

Full Length Research Paper

Induction of mutations in *Cichorium intybus* L. by base analogue 6-aminopurine (6-AP) and their detection with random amplified polymorphic DNA (RAPD) analysis

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The effect of 6-aminopurine (6-AP) on induction of mutations in *Cichorium intybus* L. (Asteraceae) in three consecutive generations (M_1 to M_3) was investigated. Three distinct mutants at 0.8% concentration were generated. These mutants were subjected to cytological analysis where 6-AP induced chromosomal aberrations such as univalents, multivalents, stickiness, laggards, bridges, etc, which reflects its potency to generate point mutations. These mutants were further analyzed using random amplified polymorphic DNA (RAPD) profiles, which differentiate the mutants genotypically, on the basis of occurrence of new bands and disappearance of old bands in combined random amplified polymorphic DNA (RAPD) profiles. The results clearly indicate that high concentrations of 6-AP induce base addition and substitution, resulting in mutations in *Cichorium*.

Key words: Mutation, *Cichorium intybus*, random amplified polymorphic DNA (RAPD), 6-aminopurine (6-AP), cytology.

INTRODUCTION

Chicory (*Cichorium intybus* L.) ($2n = 18$) is a self pollinated herb that originated from Europe and introduced in India where it grows luxuriantly. It is a well known medicinal plant used for the treatment of liver ailments. The herb grows wild and it still has low genetic variability and is self pollinated. This opens an ample room for mutation breeders to improve the crop. Mutation in plants for improvement of potential agronomic traits is important today and had become one of the most important tools in generating new varieties. Mutation breeding employing varieties of physical and chemical agents is used to explore the possibilities of developing new varieties, especially in crops having narrow genetic base. Mutation techniques are important not only for germplasm enhancement, but also for investigation of gene function, structure and mapping; focal point is the establishment of a comprehensive gene pool for crop

improvement, the genes thus produced become valuable material to plant breeders and molecular biologists for understanding not only function but also isolating and shuffling the genes between varieties (Arulbalachandran et al., 2009). The genotoxic agent used in the present study is 6-aminopurine, a base analogue of adenine causing A-T G-C and G-C A-T transitions. Mutagenesis resulted from an enhanced tendency of the analogue in comparison with adenine to exist as a disfavoured imino tautomer (Freese, 1959). In spite of lack of direct experimental evidence, the disfavoured tautomer structure is the long-established explanation for the mutagenicity of aminopurine (AP) (Sowers et al., 1986).

Plant improvement with the help of molecular markers have been routinely used for estimating genetic diversity until recently, many molecular techniques were developed into powerful tools to analyze genetic variance and genetic diversity as compared to earlier techniques (Bhat et al., 2011). Random amplified polymorphic DNA (RAPD) analysis is widely used for genetic mapping, taxonomic and phylogenetic studies of many organisms. It can also be applicable in the detection of DNA

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Table 1. Sequences of the 10-mer primers (5'-3') used in the experiments.

Primer	Sequence
OPC-02	GTCGCACGTC
OPG-03	GAGCCCTCCA
OPG-18	GGCTCATGTG
OPH-12	ACGCGCATGT
OPB-8	GTCCACACGG

alterations caused as a result of genotoxic agents (Atienzar, 2002). Chemical mutagens can produce several major types of DNA lesions such as cyclobutane-type pyrimidine dimers (Hollosoy, 2002). Other important types of DNA damages such as protein cross-links, DNA strand breaks, deletion or insertion of base pairs can also be induced by chemical mutagens. These different types of DNA damages must be detected by changes in RAPD profiles. Thus, the aim of the present study was to investigate the possibility of mutation changes in plant DNA after the treatment of various concentrations of 6-aminopurine (6-AP) using RAPD method. The 10-mer primers with single nucleotide substitutions were used in order to estimate these structural alterations in DNA following base analogue treatment. The aim of the present study was to identify the mutants of chicory using RAPD analysis.

MATERIALS AND METHODS

A set of 100 healthy seeds of chicory were pre-soaked overnight in distilled water and then subjected to different concentrations (0.02, 0.04, 0.06, 0.08 and 0.10%) of 6-AP for 12 h. After the treatment, the seeds were washed in running tap water to remove residual mutagens adhering to the seed coat and then sown in the designed field in randomized block design (RBD) of 2 x 2m plot area at a distance of 25 cm apart in three replicates. One set was soaked in distilled water and sown as control. The morpho-cytological observations were recorded from treated as well as control populations. Plants were raised from M₁ to M₃ generations by selfing and selection of the variants that deviated from the control ones like, flower mutants, height mutants (referred here to as giant mutants) and seed mutants.

Cytological studies

For cytological analysis, young flower buds from control as well as mutant plants were selected and fixed in Carnoy's fluid (alcohol : chloroform : acetic acid 6:3:1 ratio) for 40 min; transferred in propionic acid saturated with ferric acetate for 24 h and then stored in 70% alcohol. Anthers were squashed in 0.5% propionocarmine. Slides were made permanent in NBA series, mounted on Canada balsam and dried at 45°C.

RAPD analysis

Approximately, 0.20 g of plant tissue from *C. intybus* seedlings, from the control and mutants seeds were frozen in liquid nitrogen,

ground with mortar and pestle and incubated in 1.5 ml of extraction buffer (100 mM Tris-HCl pH 8, 100 mM NaCl, 50 mM EDTA pH 8, 2% SDS and 0.1 mg/ml proteinase K) for 1.5 h at 37°C. DNA was extracted twice with chloroform : isoamyl alcohol (24:1) mixture and precipitated from the aqueous phase with 2 vol. of cold isopropanol at -20°C for 48 h. DNA pellet was harvested by centrifugation, washed several times in 70% ethanol, air-dried and dissolved in deionized water. RNase treatment was performed according to Draper and Scott (1991). DNA was analyzed using agarose gel electrophoresis.

RAPD amplification involved initial denaturation of DNA template (20 ng per 25 µl reaction mix) at 94°C for 3 min, followed by 33 cycles of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C in thermocycler "Tertsik" (Russia). Amplification was finished with incubation at 72°C for 7 min. The sequences of 10-mer primers (MedBioservice) with single base substitutions are given in Table 1.

PCR amplification products were analyzed in 2.5% (w/v) agarose gel in 0.5xTBE buffer. PCR products (25 µl per sample) were mixed with 3 to 5 µl Gel Loading Dye Solution (Fermentas, Lithuania) and loaded onto the agarose gel, containing ethidium bromide (0.5 mg/ml). Gene Ruler 100 bp DNA ladder was used for each agarose gel. Electrophoresis was carried out at 80 V for 3.5 h; the results were visualized under UV light and documented using Cyber-shot digital camera.

RESULTS

In the present investigation, three mutants were isolated at 0.08% concentration, followed by selection, and these were found to be more vigorous than the control and their sibling plants with same as well as different concentrations. Moreover, these mutants were found to be true breeding in M₃ generation. The plants were differentiated on the basis of flower morphology, increased height and seed characters (Table 2).

Meiotic studies

Meiotic studies of all the four mutants were carried out to determine the fact that these are associated with point mutation or chromosomal changes. A hollow range of chromosomal anomalies were found to be induced by base analogue 6-AP. The percentage of meiotic abnormalities of control and mutants are presented in Table 3.

Pollen mother cells (PMCs) of mutants were recorded and compared with that of control. PMCs of control were perfectly normal showing normal meiotic division, while the cytology of isolated mutants exhibited various chromosomal aberrations. Percentage of abnormal PMCs varied from 13.73 to 15.32% in all the three mutants. The most common type of abnormalities observed were univalents, multivalents (Figure 1A and B), stickiness, laggards, bridges (Figure 1C), precocious separation of chromosomes, micronuclei (Figure 1D), etc. These results demonstrated in Table 3 revealed that the abnormalities were present at diakinesis, metaphase, anaphase and telophase stages of meiosis. In addition to previous common abnormalities, chiasmata frequency

Table 2. Morphological mutants screened in M₃ generation.

Code	Mutant Type	Distinguishing Character
Control		Normal plants bearing bright purple coloured flowers, having 14-16 ray florets
Mutant 1	Flower mutant	Plants having small 8 pink ray florets, having long anthers.
Mutant 2	Giant mutant	Giant and bushy mutant with long, leafy branches, thick stem, high yielding
Mutant 3	Bold seeded mutant	Hairy plant with profused branching, big flowers bearing dark bold seeds

Table 3. Effect of 6-AP in induction of cytological anomalies in isolated mutants.

Control/ mutant	Total no. of PMCs observed	Diakinesis		Metaphase			Anaphase			Telophase			Abnormal cells observed (%)	Pollen fertility	
		Univ.	Multiv.	Chiasmata frequency	Precocious separation	Stray chrom.	Stickiness	Laggards	Bridges	Laggards	Bridges	Polyads			
Control	205			17.27											97.71
Mutant 1	213	2.34	1.40	16.85	0.93	1.40	2.34	0.93	1.40	1.87	0.46	0.93	14.93		94.23
Mutant 2	218	2.75	1.83	13.21	0.91	0.91	1.43	1.37	1.37	1.37	0.91	0.91	13.73		91.65
Mutant 3	202	1.98	1.98	14.50	1.48	1.48	2.47	1.48	0.99	1.98	1.48	-	15.32		89.88

was also calculated at diakinesis stage and was found to be 17.27 chiasmata per cell in control, while in mutants, it varied from 13.21 to 16.85 chiasmata per cell (Table 3). Pollen fertility was also found to be moderately affected and was not found to interfere with seed set in all the three observed mutants.

RAPD analysis

It was shown that single nucleotide substitutions in 10-mer primers can indeed be reflected on the amplicons' profiles. The main changes observed in the RAPD profiles have resulted both in an appearance (Figure 2A) or disappearance of different bands (Figure 2B) with variation of their intensity as well. These effects might be connected with structural rearrangements in DNA caused by different types of DNA damages.

DISCUSSION

A common feature of mutation breeding in response to chemical treatment is the induction of morphological changes and increase in chromosomal anomalies. Therefore, investigation on meiotic aberrations and their genetic consequences form an integral part of most of the mutation studies and also provide a considerable clue to assess sensitivity of plants for different mutagens (Al Qurainy and Khan, 2009). The changes introduced in the mutants through application of 6-AP are due to changes in the chromosomal configuration.

Formation of chiasmata results in homologous pairing of chromosomes and controls degree of recombination. Variation in chiasmata can be considered as a means for generating new forms of recombination which influences variability within natural populations in an adaptive way (Sheidai et

al., 2006). The chiasmata frequency in mutants were lower than control, apparently, it was caused by increasing frequency of univalents, rod bivalents and multivalent, but particularly due to increased heterology induced by mutagens which directly affected the crossing over. The occurrence of univalents indicates non-homology between certain chromosomes in complement, due to failure of chromosome pairing leading to desynapsis at metaphase (Kumar et al., 2006) or due to precocious chiasmata terminalisation (Sidhu, 2008). Occurrence of multivalent suggested that translocations may have been produced due to terminal affinities of chromosomes. Bivalents clumped in single or different groups at metaphase resulting into sticky chromosomes which might have occurred as a result of improper folding of chromosome fibres and their intermingling, consequently, the chromosomes get attached to each other by

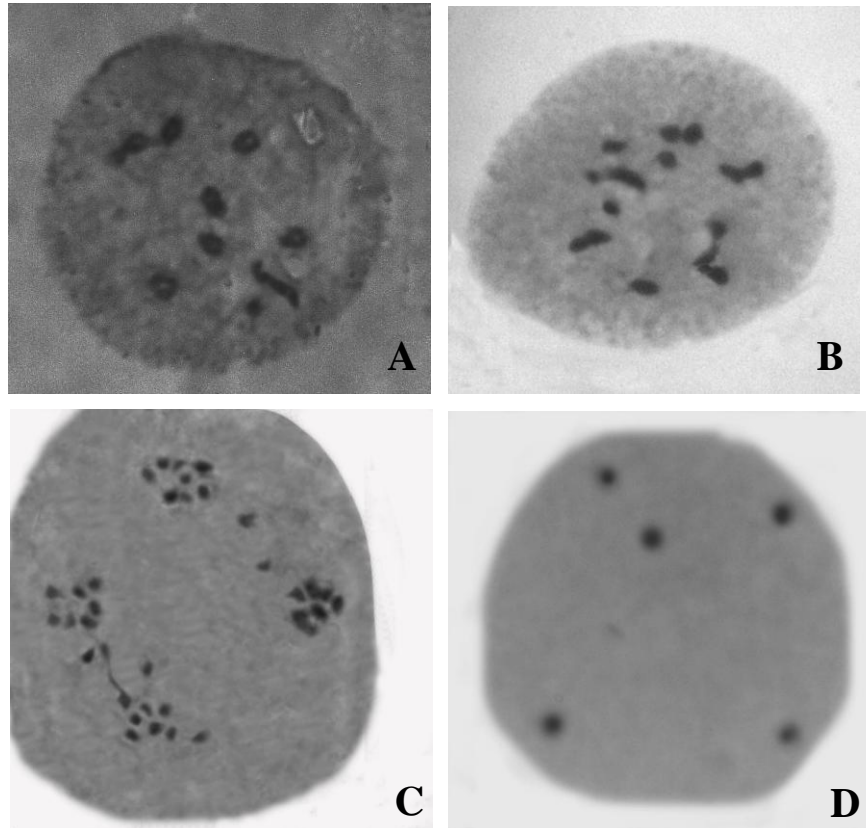


Figure 1. A: Prophase I: Diakinesis 7^{II} rings + 1^{II} rod + 2^I; B: Prophase I: Diakinesis 7^{II} rod + 2^{II} rings + 2^{II} rod + 7^I; C: Anaphase II: laggards and bridge formation; D: Telophase II: multinucleate condition.

subchromatid bridges (Myers et al., 1992). Abnormalities such as bridges and lagging chromosomes are due to spontaneous breakage and exchange rather than paracentric inversion (Lewis and John, 1966). Multinucleated condition at telophase II stage of meiosis occurred due to reorganisation of chromosome between prometaphase and anaphase II (Pozzobon and Schifino-Wittmann, 2006), laggards and non oriented chromosomes when they fail to reach the poles on time to be included in the main telophasic nucleus, form micronuclei (Utsunomiya et al., 2002). All these factors alone or together have resulted in the formation of defected microspores, which in turn lower the pollen fertility but not low enough to affect the yield (Khan et al., 2009).

Thus, RAPD method is applicable for the detection of changes in the DNA structure after different genotoxic treatments. The variation in band intensity and disappearance of some bands may correlate with level of mutants in DNA template after genotoxic treatment, which can reduce the number of binding sites for *Taq* polymerase. Appearance of new bands can be explained as the result of different DNA structural changes (breaks, transpositions, deletions, etc). The present findings are in

agreement with the previous studies on sunflower following sodium azide treatment (Mostafa and Alfrmawy, 2011). We can estimate the existence of mutation and structural alterations in plant DNA after impact of different stressful factors on the bases of DNA patterns obtained after RAPD with the set of primers. Obviously, sensitivity of the RAPD assay depends on the mutations level and it needs further investigations.

Conclusion

The use of base analogue 6-AP was found to induce mutations in *C. intybus*. At 0.08% concentration, three morphological mutants were produced which were beneficial from economical point of view. Cytological studies of isolated mutants revealed that these changes occurred as a result of chromosomal alterations and base substitutions. Our study with RAPD is very much helpful in understanding the mutation occurrence and could be validated using more primers. In future, it is expected that 6-AP can be used as a potent marker for developing new varieties in this plant with improved commercial properties suitable for Indian landscape. Further RAPD

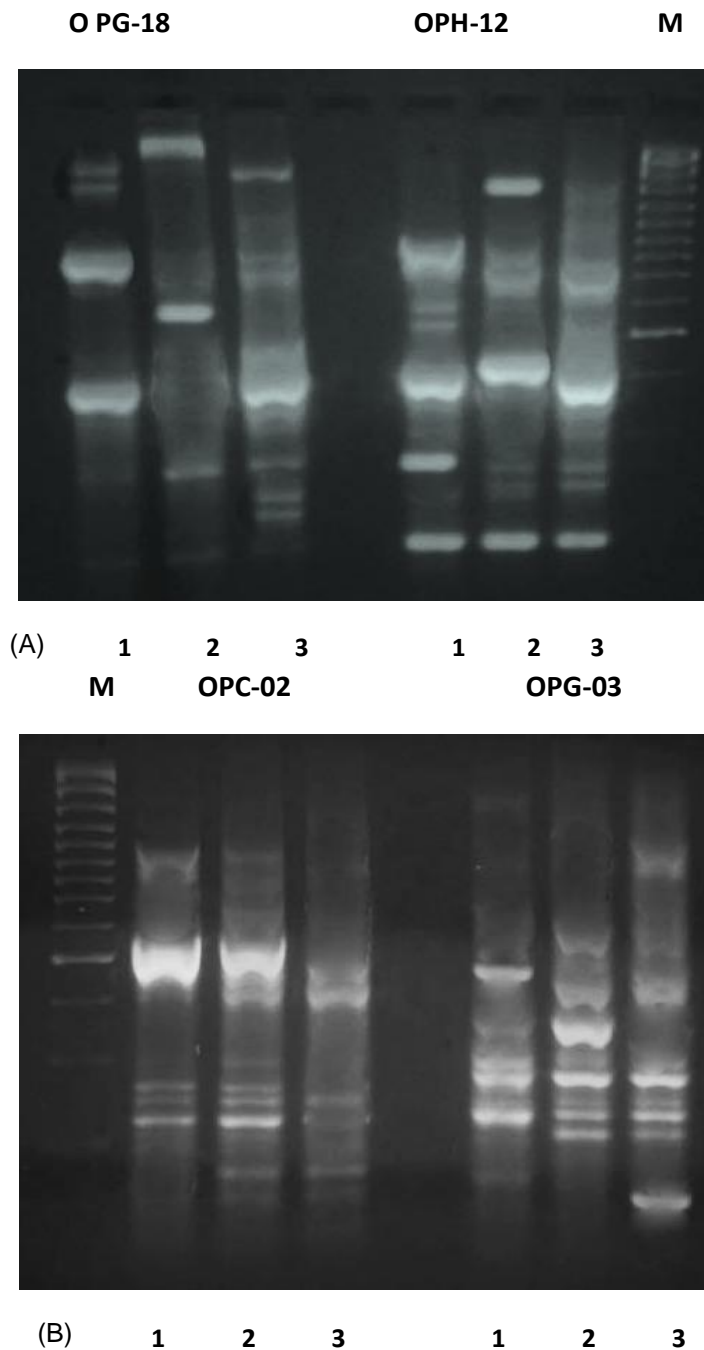


Figure 2. DNA profile of isolated mutants using RAPD.

marker technique can also be used for fingerprinting of selected mutants in crop improvement of chicory.

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