INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
11–362/AWB/2012/14–2–307–310
http://www.fspublishers.org

Full Length Article

Prospecting the Utility of Antibiotics as Lethal Selection Agents for Chloroplast Transformation in Sugarcane

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ABSTRACT

Level of sensitivity of non-transformed wild type sugarcane cells regenerating on callus induction and regeneration media supplemented with various levels of antibiotics including spectinomycin, streptomycin and kanamycin was tested in order to find out their the lethal dose for the selection of transformants. Ten different levels of each streptomycin and kanamycin (0, 25, 50, 100, 150, 200, 250, 300, 350 & 400 mg/L) and twelve different levels of spectinomycin (0, 25, 50, 100, 150, 200, 250, 300, 350, 400, 450 & 500 mg/L) were used. Five weeks old calli of HSF-240 were sub-cultured onto optimized regeneration medium supplemented with aforementioned levels of all three antibiotics. Regeneration was completely arrested at 150 mg/L kanamycin and 350 mg/L streptomycin but spectinomycin failed to arrest the regeneration. Thus studies revealed that sugarcane is naturally resistant to spectinomycin, suggesting that streptomycin and kanamycin may be used in transformation experiments.

Key Words: Sugarcane; Antibiotics; Regeneration; Chloroplast transformation

INTRODUCTION

Commercialization of genetically modified crops containing improved genetic traits will have spectacular impacts on world food production. However, the continued success with this technology depends on our abilities to introduce multigenic traits conferred by a single gene or a number of genes to obtain a higher level of expression and containment. Use of an effective antibiotic and its appropriate concentration are fundamental prerequisites for the development of successful transformation system. Since the development of first transgenic plant during early 1980s and thereafter its commercialization, antibiotic and herbicide resistance selectable marker genes have always been among the integral features of plant genetic modification endeavors (Ramessar *et al*., 2007). Selectable marker genes are principally divided into positive or negative selection markers. In accordance to their functionality the positive selection systems are further classified into conditional and non-conditional positive selection system. A conditional-positive selection system is comprised of a gene encoding an enzyme that confers resistance to a specific substrate that may either be toxic to the untransformed cells or facilitates merely the growth and proliferation of transformed cells. Non-conditional positive selection system never requires any external substrates rather it promotes the selective growth and proliferation of transformed cells (Miki & McHugh, 2004). Lethal selection system confers resistance to toxic antibiotics i.e., kanamycin, hygromycin, methotrexate, bleomycin, geneticin and phosphenothricin, whose expression in transformed cells confers the ability to survive and proliferate on the selection medium, whereas sensitive cells die (Jones *et al.,* 1987). Unlike lethal antibiotics, non-lethal antibiotics i.e., spectinomycin and streptomycin do not kill the cells/tissues, rather inhibit chlorophyll synthesis/ accumulation resulting in bleaching of the cells/tissues (Oreifig *et al.,* 2004). Among the selectable marker genes attempted for transgenic and transplastomic plant development, antibiotic resistant marker gene(s) in combination with Green Fluorescent Protein (GFP) had been the most effective selection marker(s) facilitating the extension of plastid transformation to non-green plastids as in embryogenic cells of cereal crops (Khan & Maliga, 1999).

While selecting a selection system, the resistance mechanism either detoxification of the selective agent or modification of the target enzyme should be considered as the detoxification of selective agent by expression of a modified enzyme in the transformed cells can enable untransformed cells, to escape. This never happens if resistance is based on the production of a modified target enzyme (Brasileiro & Aragao, 2001). Thus selectable marker genes have always been of pivotal importance for the development of plant transformation system as they are core agents in transgene selection (Miki & McHugh, 2004). Different types of selection systems are available including genes conferring resistance to antibiotics, antimetabolites,

To cite this paper: Mustafa, G. and M.S. Khan, 2012. Prospecting the utility of antibiotics as lethal selection agents for chloroplast transformation in sugarcane. *Int. J. Agric. Biol*., 14: 307–310

herbicides, hormone biosynthesis and toxic levels of amino acids and their analogs. The genes conferring resistance to various antibiotics (SPT) for streptomycin, (*aph2, nptII or neo*) for kanamycin, (*aphIV=hpt*) for hygromycin, [*acc(3)-I*] for gentamycin, G418 (*aph2*) for geneticin (derivative of genetamycin), (*aadA*) for both spectinomycin and streptomycin, (*dhfr*) for methotrexate resistance (Irdani *et al*., 1998). The herbicide resistance genes include phosphinothricin resistance genes (bar, pat), 2,4-D resistance gene (*tfdA*), glyphosate resistance genes (*aroA*, EPSPS), sulfonylurease resistance genes (*csr1-1*) and bromoxynil resistance genes (bxn). Stringency of the selection agent, its mode of action, mechanism of transport into plant cells and associated signaling pathways determine the worth of a particular selection system (Wilmink & Dons, 1993). Furthermore, transformation efficiency, biosafety and zero health hazards should also be the fundamental norms of a selection system for commercialization of GM crops (Sundar & Sakthivel, 2008).

Keeping in view the significance and sensitivity of the problem, pilot experiments were conducted to figure out the natural resistance and optimal level of antibiotics for the selection of putative transformation events.

MATERIALS AND METHODS

Sugarcane leaf roll discs were cultured on the callus induction medium (MS3) supplemented with different levels of the antibiotics spectinomycin (0, 25, 50, 100, 150, 200, 250, 300, 350, 400, 450 & 500 mg/L), streptomycin (0, 25, 50, 100, 150, 200, 250, 300, 350 & 400 mg/L) and kanamycin (0, 25, 50, 100, 150, 200, 250, 300, 350 & 400 mg/L). The callus initiation and proliferation was observed on this antibiotic containing medium in the light as well as in dark regimes. The dark proliferated, antibiotic exposed calli were transferred onto regeneration media having different levels of antibiotics (as cited above) and were incubated in light with photon flux density of 30 µmol $m^{-2}s^{-1}$. Antibiotic sensitivity of callus was evaluated by survival rate and regeneration response.

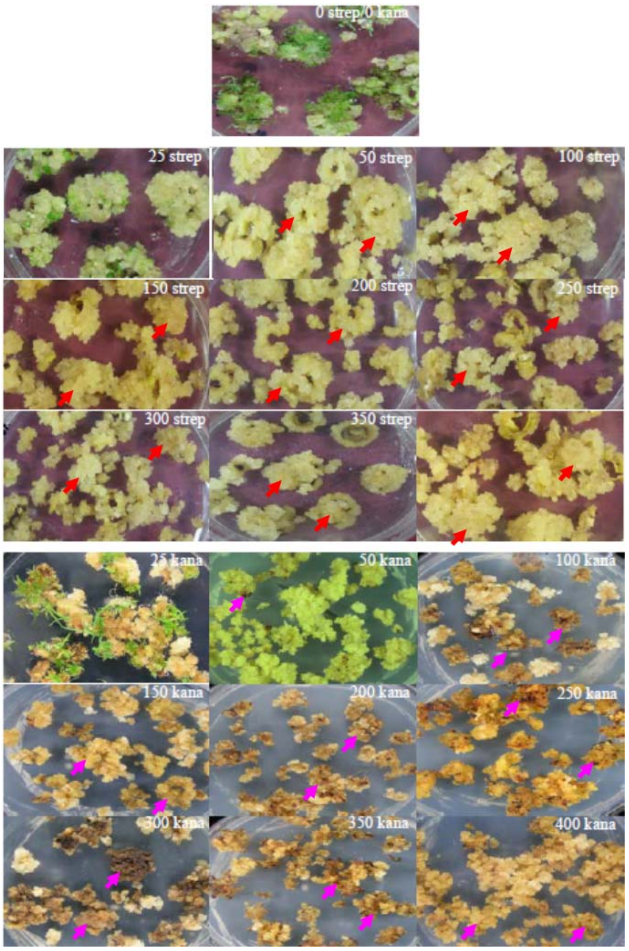
RESULTS AND DISCUSSION

Impact of streptomycin: The effect of streptomycin on the development of embryogenic culture of sugarcane was determined both under propagative (callus culture medium) and regenerative (regeneration medium) conditions. Callus development and proliferation from sugarcane leaf roll discs was observed in abovementioned levels of streptomycin containing medium both in the light as well as in dark. In the light proliferated, antibiotic exposed calli, no evident results could be attained perhaps, because of the reason that light itself poses an inhibitory effect on callus proliferation. Up to 350 mg/L of streptomycin the callus proliferation and regeneration was severely arrested. The dark proliferated, antibiotic exposed calli were transferred onto regeneration media having different levels of antibiotics and incubated in light. The regenerative phase was severely affected by streptomycin and at a concentration of 350 mg/L regeneration was absolutely diminished (Fig. 1). Moreover, chlorophyll degradation and bleaching was also observed and found to be directly proportional to the levels of the streptomycin. Thus, chlorosis appeared to be the most fundamental factor in the development of etiolated shoots, which makes a visible difference among the transformed and untransformed plants (Larkin *et al.,* 1996). This makes streptomycin a more suitable selective agent as it causes bleaching rather than cell death (Klee *et al.,* 1987), thus facilitating selection for the contrast between green tissue and chlorotic tissue rather than for survival and growth (Maliga *et al*., 1988). Literature suggests that streptomycin irreversibly binds to the 30S ribosome and freezes the 30S initiation complex (30S-mRNA-tRNA), so that no further initiation can occur (Gale *et al.,* 1981). It also slows down protein synthesis and hence induces misreading of the mRNA. Hence, chlorosis resulted from the exposure of calli to streptomycin is the result of a halt in protein synthesis (Svab *et al*., 1990). Further, European Food Safety Authority has also reaffirmed that *aadA* gene encoding resistance against spectinomycin/streptomycin pose no threat to humans or to the environment, thus a potential selectable marker gene for plant transformation (EFSA, 2009).

Impact of kanamycin: The consequences of kanamycin on sugarcane embryogenic system were also determined for the establishment of optimal levels of antibiotic for selection purpose. Leaf roll discs of wild type sugarcane were cultured on the callus induction medium (MS3) supplemented with increased levels of kanamycin. Callus proliferation and growth was less affected in dark as compared with light. In order to explore regeneration stringency by kanamycin, five weeks old, dark proliferated, antibiotic exposed calli were cultured onto regeneration media having different levels of antibiotics and incubated in light with photon flux density of 30 µmol $m^{-2}s^{-1}$. Arrest of regeneration began at 50 mg/L of kanamycin and complete necrosis of tissues was observed at 150 mg/L of kanamycin concentration. Starting from kanamycin concentration of 100 mg/L to higher, no green regenerants were observed (Fig. 1). Thus, kanamycin resulted into dead embryoids being lethal in its activity and resulted into complete embarrassment of calli proliferation and regeneration. Brasileiro (1998) reported that deleterious effect of kanamycin is due to its potential to inhibit protein synthesis by its binding to the 30S subunit of the ribosome, blocking the formation of initiation complexes and decreasing the fidelity of translation. It may even be toxic to untransformed tissues by secreting inhibitors or preventing transport of nutrients to the living transformed cells (Haldrup *et al*., 1998). The kanamycin resistant gene (*nptII*) is one of the most frequently used selectable marker genes for the development of transgenic plants and has been used to engineer dicots (Bevan *et al*., 1983; Fraley *et al*., 1983) as

Fig. 1: Sugarcane cultivar HSF-240 non-transformed callus proliferated on MS3 medium having MS salts, MS vitamins, 30 g/L sucrose, 3 mg/L 2,4-D and then cultured onto regeneration medium having different levels of antibiotic streptomycin and kanamycin each (0, 25, 50, 100, 150, 200, 250, 300, 350 & 400 mg/L)

Note that streptomycin, being non lethal selectable marker caused bleaching of the exposed tissues/calli (red arrows), whereas kanamycin, being lethal in its mode of action resulted in necrosis of the exposed tissues (pink arrows)

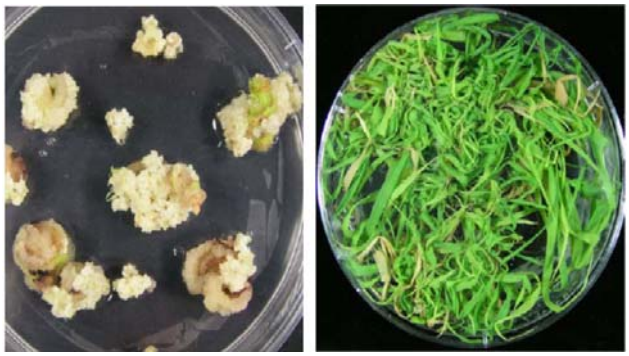


well as monocots (Cui *et al*., 2011). It is known that 44–77% of the transformation studies used *nptII* gene as the selectable marker (Miki & McHugh, 2004). International regulatory agencies have also approved the commercial release of transgenic oilseed rape, potato, corn, flax, tomato, cotton and chicory having *nptII* gene. NPT II proteins, expressed by the *nptII* marker gene have proved to be non-toxic and non-allergenic thus having no adverse effects on animals, humans and environment making it an ideal choice for transgenics (Flavell *et al*., 1992; Fuchs *et al.*, 1993).

Impact of spectinomycin: For this purpose wild type leaf roll discs were cultured on the callus induction medium (MS3) supplemented with above mentioned levels of spectinomycin. There was no detrimental effect of exposure of spectinomycin on callus proliferation and regeneration

Fig. 2: Callus induction and regeneration in genotype HSF-240 on spectinomycin concentration of 500 mg/L

Callus proliferative and regenerative phase showed insensitivity to the antibiotic and responded similar to non-transformed explant



hence, it was found to be exactly similar to antibiotic free medium (Fig. 2). Thus sugarcane calli were found to be resistant to spectinomycin. Most of the dicot species are sensitive to spectinomycin and have successfully been transformed for nuclear as well plastid transformation (Day & Goldschmidt-Clermont, 2011). It is well known as a valuable selection marker and has successfully been used for the transformation of tobacco, tomato, potato and brinjal (Singh *et al*., 2010).

Antibiotics differ in stringency depending upon their mode of action that ultimately decides its value for the selection of transformants (Wilmink & Dons, 1993). Among the antibiotics we tested, streptomycin appeared to be most stringent as for as its mode of action is concerned, while spectinomycin had no effect on sugarcane regeneration. Selectable marker genes are of core importance for the development of plant transformation system but stringency, transformation frequency and biosafety should always be the fundamental considerations before the choice of a selection system.

Scientists had always been striving for the development of marker free transgenic plants for commercialization in order to attract consumer acceptance so ultimate wish is to use a human friendly selection system. Thus antibiotic resistance genes, present in the GM foods should either be removed or replaced by some non antibiotic selectable marker systems or plant based selection systems (EFSA, 2009) to combat all of these concerns. Yemets *et al.* (2008) employed a goose grass derived α-tubulin gene for the transformation of flax, soybean and tobacco. Similarly a tobacco derived anthranilate synthase gene (Song *et al.*, 1998), sweet pepper derived ferredoxin like protein (*pflp*) gene (Chan *et al.*, 2005) and maize derived knotted1 (*kn1*) gene (Luo *et al.*, 2006) had been used for the development of biosafe GM foods so that potential risks relevant to the traditional selection marker genes are eliminated. Furthermore, the intrinsic tolerance of sugarcane to spectinomycin indicates the presence of natural antibiotic resistance mechanisms in plants. If explored, this would

pave a way towards the usage of plant based antibiotic resistance mechanisms for the selection of transgenes rather than bacteria based genes, for the production of more biosafe and human friendly transgenic plants without requiring marker gene elimination.

Acknowledgment: The authors would like to thank Ministry of Science and Technology for funds to MSK, Higher Education Commission, Pakistan for providing funds for the completion of PhD studies and Director, Sugarcane Research Station AARI, Faisalabad for explant provision.

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(Received 09 June 2011; Accepted 25 October 2011)