Scientia Horticulturae 125 (2010) 461–468

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Agrobacterium × plant factors influencing transformation of 'Joseph's coat' (*Amaranthus tricolor* L.)

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ARTICLE INFO

Article history:
Received 1 March 2010
Received in revised form 16 April 2010
Accepted 17 April 2010

Keywords:
Amaranthus tricolor L.
Agrobacterium rhizogenes
Rhizoclones
rolB gene amplification
Opine assay

ABSTRACT

A protocol is developed for *Agrobacterium*-mediated genetic transformation of *Amaranthus tricolor* via explant co-cultivation with *Agrobacterium rhizogenes*. Bacteria-plant specific factors which influenced transformation were optimized. Of the two *Agrobacterium* strains employed, LBA9402 was more infectious compared to A4. Bacterial suspensions grown overnight with 100 μM acetosyringone and experiencing $O.D._{660} = 0.6$ followed by dilution to a density of 10^9 cells ml^{-1} were the most effective. Explants from garden-grown plants were more responsive than those from *in vitro* cultures; stem internodes being better than leaves. Immersion of the pre-pricked explants in bacterial suspension resulted in a markedly higher transformation frequency compared to the direct injection method. The infection of internode explants with the LBA9402 strain followed by co-cultivation on growth regulator-free MS medium (MS0) for 5 days resulted in emergence of hairy roots up to a maximum frequency of 97.22%. Roots were individually cultured in MS0, but fortified with bactericidal antibiotic (500 $\mu g\,ml^{-1}$ cefotaxime). Rhizoclones showing prolific growth were renewed through successive subcultures in MS0. Opine gene expression was revealed by positive agropine and mannopine synthesis in all selected transformed rhizoclones. Shoot regeneration from root clones, capable of auxin-independent growth and opine proficiency, was stimulated in MS augmented with 2.0 $mg\,l^{-1}$ zeatin. pRi *TL*–DNA *rol*B and pRi *TR*–DNA *man*2 ORF were detected in leaf tissues of regenerated plants from selected hairy root clones through PCR amplification. The implication of such findings is discussed on the possibility of conferring protection to crop amaranths against biotic stress challenges, particularly due to insects, viruses or fungal pathogens.

1. Introduction

The amaranths represent an horticulturally important group of annual herbaceous plants represented by about 20–25 species occurring in India of a total of 60 species in the world flora under *Amaranthus* L., which is the fifth largest genus in the Family: Amaranthaceae of order: *Caryophyllales* (The Wealth of India, 1948). *Amaranthus* species are grown in several parts of the world including Mexico, Central and South America, India and Africa where they are mostly consumed as leafy vegetables ('green amaranths') or grain crops ('grain amaranths'). Green amaranths serve as one of the most delicious leafy vegetables and are a rich source of protein (up to 5.6% on fresh weight basis), requisite vitamins (A, B, C, folic acid), minerals (Ca, Mg, K, P, Na, N, Fe, Mn, Zn) and fibres (5.25%) (Elias, 1977; Flores and Teutonico, 1986). Besides, they contain other biologically pro-health compounds such as the antioxidant squalene and carotenoids (Kumari and Prakash, 2005) and thus, are recommended as a nutritious food with medicinal properties for young children, lactating mothers and for patients with fever, hemorrhage, anemia or kidney complaints. Of several species cultivated in tropical and sub-tropical areas of India *Amaranthus tricolor* is probably the highest yielding leafy vegetable crop species with its excellent nutraceutical value. On the other hand, the attraction of the grain amaranths (*A. hypochondriacus*, *A. cruentus* and to some extent *A. caudatus*) to both earlier civilizations and modern consumers is due to the thousands of tiny but highly nutritious pinkish white or golden seeds that are unusually high in protein (14–16%) with a well-balanced amino acid composition and particularly an elevated lysine content. Thus, amaranths qualify as an ideal food source for people of low income-food deficit countries (LIFDC). Besides, some *Amaranthus* species (e.g. *A. caudatus*, 'love-lies-bleeding'; *A. hypochondriacus*, 'Prince's Feather'; *A. tricolor*, 'Joseph's coat') are valued as ornamentals being gifted either with floral splendor due to their radiantly coloured inflorescence or variegated foliage. Owing to their high C_4-type photosynthetic efficiency and high yielding ability coupled with minimum of cultivation constraints and moderate tolerance to drought, salinity and heat, amaranths have evolved as a nutritionally rich food crop to serve as an essential component of the sustainable horticulture

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doi:10.1016/j.scienta.2010.04.034

system in tropics (Flores and Teutonico, 1986; Gajdosova et al., 2003).

Unfortunately, amaranths are susceptible to damage by foliar insects such as leaf miners, leaf rollers, cutworms, aphids, flea beetles and mites (Metcalf and Metcalf, 1993). Blister beetles and alfalfa webworm are the two leaf feeders that have caused substantial economic yield loss. Besides, a majority of grain, green and ornamental amaranths are susceptible to two common viruses namely Amaranthus leaf mottle potyvirus and Amaranthus mosaic potyvirus. In addition, *A. tricolor* is also susceptible to Apple mosaic ilavirus. Several *Amarathus* species are also known to be challenged by fungal pathogens including *Fusarium* (*F. equisetti* and *F. moniliforme*), *Aspergillus flavus, A. parasiticus, A. versicolor, Penicillium viridicatum, P. puberulum, P. crustosum, P. citrinum, P. expansum* and *F. solani* (Bresler et al., 1991).

Therefore, it was imperative to intensify investigations aimed at causing genetic improvement of this hitherto under-exploited crop via genetic engineering using suitable constructs of transgenes to confer resistance to biotic stress due to pests, viruses or fungal pathogens. Success in transgenic manipulation of amaranths is only limited to the grain-type *A. hypochondriacus* using *Agrobacterium tumefaciens* to study the expression of a light-harvesting chlorophyll a/b-binding protein gene promoter (Jofre-Garfias et al., 1997). To date, there is no report on genetic transformation of the most important vegetable amaranth *A. tricolor*. This led to the present work that was broadly envisioned at exploring the possibility of *Agrobacterium*-mediated genetic transformation of *A. tricolor*. A pre-requisite to this is the succinct evaluation and standardization of *Agrobacterium* × plant factors influencing genetic transformation. These would include bacterial strains, growth phase of culture, cell density, virulence inducer, explant type, infection strategy, co-cultivation period, bactericidal antibiotic, etc. Therefore, in the present study, efforts were made to optimize each one of them with a view to maximizing the transformation efficiency and thereby developing a reproducible protocol for genetic transformation of amaranths.

2. Materials and methods

2.1. Plant material

Stem internode and leaf explants were collected from plants of *A. tricolor* L. (NBPGR Accession No. IC-447684) grown in the experimental plots in the Department of Botany, Utkal University, Bhubaneswar (India). Explants were placed under running tap water (15 min) followed by treatment with 7.5% (v/v) lizol (Reckitt Benckiser, India) for 30 min and rinsed in autoclaved tap water (5–6 changes). These were surface-disinfected by treatment with 0.1% mercuric chloride (8 min) followed by rinsing with autoclaved distilled water (5–6 changes). Seeds were surface-disinfected by dipping them in 70% (v/v) ethyl alcohol (10 s), followed by treatment with lizol (7.5%, v/v; Reckitt Benckiser, India) and mercuric chloride (0.1%, 12 min) and, thereafter, rinsing in autoclaved distilled water as for outdoor explants. Surface-disinfected seeds were placed in 0.8% (w/v) agar-solidified Murashige and Skoog's (1962) basal medium (MS) without added growth regulators (MS0) for *in vitro* germination. Seedlings were maintained inside the culture room (25 ± 1 °C, 35–40 μmol m^{-2} s^{-1} photon flux density [PFD], 60% R.H.) where axenic plants were eventually established in 300 ml screw-capped glass jars (Excel Corporation, Alleppey, Kerala, India.) containing MS0 (20 ml per jar).

2.2. Bacterial strains and culture media

The wild type A4 strain of *Agrobacterium rhizogenes* (kind gift from D. Tepfer, Laboratoire de Biologie de la Rhizosphere, Institut National de la Recherche Agronomique, Versailles, Cedex, France) harbours an agropine-type pRiA4 while LBA 9402 (kind gift from M.R. Davey, School of Biological Sciences, University of Nottingham, England, U.K.) is a rifampicin resistant strain possessing an agropine-type Ri plasmid pRi1855. LBA 9402 strains were grown at 26–28 °C in modified YEB medium (5 g l^{-1} nutrient broth, 1 g l^{-1} yeast extract, 5 g l^{-1} peptone, 5 g l^{-1} sucrose, 15 g l^{-1} agar; pH 7.4). The pH of the medium was adjusted prior to autoclaving and the medium cooled to 40 °C in water bath. Thereafter, 2 ml of 1 M $MgSO_4$, $7H_2O$ and 50 mg l^{-1} rifampicin from respective filter-sterilized stock solutions were added to it. A4 strains were grown in MYA medium (5 g l^{-1} yeast extract, 0.5 g l^{-1} casamino acids, 8 g l^{-1} mannitol, 2 g l^{-1} $(NH_4)_2SO_4$, 5 g l^{-1} NaCl; pH 6.6). For explant infection a loop-full of bacteria from single colony was inoculated into 20 ml liquid medium in a 50 ml Erlenmeyer flask and incubated on a reciprocal shaker (120 rpm) at 28 °C. A 100 μl aliquot of the overnight suspension was re-inoculated into fresh medium (10 ml/25 ml Erlenmeyer flask). Prior to bacterial inoculation, acetosyringone (Sigma, USA; 100 mM stock solution in DMSO) was added to the culture medium at final concentrations of 50, 75, 100, 150, 200 μM. The cultures were grown for 16–18 h on a reciprocal shaker (120 rpm) at 28 °C.

2.3. Transformation

Leaves and stem internodal explants from *in vitro*-grown axenic plantlets (3–4 weeks after germination) or those from outdoor-grown plants after surface-disinfection were used for transformation. Apical portions of the cut internodal surfaces and leaf midribs were inoculated with 10–30 μl of an overnight culture of *Agrobacterium* ($O.D._{660}$ = 0.6) by means of a sterile hypodermic needle in two ways. The explants were either injected with bacterial solution directly by hypodermic syringe or pre-wounded by manual pricking with the sterile needle and thereafter immersed in the bacterial suspension (10–20 min). Third method was also attempted in which explants were floated in the *Agrobacterium* suspension without a precocious wounding. *Agrobacterium*-treated explants were plunged in 300 ml screw-capped jars containing MS0 medium (20 ml per jar) and the jars were kept inside the culture room (25 ± 1 °C) under diffuse light with a low PFD (10–15 μmol m^{-2} s^{-1}). Following co-cultivation (2–8 days) explants were transferred to 0.8% (w/v) agar-solidified MS0 supplemented with a bactericidal antibiotic. Three different antibiotics were tested, namely sporidex (Ranbaxy, Mumbai, India), carbenicillin (Sigma, USA) and cefotaxime (Sigma, USA) at a range of concentrations (250, 500, 750, 1000 μg ml^{-1}). Control cultures, containing similar explants but wounded with the hypodermic needle without bacteria, were maintained under similar light and temperature conditions as for inoculated cultures.

2.4. Statistical analysis

All transformation experiments were set up in a completely randomized design (CRD). Each treatment consisted of 10 replicate jars, each containing 1–2 leaf explants or 3–4 internodes. Each experiment was repeated 7 times. Data were analyzed using analysis of variance (ANOVA) for a completely randomized design. Duncan's new multiple range test (DMRT) (Gomez and Gomez, 1984) was used to separate the mean of significant effect.

2.5. Establishment of transformed root cultures

Individual roots (1.0–1.5 cm) that were developed along the inoculated surface were excised and each transferred to 50 mm × 11 mm transparent plastic TPX Petri dish (Tarsons, India) containing 6.0–8.0 ml of MS0 agar medium. The medium was sup-