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REGENERATION OF TRANSGENIC EGGPLANTS *(SOLANUM MELONGENA* L.) FOR A CYSTEINE PROTEINASE INHIBITOR

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ABSTRACT

Transgenic eggplants *(Solanum melongena* L.) were recovered as a results of *Agrobacterium tumefaciens* mediated transformation performed with ERA101 strain carrying the binary plasmid pCYS. Plasmid pCYS contains a cysteine proteinase inhibitor gene derived from soybean *(Glycine max* L.). The effect of growth regulators and antibiotics on eggplant transformation was also studied and optimised. A total of 25 independent transgenic lines were obtained from 300 co-cultivated explants of five eggplant lines. Specific PCR analysis of the putative transformants demonstrates the presence of fragment corresponding to the kanamycin selective marker NPTII. Protease inhibitors are an important element of plant defence response to insect --redation. The role of '"Iroteinase inhibitors genes is discussed in the context of multigene resistance to develop an effective strategy to achieve and maintain an higher and durable level of pest resistance when combined with Bt gene.

INTRODUCTION Eggplant *(Solanum melongena* L.), with the world production of 9 million metric tons (FAO, 1995), is one of the most important Solanaceous crop in Asia and Mediterranean basin and it represents a popular vegetable in the diet of the inhabitants of these countries. Among the European countries Italy is the first producer of eggplants with 11.000 ha of cultivation and an annual production over 3 hundred thousands tons (Setti, 1997).

The commercial production of eggplant is extensively hampered by devastating attacks of the coleopteran insect, Colorado Potato beetle (CPB) *(Leptinotarsa decemlineata* Say) in western countries (Cotty & Lashomb, 1981), Glasshouse whitefly *(Traleurode vaporiariorum* Westw) and fruit and shoot borer *(Leucinodes orbonalis.* Guenee) in Asian countries (Atwal, 1986). In the first cases larvae feeds the leaves, in the latter case they upon hatching, bore into the fruits and shoots causing severe damage which make the fruits non-martable or inedible.

In particular CPB represents the most important insect pest of thi. ...rop in Europe and America. In the absence of an effective pest control program, this insect may cause total destruction of eggplant crop (Cotty & Lashomb, 1982; Maini *et al.,* 1990; Arpaia *et al.,* 1995). Chemical pesticide applications are estimated to be expensive and to have a strong environmental impact (Dulmage, 1980). Conventional breeding programmes to develop high-quality and pest resistance varieties often require 12 or more years of sustained, cooperative effort between plant breeders and entomologist to be£in producing resistant varieties (Roberts *et al.,* 1988; Stoner, 1992). Eggplant gene pool lacks of valuable resistance gene to CPB.

The ability to efficiently introduce useful foreign genes into plants is the key to the success of plant biotechnology industry. Genetic engineering is becoming a routine process for an increasing number of horticultural crop plants, resulting in a number of transgenic products which are ready for or close to market introduction (Grumet, 1995; Mol *et al.,* 1995; Woodson, 1997).

The insecticide crystal protein genes of *Bacillus thuringiensis* (Bt) have emerged as an important gene family in the biotechnological manipulation of insect resistance in cultivated plant species (McGaughey & Whalon, 1992; Flischhoff, 1996). Endotoxins have been identified with activity against Lepidoptera, Diptera, Coleoptera, and both Lepidoptera and Diptera (Peferoen *et al.,* 1990; Gill *et al.,* 1992), and various strains and formulations of *B. thuringiensis* are currently used as microbial pesticides against insects in these three taxonomic orders.

The development of plant regeneration (Gleddie *et al.,* 1983; Rotino *et al.,* 1987) and transformation technique in eggplant (Rotino & Gleddie, 1990) provides the opportunity to transfer new specific traits of interest (i.e. those for insect pest resistance) into valuable genotypes. Moreover, transgenic eggplants re~istant to Colorado Potato Beetle by means of *B. thuringiensis* endotoxin have being successful obtained (Arpaia *et al.,* 1997; Iannacone *et al.,* 1997).
The other type of gene for plant resistance that has been field tested codes a protein inhibiting proteases - in animal digestive systems (Ryan, 1990). The proteases are essential enzymes mediator for the digestion of plant proteins by herbivores.
Several inhibitor protease genes have been tested. They are identified as serine, cysteine, aspartic or metallo-proteases based on the active aminoacid in the reaction centre (Ryan, 1990). Insects mainly use one or a combination of serine, cysteine and aspartic proteases as major digestive proteolytic enzymes (Ryan *et al.,* 1981). Plant cysteine protease inhibitors are typified by the phytocystatins, which inhibit proteases of the papain superfamily. In some Coleopteran and Hemipteran insects, major digestive proteolytic activities are apparently the result of papain-like cysteine proteases that are susceptible to inhibition by plant cysteine protease inhibitors. Transgenic plants expressing cysteine protease inhibitors show enhanced resistance to predation by pests, indicating the useful function of these inhibitors (Johnson *et al.,* 1990; Urwin *et al.,* 1995; Bolter & Latoszekgreen, 1997).
In the present study we transfofDled eggplant using cysteine inhibitor from Soya bean gene to confer pest resistance in this Solanaceous crop. Our fmal aim is to combine in the same line such resistance with the already obtained *B. thuringiensis* endotoxin resistance in eggplant in order to produce a synergetic effect to maintain an higher and durable level of resistance to CPB.

MATERIAL AND METHODS

The lines Tina, Tal 8-1, Tall-I, SM5-44, and DR2, were employed. Seeds for *in vitro-grown* plants were surfaced-sterilised by dipping in 70% ethanol for 30 seconds, followed by 20 minutes in 7% calcium hypochlorite and fmally rinsing three times in sterile water. The sterile seeds were germinated in petri dishes (25-30 per plate) on a filter paper soaked with sterile water and incubated in the darkness at 28°C. After 7-10 days the germinated seeds were transferred into sterile GA7 boxes (Magenta Corp.) containing 40 ml of V3 medium supplemented with 2% (w/v) sucrose and 0.6% agar, pH 5,8 (0,2 N KOH prior to ! autoclaving). Plants were maintained in a growth room at 25°C with a 16 h day-length under fluorescent I light (50 uEm-2 sec-I).
The procedure for eggplant transformation was essentially as described by Rotino & Gleddie (1990) and Rotino *et al.* (1992) with modifications. Leaf, cotyledon and hypocotyl explants were precultured for 2 days in MS macro- and micro-nutrients, Gamborg vitamins (1968), 0,5 gl-l of MES, 20 11M of acetosyringone supplemented with the growth regulators (mgl-l) 0,5 ZEA, 0,3 BAP, 0,2 KIN and 0,1 NAA; media were solidified with 2 gl-1 of Phytagel (Sigma), pH 5,8. For explant infection, an overnight liquid culture of *Agrobacterium tumefaciens* was centrifuged and the pellet re-suspended at 0,1 *OD600* density in MS basal medium, 2% glucose, 200 11M of acetosyringone, pH 5,5. The cut edges of the hypocotyls were cut again and all the explants were infected by dipping in the bacterial suspension for 5 minutes, blotted dry onto sterile filter paper and then placed back in the same plates. After 48 h of co-cultivation the explants were transferred to selective medium (described above) without acetosyringone and supplemented with 50 mgl-l of kanamycin and 500 mgl-l of cefotaxime. Shoot-buds differentiation and shoot elongation was achieved by transferring calli with compact green nodules to the same selective medium without NAA. Shoots were rooted and propagated in V3 medium (Arpaia *et al.,* 1997) without antibiotics. Regenerated plants were labelled according to the original callus (first no.) and shoot (second no.). Transgenic plantlets were grown m the greenhouse and flower buds were covered with paper bags for sclf-pollination.
Alternatively, a second protocol was compared with the above described one. This protocol was set up for "Hibush" eggplant cv. by Billings *et al.* (1997) and it differs from the first one for the culture regeneration culture medium which contains 0,1 11M thidiazuron (TDZ) combined with 10 to 20 11M N-6- (isopentyl) adenine (2iP). Augmentin at 300 mgl-1 was used after co-cultivation to eliminate *A. tumefaciens* instead of cefotaxime. For each of these two protocols 300 explants were used.

Leaf-discs from putative transformants were cultured on regeneration medium containing 30 mgl-1 of kanamycin to verify their ability to produce callus. Expression of NPTII marker gene was also monitored just after plantlet acclimatisation by spraying with a 300 mgl-1 kanamycin solution according to Sunseri *et al.* (1993).
Plant DNA was isolated from young leaves according to Doyle & Doyle (1990). PCR analysis was performed using the primers which amplified a 839-bp fragment of the NPTII cc-ding regIon (Arpaia *et al.,* 1997). PCR reaction was performed using 400ng of template DNA in 50 1.11 of 50mM KCl, 10 mM Tris- HCl (pH 8,3), 1,5mM MgC12, 0.001% (w/v) gelatine, 200 I.1M dNTPs, 50 pM of each primer and 1 U *AmpliTaq* polymerase (Perkin Elmer). Amplification was carried out in a thermocycler (Perkin Elmer)- programmed for: one cycle of 5 min at 95°C; 35 cycles of 15 sec at 95°C, 1 min at 60°C, 3 min at 72°C; and one fmal cycle of 10 min at 72°C. PCR products were subjected to electrophoresis in a 1% (w/v) agarose gel containing 0,1 I.1grnl-1 of ethidium bromide and analysed under UV light.

RESULTS AND DISCUSSION

With the flrst treatment explants following co-cultivation formed callus and shoots on selective media. White, friable callus was visible along the cut edges of the explants within 3-4 weeks from the infection. No shoot or bud differentiation was observed at this stage. After three or more subcultures on selective medium in constant presence of kanamycin (50 mgl-I), despite the colour of the explants was yellowish, some regions of the calli turned green and compact green nodules were formed. Thirty-seven resistant calli were selected and shoot primordia differentiated from most of the nodules.
Twenty-five putative transgenic plant lines were obtained from 300 co-cultivated leaf explants with a _.lnsformation frequency of 8,3%. No callus formation or shoot organogenesis was observed in control explants cultured on selective regeneration medium. This result indicate that kanamycin at 50 mgl-1 was an efficient level of selection. After co-cultivation, the explants subjected to the Billings treatment enlarged, showed a very brilliant green colour and formed abundant mass of callus. But they suddenly necrotised and died after two subculture. The different genotypes employed may explain the contrasting results obtained.
The DNA amplification (PCR analysis) clearly showed the presence of strong fragment NPTII marker gene for all the transformant events. The presence of an active NPTII gene was also confIrmed by the absence of chlorosis after *in vivo* spray of kanamycin solution The rooted transformed plants are now growing in the greenhouse to perform molecular and biochemical analyses and inse~t bioassays.
The ability of insects populations to overcome plant resistance has been a continuing problem for pest resistance breeding. This has been true for conventionally bred genotypes resistant to insects (Gracen, 1985), and it is expected to happen for genetically engineered resistance, too. Other species of caterpillar have evolved populations resistant to *B. thuringiensis* in place where it was used intensively as a spray (McGaughey, 1985; Shelton *et al.,* 1993). Transgenic eggplants containing cystaine protease inhibitor gene from dicot plant, soybean, were regenerated and after a further experimental field trial, to detenninate the real resistance level, it can be used to cross with lines transformed for *B. thuringiensis* endotoxin with the final aim to accomplish the two different genes into a multigene resistance line with a possible synergetic effect. An appropriate strategy of resistant varieties deployment should be also evaluated to preserve the resistance traits.

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