Pak. J. Bot., 43(4): 2183-2186, 2011.

DETECTION OF SODIUM AZIDE-INDUCED MUTAGENICITY IN THE REGENERATED SHOOTS OF *ARTEMISIA ANNUA* L., USING INTERNAL TRANSCRIBED SPACER (ITS) SEQUENCES OF nrDNA

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Abstract

Sodium azide (NaN_3) is a well known chemical mutagen which can effectively cause point mutation in plant genome. The mutagenicity by this potential mutagen was assessed in the regenerated mutant shoots of *Artemisia annua* using internal transcribed spacer (ITS) sequences of nrDNA. Insertions and/or deletions were detected in nrDNA-ITS sequences of all mutant shoots and compared with control ones using the ClustalX program. The regenerated shoots TS1 and TS2 had deleted bases, whereas TS3, TS4 and TS5 had insertions, because NaN_3 replaced the cytosine (C) by thymine (T) (C→T) (shoots; TS1and TS4) and thymine (T) replaced by guanine (G) (T→G) (shoot; TS5), respectively. Artemisinin content was also measured in the regenerated six-week-old shoots of *A. annua.* All regenerated shoots had enhanced level of this compound as compared to that in the controls, being highest in the regenerated shoot TS3.

Introduction

Chemical mutagens generally produce induced mutations which cause base pair substitutions, especially G.C→A.T which results in amino acid changes thereby modifying the function of proteins, but they do not eliminate their functions as occur in deletions or frame shift mutations (Van der Veen, 1966). The mutants produced by mutagens of any type are useful for the isolation, identification and cloning of potential genes, which play a role in improving crop yield, stress tolerance and a number of other qualitative and quantitative traits (Ahloowalia & Maluszynski, 2001).

Sodium azide (NaN_3) is one of the potential chemical mutagens being frequently used for improving crop yield and quality (Al-Qurainy & Khan, 2009). The mutagenic capability of sodium azide has been reported to occur due to the generation of an organic metabolite of azide compound (Owais & Kleinhofs, 1988) which interacts with cellular enzymes and DNA. Being a powerful mutagen, it can affect different parts of the plants by affecting a variety of metabolic phenomena involved in growth and development (Al-Qurainy & Khan, 2009).

Artemisia annua L., is commonly grown in many countries of the world as an aromatic and medicinal plant (Al-Qurainy & Khan, 2010). Although this plant comprises a variety of active ingredients, the most active compound is 'artemisinin' (a sesquiterpene lactone), which is being widely used to counteract malaria (Dhingra *et al.*, *2000*). Furthermore, this compound has been found very effective to counteract a variety of viruses including hepatitis B, C and many others (Efferth *et al*., 2008).

The biosynthesis of artemisinin in *A. annua* depends on the level of gene expression such as squalene synthase, one of the major enzymes of sterol pathway (Zhang *et al*., 2009). Other genes such as cytochrome P450 and its associated reductase enzyme have been shown to catalyze a number of steps in the biochemical pathway of artemisinin production (Arsenault *et al*., 2008).

Recently, it has been shown that artemisinin can be obtained in appreciable amounts from differentiated shoot cultures treated with sodium azide (Al-Qurainy & Khan, 2010). The content of this compound from *A. annua* is highly variable, ranging between 0.01% and 1%, depending on the type of a variety and environmental conditions (Brown, 2010). Its production by means of cell, tissue or organ cultures is generally very low so for enhanced artemisinin production through mutational approach using chemical mutagens has been considered a viable approach in a few studies (Jha *et al*., 1988; Tawfiq *et al*., 1989; Al-Qurainy & Khan, 2010).

Although a variety of protocols are employed to detect mutation caused by mutagens, few molecular markers such as amplified fragment length polymorphism (AFLP) (Papadopoulou *et al*., 1996), random amplified polymorphic DNA (RAPD) (Khawale *et al*., 2007; Atak *et al*., 2011), cleaved amplified polymorphic sequence (CAPS) marker (Knoll *et al.,* 2011) have been employed to detect mutation. Internal transcribed spacer sequences of nrDNA have the potential to resolve phylogenetic relationships in different plant species (Gulbitti-Onarici *et al*., 2009), genetic diversity assessment (Mondini *et al*., 2009), authentication of plant species (Al-Qurainy *et al.,* 2011) and intra-specific variation (Haque *et al*., 2009). The nrDNA region has frequent insertions/deletions which can be phylogenetically informative (Baldwin *et al*., 1995). Thus, in the present study, the NaN_3 mutagenicity in *A. annua* was assessed using nrDNA-ITS markers.

Materials and methods

Callus development: The processes of seed germination and callus development as described in Al-Qurainy and Khan (2010) were followed. The sterilized seeds of *A. annua* were germinated on sterilized soil containing half MS liquid basal media (Murashige & Skoog, 1962). The callus was raised from the leaves of two-week-old seedlings on the MS media containing α-naphthalene acetic acid (NAA) and kinetin (each 1.0- 2.5 mg/L) in different combinations and kept it at 25 ± 2ºC in a growth chamber at 45 µmol $m^{-2}s^{-1}$ photosynthetic photon flux density (PPFD) for 16 h daily. Finally, the calluses were produced in bulk on the MS medium supplemented with NAA and kinetin (each 0.5 mg/L).

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Mutant callus development from normal callus: We employed the protocol of callus treatment with sodium azide as reported in Al-Qurainy & Khan (2010) for the development of mutant calli in *A. annua*. The seven-week-old calli were treated with varying concentrations of NaN_3 ranging from 1-5 mM. They were placed on fresh same callusing media as used for normal callus development after treatment and washing, and further sub-cultured on fresh media at two-week time-intervals to regain the proper growth.

Shoot regeneration from callus: The callus was transferred on shoot regeneration media containing NAA (0.5 mg/L) and BAP (1.5 mg/L) for the development of the shoots. Shoot regeneration occurred after four-weeks of inoculation. For each treatment, three-replicates were taken for statistical analysis and also for reproducibility of the results. Six-week-old mutant shoots regenerated from calli (TS1, TS2, TS3, TS4, TS5) were employed for artemisinin extraction along with control shoot (TC).

Mutation detection using nrDNA-ITS sequences

Genomic DNA extraction, PCR and sequencing: The total genomic DNA was extracted from the regenerated shoots of *Artemisia annua* following the modified CTAB method (Khan *et al.,* 2007). ITS sequences of nrDNA were amplified using PCR bead (GE healthcare, UK) and a single reaction consisted of 20 µl of deionized sterile water, 25 ng DNA per reaction volume and 10 pm of each forward and reverse primer (ITS1/4 primers) following White *et al*., (1990). Purification of the PCR products was carried out with the SolGent PCR purification Kit-Ultra (SolGent, Daejeon, South Korea) before sequencing. Cycle sequencing was conducted using the same primers as used for amplification through an ABI PRISM 3100 DNA Analyzer (Perkin-Elmer, Applied Biosystems). Sequence alignment was performed using the ClustalX version 1.81 (Thompson *et al*., 1997) for all sequences. *Artemisia apiacea* (accession no: AM398848) was used to confirm our ITS sequences through sequence alignment. All sequences generated in the present study were deposited in the GenBank and the accession numbers assigned are given in Table 1.

Table 1. Artemisinin content in various mutant shoots of *Artemisia annua*.

Samples	GenBank Accession no.	Artemisinin content (µg/g dw) mean ± SD
TC		625.03 ± 3.56
TS1	HQ735410	676.74 ± 6.97 [a]
TS2	HQ735411	723.72 ±3.03 [a]
TS3	HQ735412	790.26 ± 4.89 [a]
TS4	HQ735413	765.18 ± 4.94 [a]
TS5	HQ735414	738.06 ± 6.99 [a]

Values are mean ± SD for three replicate (at $p<0.01$)

Estimation of artemisinin in regenerated shoots with high performance liquid chromatography (HPLC): The artemisinin was extracted according to the method followed by Al-Qurainy & Khan (2010). The extracted artemisinin was quantified using HPLC equipped with a C18 reverse phase column (5 µm, 4.6*250 mm) using premix methanol: 100 mM potassium-phosphate buffer (pH 6.5) in the ratio of 60: 40 as a mobile phase at a flow rate of 1 ml/min. The detector wavelength was set at 260 nm. Artemisinin was quantified from a standard curve prepared using varying concentrations of pure artemisinin.

Results and discussion

Varying doses of NaN_3 showed mutagenic effect on percent seed germination, and root and shoot length of *A. annua.* The mutagenic effect was found to be dose dependent. However, the mutagenicity of this mutagen has been reported to depend on its concentration, dilution factors, pH of the phosphate buffer solution and incubation period (Al-Qurainy & Khan, 2009; 2010).

Sodium azide (NaN_3) was employed on the callus cells to create mutation and further mutant shoots were regenerated from these treated calluses on MS media containing NAA (0.5 mg/L) and BAP (1.5 mg/L). Since, NaN_3 is a point mutagen and creates insertions and/or deletions so its mutagenicity was examined using the internal transcribed spacer (ITS) sequences of nrDNA (Fig. 1). The mutant shoots (TS 1 and TS2) of *A. annua* regenerated at 1 and 2 mM NaN_3 had deleted base on nrDNA-ITS sequences at position 114, whereas base insertion was found at position 115 in the mutant shoots (TS3) at 3 mM, 479 in mutant TS5 at 5 mM and 508 in mutant TS4 at 4 mM, respectively (Fig. 1). The mechanism of sodium azide-induced mutation is still unknown in the literature (Khan *et al*., 2009), however, its mutagenicity was tested using nrDNA-ITS markers, but the rate of mutation was found very low in this gene.

Besides, mutagenicity of this mutagen, the effect of this mutagen was also studied on the biosynthesis of artemisinin in the regenerated mutant shoots of *A. annua*. All mutant shoots developed from normal calli at various concentrations of NaN_3 showed enhanced content of artemisinin as compared to those of the normal shoot. The content of this compound in the regenerated mutant shoots, i.e., TS1, TS2, TS3, TS4 and TS5 obtained upon callus treatment with NaN_3 at 1, 2, 3, 4 and 5 mM was 676.74 ± 6.97, 723.72 ± 3.03, 790.26 ± 4.89, 765.18 ± 4.94 and 738.06 ± 6.99 µg/g DW as compared to normal shoot (TC) which had 625.03 ± 3.56 µg/g DW (Table 1). The enhancement in artemisinin content was found to be dose dependent up to 3 mM of NaN_3, however, at higher concentrations of this mutagen, the results were not satisfactory. Such unpredictable results may have been due to some random mutations at various loci.

The maximum artemisinin content was found in the shoots of mutant TS3 which was produced with 3 mM of NaN_3. Similar results were found from the calli of *A. annua* (Al-Qurainy & Khan, 2010) when the calli were produced from foliage leaves of germinated seeds treated with 3 mM of NaN_3. The variation in the artemisinin biosynthesis among various parts of the plant has been examined also at nutritional content level (Brisibe *et al*., 2009).

In conclusion, sodium azide is a good mutagen for causing point mutation. However, the mutagenicity of this mutagen was not found according to the concentrations used on the cells of *A. annua* callus. The frame shift mutation or deletion (more than one base) was not found in any of the regenerated mutant shoots.

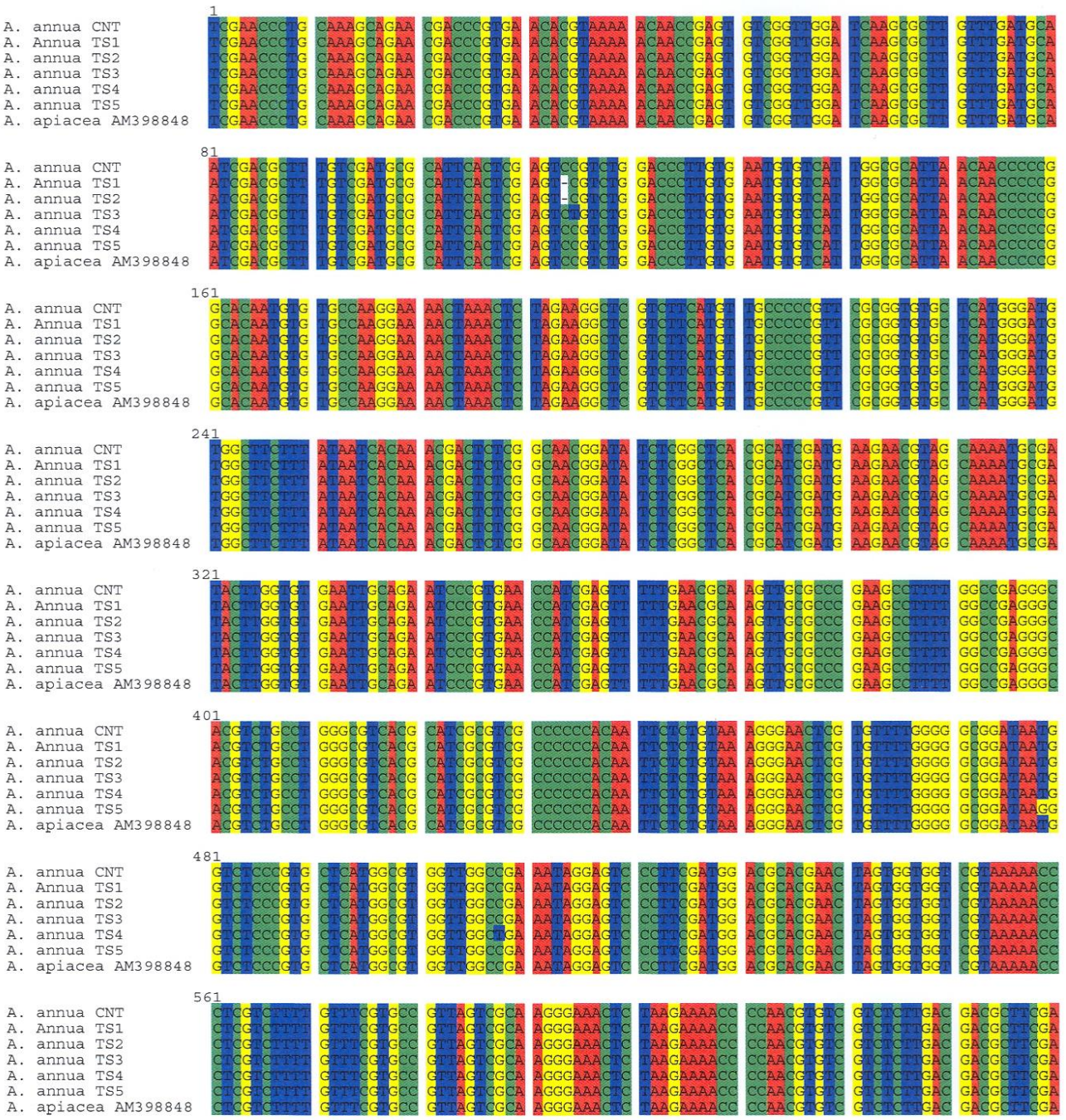

Fig. 1. Sequence alignment for ITS sequences of nrDNA for mutant and normal shoots.

Acknowledgement

The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding the work through the research group Project No, RGP-VPP-014.

References

Ahloowalia, B.S. and M. Maluszynski. 2001. Induced mutation. A new paradigm in plant breeding. *Euphytica*., 118(2): 167-173.

Al-Qurainy, F., S, Khan, M. A. Ali, F.M. Al-Hemaid, M. Tarroum and M. Ashraf. 2011. Authentication of *Ruta graveolens* and its adulterant using internal transcribed

spacer (ITS) sequences of nuclear ribosomal DNA. *Pak. J. Bot*., 43(3): 1613-1620.

Al-Qurainy, F. and S. Khan. 2010. Mutational approach for enhancement of artemisinin in *Artemisia annua*. *J. Med. Plant Res.*, 4(17): 1714-1726.

Al-Qurainy, F. and S. Khan., 2009. Mutagenic effects of sodium azide and its application in crop improvement. *World Appl. Sci. J.*, 6(12): 1589-1601.

Arsenault, P.R., K.K. Wobbe and P.J. Weathers. 2008. Recent advances in artemisinin production through heterologous expression. *Curr. Med. Chem.*, 15(27): 2886-2896.

Atak, C., O. Celik and L. Acik. 2011. Genetic analysis of rhododendron mutants using random amplified polymorphic DNA (RAPD). *Pak. J. Bot*., 43(2): 1173-1182.

Baldwin, B.G., M.J. Sanderson, J.M. Porter, M.F. Wojciechowski, C.S. Campbell and M.J. Donoghue. 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. *Ann. Missouri Bot. Gard.*, 82: 247-277.

Brisibe, E.A., U.E. Umoren, F. Brisibe, P.M. Magalhaes, J.F.S. Ferreira, D. Luthria, X. Wuh and R.L. Prior. 2009. Nutritional characterization and anti-oxidant capacity of different tissues of *Artemisia annua* L. *Food Chem.*, 115: 1240-1246.

Brown, G.D. 2010. The biosynthesis of artemisinin (Qinghaosu) and the phytochemistry of *Artemisia annua* L. (Qinghao). *Molecules*., 15: 7603-7698.

Dhingra, V., V.M. Rao and L. Narasu, 2000. Current status of artemisinin and its derivatives as antimalarial drugs. *Life Sci*., 66: 279-300.

Efferth, T., M.R. Romero, D.G. Wolf, T. Stamminger, J.J.G. Marin and M. Marschall. 2008. The anti-viral activities of artemisinin and artesunate. *Clin. Infect Dis*., 47: 804-811.

Gulbitti-Onarici, S., C. Sancak, S. Sumer and S. Ozcan. 2009. Phylogenetic relationships of some wild wheat species based on the internal transcribed spacer sequences of nrDNA. *Curr Sci*., 96: 794-800.

Haque, I., R. Bandopadhyay and K. Mukhopadhyay. 2009. Intra-specific variation in *Commiphora wightii* populations based on internal transcribed spacer (ITS1-5.8S-ITS2) sequences of rDNA. *Diversity*., 1: 89-101.

Jha, J., T.B Jha, S.B and Mahato, 1988. Tissue culture of *Artemisia annua* L.: A potential source of an antimalarial drug. *Curr. Sci*., 57: 344-346.

Khan, S., F. Al-Qurainy and F. Anwar. 2009. Sodium azide: a chemical mutagen for enhancement of agronomic traits of crop plants. *Environ. We Int. J. Sci. Tech.*, 4: 1-21.

Khan, S., M.I. Qureshi, M. Kamaluddin, T. Alam and M.Z. Abdin. 2007. Protocol for isolation of genomic DNA from dry and fresh roots of medicinal plants suitable for RAPD and restriction digestion. *Afr. J. Biotechnol.*, 6(3): 175-178.

Khawale, R.N., V. Yerramilli and S.K. Singh. 2007. Molecular marker-assisted selection of *in vitro* chemical mutagen-induced grapevine mutants. *Curr. Sci*., 92 (8): 1056-1060.

Knoll, J.E., M.L. Ramos, Y. Zeng, C.C. Holbrook, M. Chow,S. Chen, S. Maleki, A. Bhattacharya, and P. Ozias-Akins. 2011. TILLING for allergen reduction and improvement of quality traits in peanut (*Arachis hypogaea* L.). *BMC Plant Biol*., 11: 81.

Mondini, L., A. Noorani and M.A. Pagnotta. 2009. Assessing plant genetic diversity by molecular tools. *Diversity*, 1: 19-35.

Murashige, T and F.A. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol Plant*., 15: 473-479.

Owais, W.M. and A. Kleinhofs. 1988. Metabolic activation of the mutagen azide in biological systems. *Mutat Res*., 197: 313-323.

Papadopoulou, K., R.E. Melton, M. Leggett, M.J. Daniels and A.E. Osbourn. 1996. Compromised disease resistance in saponin deficient plant. *PNAS.,* 96: 12923-12928.

Tawfiq, N.K., L.A. Anderson, M.F. Roberts, J.D. Phillipson, D.H. Bray and D.C. Warhurst. 1989. Antiplasmodial activity of *Artemisia annua* plant cell cultures. *Plant Cell Rep*., 8: 425-428.

Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin and G.D. Higgins. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl. Acids Res*., 24: 4876-4882.

Van der Veen, J.H., 1966. Arabidopsis Information Service, 3: 26.

White, T.J., T. Bruns, S. Lee and J. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: (Eds.): M. Innis, D. Gelfand, J. Sninsky & T. White. PCR Protocols: *A Guide to Methods and Applications*, Academic Press, San Diego, CA, USA, 315-322.

Zhang, L., F. Jing, F. Li, M. Li, Y. Wang, G. Wang, X. Sun and K. Tang. 2009. Development of transgenic *Artemisia annua* (Chinese wormwood) plants with an enhanced content of artemisinin, an effective anti-malarial drug, by hairpin-RNA-mediated gene silencing. *Biotechnol. Appl. Biochem.*, 52: 199-207.

(Received for publication 6 January 2011)