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Screening for Putative Transgenic Rice and Cotton Plants: A Simple and Easy Method

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Abstract: A simple leaf tip assay was used for screening of putative transgenic plants expressing the hygromycin resistance gene (*hph*) or kanamycin resistance gene (*nptII*). Leaf tips were excised from *in vitro* or *in vivo* transgenic plants and cultured on MS medium without phytohormons containing the suitable concentrations of hygromycin and kanamycin. Leaf tips of plants transformed with the marker gene showed no significant effect of the appropriate drug for at least two weeks. While non-transgenic leaf tips had noticeable symptoms of bleaching, necrosis or browning after 3-4 days of selection. This method is simple, rapid and allows clear distinction between transformed and non-transformed plants both, in monocots and dicots.

Key words: Leaf tips, cotton, rice, marker genes

Introduction

Delivery of DNA and its expression in plant cells are essential steps to get a transformed plant. One of the most important aspects of plant transformation is the preferential selection and growth of transformed cells, generally achieved by introducing a gene for antibiotic/herbicide/drug resistance. Neomycin phosphotransferase *(nptII* gene) confers resistance to kanamycin (Uchimiya *et al*., 1986) and hygromycin phosphotransferase *(hpt* also called *hph)* to hygromycin (Blochlinger and Diggelmann, 1984; Gritz and Davies, 1983).

Identification of transgenic plants immediately after the transformation process and the inheritance of the transgenes in its progeny is usually time consuming, laborious and often uses expensive procedures, such as Southern or Northern hybridization, dot blot analysis, enzymatic assay, polymerase chain reaction (PCR) and GUS assay. Although PCR is fast and sensitive method, it is susceptible to cross-contamination. GUS assay (beta glucuronidase assay) is an easy method to determine the delivery of the foreign gene into the plant after 16-48 hrs of the entry of DNA, by transient expression of the Reporter gene (Jefferson *et al*., 1986). This assay is based on fluorescence emitted by the product of GUS substrate x-gluc, which makes it very expensive. Direct in-plant assays for selectable marker gene activity such as spraying on the whole plants or leaf paintings with herbicide (Datta *et al*., 1992) or germination of seeds on selective media (Hiei *et al*., 1994) are being used. However, simple leaf painting and germination tests are not suitable for early identification of regenerated transgenic plants, spraying the whole plant might damage the subject plant.

In this communication, we report a simple method to identify and screen Basmati transgenic rice and cotton plants expressing the hygromycin resistance gene (*hph*) or kanamycin resistance gene (*nptII*), the two most widely used selectable marker genes in plant transformation. This assay might be also effective for identifying other transgenic plants in both dicots and monocots. The method is simple, rapid, requires less amount of plant material and allows clear discrimination between transformed and nontransformed plants. This procedure is very simple and can be used as an alternative to bioassay, leaf-painting etc. for preliminary test of transgenic plants to confirm the expression of transgene in plants. Moreover, more than one assays will further help to draw a correct conclusion.

Materials and Methods

Plants Material: Transgenic plants of rice (*Oryza sativa* cv. Basmati-370) containing *hph* gene were produced by biolistic transformation method reported by Husnain *et al*. (1995). Cotton plants (*Gossypium hirsutum* cv. MNI-1,93) containing *nptII* gene were produced by a combination of biolistic and *Agrobacterium* mediated transformation (Haris *et al*., 1998, 1999). The primary transformants, their progeny and control plants were grown in green house for 1-3 months before using them in this assay.

Sterilization: Leaves of one-month-old transgenic cotton and rice plants grown *in vitro* conditions were used as the source material. In the case of the greenhose plants, even two months old leaves were used. Cotton leaves were surface sterilized with 70% ethanol for 2 min, followed by commercial bleach containing 10% sodium hypochlorite for 5 min with vigorous agitation and 0.1% $HgCl_2$ for 2 min. The treated leaves were washed several times with autoclaved distilled water. Rice leaf tips from green house were also surface sterilized with the same procedure except commercial bleach treatment.

Leaf Tip Assay: From *in vitro* grown or green house plants, leaf tips (about 1-3 cm long) were excised with the help of fine sterilized blade from all the transgenic and control plants and immediately placed with the cut ends embedded in the medium in petriplate, to allow good contact with the media. The medium was composed of Murashige and Skoog Salts (Murashige and Skoog, 1962), pH-5.8, 1 50 mg/l citric acid, 1.5 mg/l gelrite, 30 gm/l sucrose, MS vitamins 100 mg/l ascorbic acid and approviate concentration of hygromycin or kanamycin.

For rice, MS medium containing different concentrations of hygromycin (10-100 μg/ml) were used. While in the case of cotton plants leaf tips were placed in MS medium containing 20-50 μg/ml hygromycin and 40-120 μg/ml kanamycin. All the cotton leaf tips were kept at 27°C. All assays were carried out under light for 16 hrs and then placed in dark for 8 hrs.

Results and Discussion

Rice Leaf tip Assay with Hygromycin: Twenty different transgenic rice plants derived from hygromycin selection were used in this assay. Expression levels of the *hph* gene in these plants were not determined but some of these

Table 1: Leaf tip assay of Rice (*Oryza Sativa*) for Hygromycin Resistant gene

Rice plant	Leaf assay	Other observations
Control-I	-	Green, Healthy
Control-II	-	Dead, Uniform Necrotic Tips, Blackened
CAMB-50	+	Green with Less Pronounced Bleaching
CAMB-445	-	Bleached Uniformly Green Strips
CAMB-623	+	Green with Less Pronounced Bleaching
CAMB-50-1	+	Greenish Yellow
CAMB-402	+	Green with Yellow Necrosis at Tips
CAMB-632	+	Green with Localized Bleaching
CAMB-615	+	Green Yellow
SAM-1	+	Green, Healthy, Brownish Yellow Patches
CAMB-622	-	Dark Brown Striped and Bleached
CAMB-472	-	Bleached, 10% Green
CAMB-536	+	Green, Bleached Strips
ANW-1	+	Green, Healthy
SAM-7	+	Green with Localized Patches
CAMB-470	-	Bleached Uniformly
SAM-5	+	Green, Localized Bleaching

Control-I = Rice leaf tips of control on MS
Control-II = Rice leaf tips of control on MS medium supplemented with Hygromycin

Table 2: Leaf tip assay of cotton plants for hygromycin resistance

Plant Number	Results	Observations after One Week
CAMB-304	-	Dead, Localized Bleaching
CAMB-306	-	Dead, Localized Bleaching
CAMB-314	-	Dead, Curled, Necrotic
CAMB-317	+*	Green, Localized Bleaching
CAMB-318	+	Green, Healthy
CAMS-323	-	Dead, Necrotic
CAMB-324	-	Dead, Necrotic
CAMB-328	-	Dead, Necrotic
CAMB-330	-	Dead, Necrotic
CAMB-336	-	Dead, Necrotic
Cam8-337	+	Green, Marginal Bleaching
CAMB-338	-	Dead, Black Bleashed
CAMB-340	-	Dead, Black Bleashed
CAMB-343	-	Dead, Necrotic
CAMB-344	+**	Green, Healthy
CAMB-347	-	Dead
CAMB-347	-	Dead, Necrotic
CAMB-348	-	Localized Bleaching
CAMB-349	+*	Green, Small Necrotic Patches
CAMB-351	+	Green, Marginal Bleaching
CAMS-353	-	Localized Bleaching
CAMB-354	+	Green, Chlorosis
CAMB 356	-	Dead, Black
CAMB-357	-	Dead, Chlorotic
CAMB 360	+*	Green, Curled
CAMB 361	+	Green, Marginal Bleaching
CAMB-362	-	Dead, Black
CAMF1-366	-	Dead Main Vien Bleached
CAMB-368		Dead Chlorotic
CAMB-369	-	Dead, Necrotic
CAMB-372	-	Dead, Necrotic
CAMB-373	-	Dead, Browinsh Black
CAMB-379	-	Dead, Chlorotic
CAMB-380	+**	Green, Necrotic Patches at the Tip
CAMB-383	+*	Green, Healthy
CAMB-384	+	Green, Yellow Patches
CAMB-385	-	Dead, Brown
CAMB-386	-	Dead, Chlorotic
CAMB-387	-	Dead Chlorotic
CAMB-388	-	Dead, Black
CAMB 391	-	Dead, Browinsh Black Strips
CAMB-392	-	Dead, Black, Patches
Control-I	-	Green, Healthy
Control-II	-	Dead, Blackish Brown and Necrotic Tips

Control-I = Leaf tips of control on MS, *Low expression
Control-II = Leaf tips at control on MSH-30, **High expression

plants have shown positive results in PCR against specific primers of hygromycin resistance gene. Two controls were used in this experiment, one from a non-transgenic rice plant without bombardment/*Agrobacterium* and another from a non-transgenic plant bombarded with tungsten particles only. Leaf tips of transgenic and control rice plants were placed on media containing different concentrations of hygromycin.

Non-transgenic rice leaf tips showed necrosis, dark brown strips or bleached tips, after 3 days on 40 μg/ml hygromycin, Hygromycin 40 μg/ml resulted in necrosis or bleached leaf tips of non-transgenic rice plants. However,

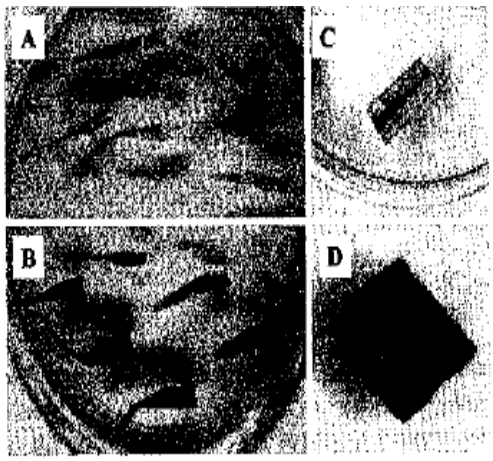

Fig. 1: Leaf tip assay for hygromycin resistane in rice plant
a. Control leaf tips growing on MS medium supplerneted with 40 μg/ml hygromycin
b. Transgenic rice leaf tips growing on MS *medium* supplemented with 40 μg/ml hygromycin
c. Control leaf piece growing on MS medium supplemented with 50 μg/ml hygromycin
d. Leaf pieces of transgeni plants growing on MS medium supplemented with 50 μg/ml hygromycin

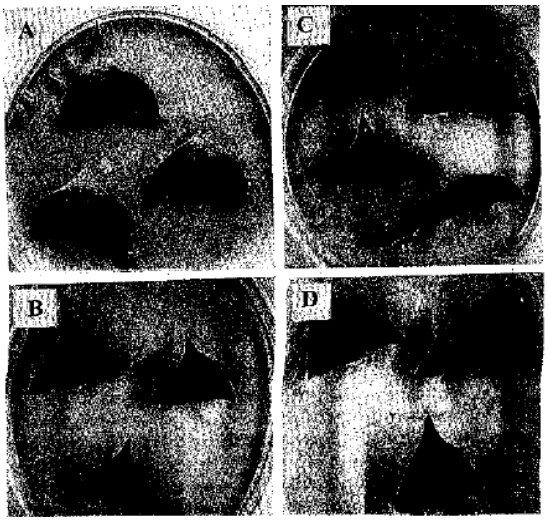

Fig. 2(a-d): Leaf tip assay for hygromycin and kanamycin resistance in cotton plants
a. Leaf tips of control cotton plants growing on MS medium supplemented with 30 μg/ml hygromycin
b. Leaf tips of transgenic cotton plants growing on MS medium supplemented with 30 μg/ml hygromycin
c. Leaf tips of control cotton plants growing on MS medium
d. Leaf tips of control cotton plants growing on MS medium supplemented with 120 μg/ml kanamycin

higher concentration of Hygrormycin (40 μg/ml or greater) resulted in more pronounced and widespread symptoms. Leaf tips of all the transgenic rice plants remained healthy

and green in the assay for at least 15 days (Fig. 1b). Some of the transgenic rice plants showed other kinds of symptoms e.g., localized bleaching, others turned blackish brown from tip and still others showed bleaching in strips etc (Table 1). From all of the above described symptoms, rice leaf tips could be distinguished from the non-transgenic leaf tips (Fig. 1a,b). These different kinds of symptoms may be due to different copy number of *hph* gene because this leaf assay could give a qualitative discrimination between plants with minimal levels of *hph* gene expression and non-transgenic plants. In some experiments, 1 cm square leaf pieces of mature rice plants were used as they gave better results in MS medium supplemented with hygromycin (50 mg/ml) for seven days. Pieces of control rice leaf turned yellow and bleached while pieces of transformed rice leaf remained green and healthy (Fig. 1c, d).

Cotton Leaf Tip Assay with Hygromycin and kanamycin: About, seventy transgenic cotton plants were assayed in the same way as cereal leaf tips. Leaf tips from transgenic cotton plants expressing the *hph* gene (hygromycin resistance gene) and the *nptII* gene (confers kanamycin resistance) were placed on media containing different concentration of hygromycin and kanamycin (Table 2). Nontransgenic cotton leaf tips showed partial bleaching in the medium containing 30 μg/ml hygromycin after four days and complete bleaching after 8-days (Fig. 2a). At lower concentrations of hygromycin, bleaching was less rapid and vice versa. Transgenic cotton leaf tips remained green and healthy for more than two weeks at all levels of hygromycin (Fig. 2b). Different kinds of symptoms due to the effect of hygromycin on transgenic plants and may be due to different expression levels are shown in the Table 2. It was observed that leaf tips of control cotton plants remained green and healthy on MS medium, even after one month (Fig. 2c).

The same assay was repeated with non-transgenic (control) cotton leaf tips placed on media containing different concentrations of kanamycin. Almost, all of the leaf tips remained green and were unaffected for more than two weeks, while in one or two leaf tips, radish, necrotic areas were observed after two weeks on 120 μg/ml of kanamycin (Fig. 2d). This concentration was too high to damage the leaf tissues. But no significant difference was observed after 10-15 days. These unexpected results may be due to differences in the bleaching effect of Kanamycin and hygromycin. Therefore, this assay can not distinguishe between *nptII* expressing and non-transgenic plants. All the above mentioned results of this assay were supported by other assays, such as, PCR, western blot analysis and ELISA etc (data not shown).

In conclusion, the leaf tip assay can be used as an easy and inexpensive method to distinguished between transgenic and non-transgenic plants of cotton and rice. The main advantage of the method is the use of minimal amount of transgenic plant tissues, as well as chemicals which cause no damage to the whole plant. It can also be used for screening large numbers of segregating population of transgenic plants and preliminary selection of transgenes. This method may also be used with other selectable marker genes like herbicide resistance gene *(bar).* This is the first report of such work on Basmati rice and cotton cultivars. Aromatic indica Basmati rice was used as a monocot plant and recalcitrant cotton as a representative of dicots plants.

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