étude comparative de la distribution florale dans une portion des Alpes et des Jura. *Bulletin del la Société Vaudoise des Sciences Naturelles*, vol. 37, p. 547-579.

MORENO, S.; MARTÍN, J.P. and ORTIZ, J.M. (1998). Inter-simple sequence repeats PCR for characterization of closely related *grapevine germplasm. Euphytica,* vol. 101, no. 1, p. 117-125. **[CrossRef]**

MUMINOVIC, J.; MERZ, A.; MELCHINGER, A.E. and LUBBERSTEDT, T. (2005). Genetic structure and diversity among radish varieties as inferred from AFLP and ISSR analyses. *Journal of the American Society for Horticultural Science*, vol. 130, no. 1, p. 79-87.

PAVLOVIC, S.; VINTERHALTER, B.; MITIC, N.; ADZIC, S.; PAVLOVIC, N.; ZDRAVKOVIC, M. and VINTERHALTER, D. (2010). *In vitro* shoot regeneration from seedling explants in *Brassica* vegetables: Red cabbage, broccoli, savoy cabbage and cauliflower. *Archives of Biological Sciences*, vol. 62, no. 2, p. 337-345. **[CrossRef]**

QIN, Y.; LI, H.L. and GUO, Y.D. (2007). High-frequency embryogenesis, regeneration of broccoli (*Brassica oleracea* var. *italica*) and analysis of genetic stability by RAPD. *Scientia Horticulturae*, vol. 111, no. 3, p. 203-208. **[CrossRef]**

RAVANFAR, S.A.; AZIZ, M.A.; KADIR, M.A.; RASHID, A.A. and HADDADI, F. (2011). *In vitro* adventitious shoot regeneration and acclimatisation of *Brassica oleracea* subsp. *italica* cv. Green Marvel. *African Journal of Biotechnology*, vol. 10, no. 29, p. 5614-5619.

ROBSON, P.R.H.; DONNISON, I.S.; WANG, K.; FRAME, B.; PEGG, S.E.; THOMAS, A. and THOMAS, H. (2004). Leaf senescence is delayed in maize expressing the *Agrobacterium IPT* gene under the control of a novel maize senescence-enhanced promoter. *Plant Biotechnology Journal*, vol. 2, no. 2, p. 101-112. **[CrossRef]**

ROHLF, F.J. (2002). NTSYS-pc: Numerical Taxonomy System, version 2.1 Exeter Publishing. Ltd., Setauket, New York, USA.

RUIZ, J.M. and BLUMWALD, E. (2002). Salinity-induced glutathione synthesis in *Brassica napus*. *Planta*, vol. 214, no. 6, p. 965-969. **[CrossRef]**

SCHIRMER, E.C.; LINDQUIST, S. and VIERLING, E. (1994). An Arabidopsis heat shock protein complements a thermotolerance defect in yeast. *Plant Cell*, vol. 6, no. 12, p. 1899-1909. **[CrossRef]**

WANG, Q.M.; GAO, F.Z.; GAO, X.; ZOU, F.Y.; SUI, X.; WANG, M.; HUI, Y.J. and WANG, L. (2012). Regeneration of *Clivia miniata* and assessment of clonal fidelity of plantlets. *Plant Cell, Tissue and Organ Culture*, vol. 109, no. 2, p. 191-200. **[CrossRef]**

ZIETKIEWICZ, E.; RAFALSKI, A. and LABUDA, D. (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics*, vol. 20, no. 2, p. 176-183. **[CrossRef]**

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Figures

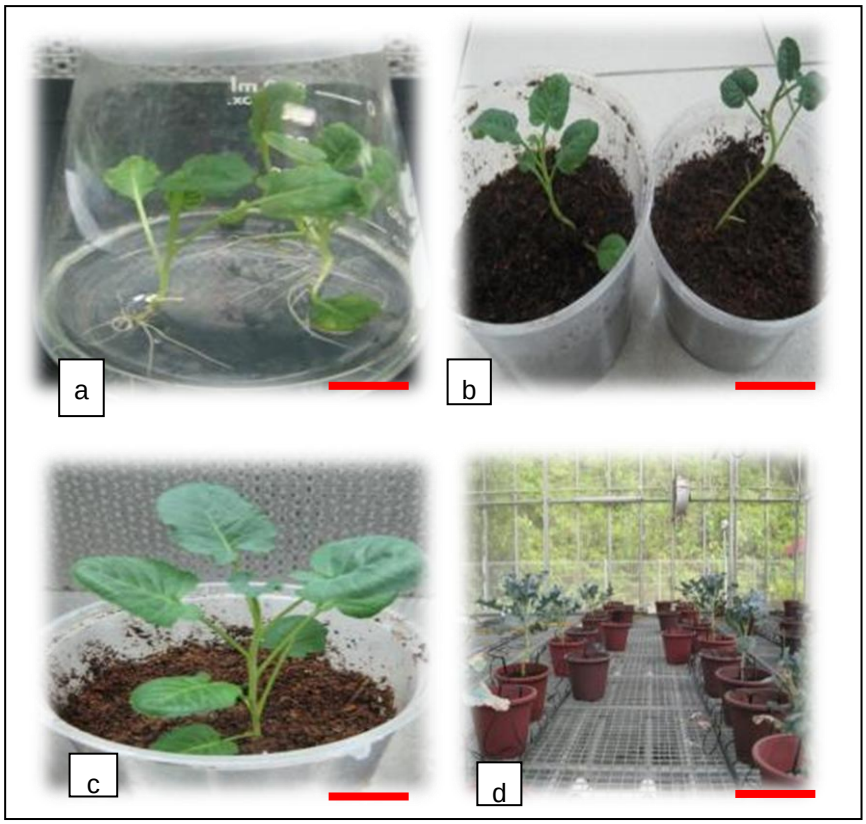

Fig. 1 Acclimatization of broccoli transformants (a) rooted transgenic broccoli cv. Green Marvel in a culture flask, (b and c) after 1 month in plastic pots, and (d) after three months acclimatization at 34ºC in the transgenic greenhouse. (a) and (c) Bar = 30 mm; (b) Bar = 40; and (d) Bar = 50 mm.

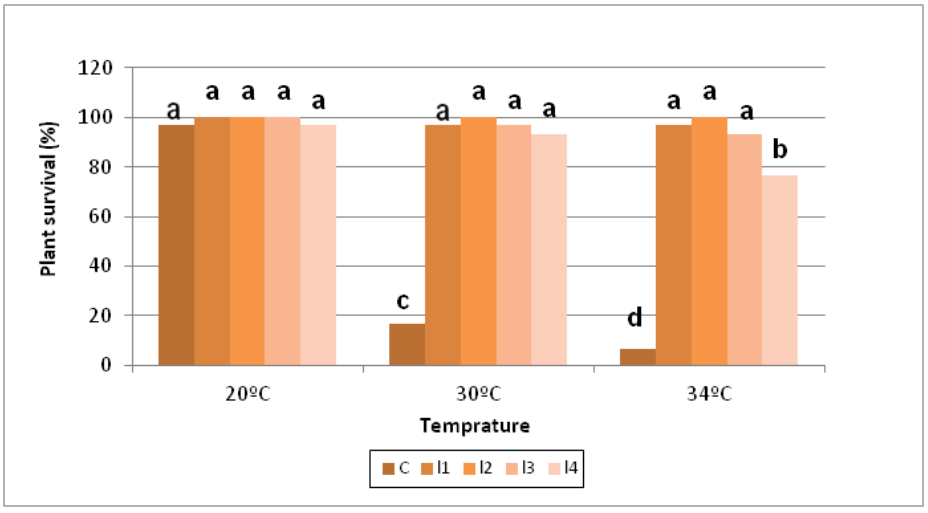

Fig. 2 Percentage of transgenic broccoli survival after four months of acclimatization at different temperatures. Means followed by the same letter(s) are not significantly different based on DMNRT (p = 0.05). (C: control; l1: transgenic line 1; l2: transgenic line 2; l3: transgenic line 3; l4: transgenic line 4).

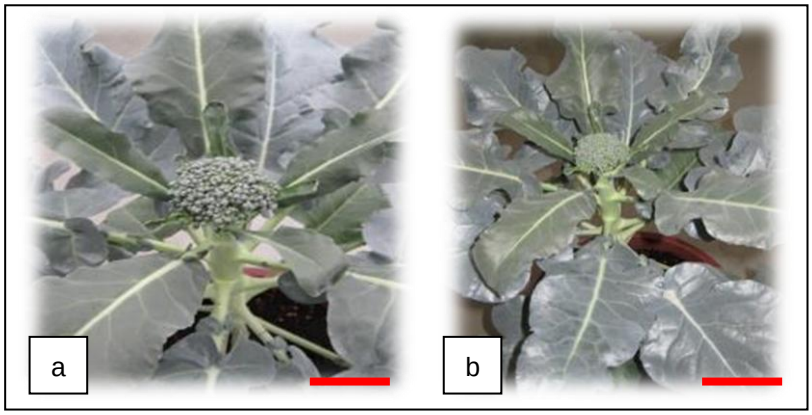

Figure 3. Flowering of two broccoli plants after four months of acclimatization in the transgenic greenhouse (a) non-transgenic and (b) transgenic broccoli plants. Bar = 30 mm.

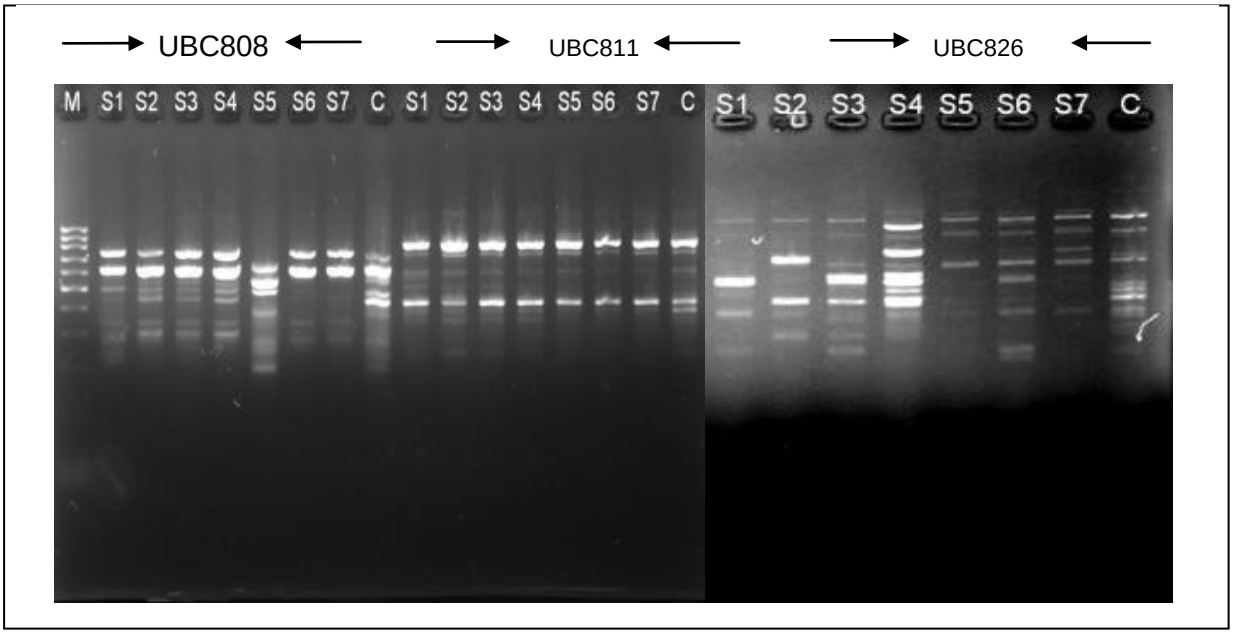

Fig. 4 Agarose gel electrophoresis of ISSR amplifications of transgenic broccoli lines and the non-transgenic with primers UBC808, UBC811 and UBC826. M: DNA marker (100 to 1000 bp); lanes S1-S7: transgenic broccoli lines; and lane C: non-transgenic broccoli.

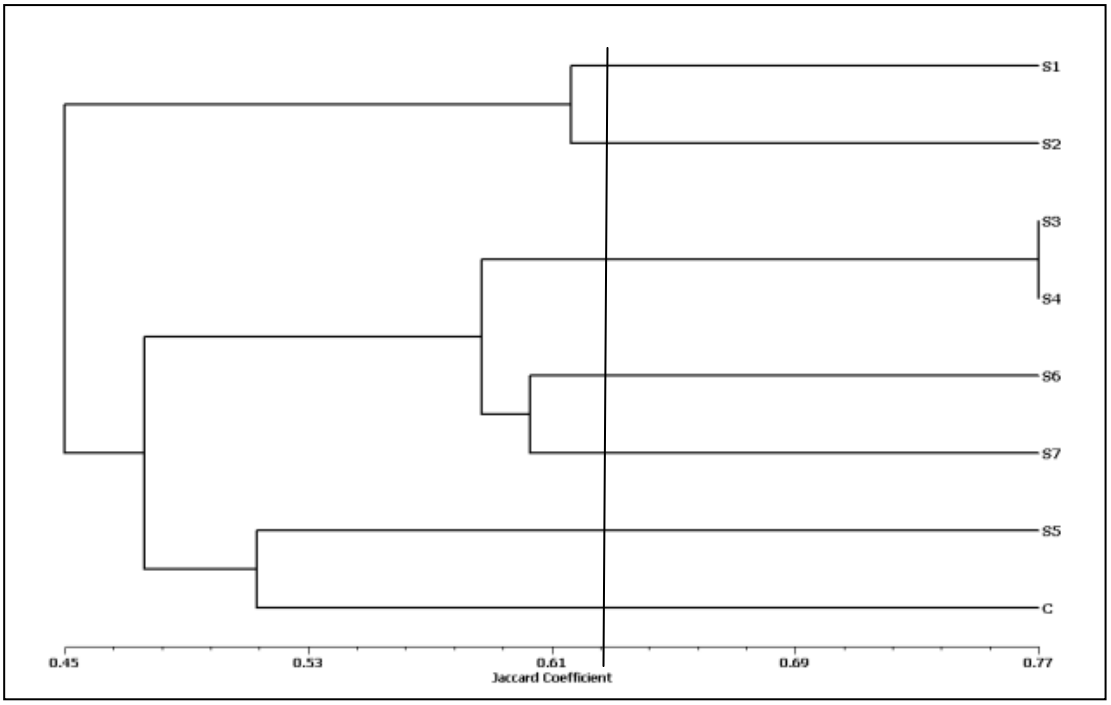

Fig. 5 UPGMA cluster analysis for the transgenic lines (S1-S7) and non-transgenic (C) *Brassica oleracea* subsp. *italica* based on genetic distance of ISSR marker.