Acetosyringone and osmoprotectants like betaine or proline synergistically enhance Agrobacterium-mediated transformation of apple

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ABSTRACT: The effects of the plant signal molecule acetosyringone (AS) and the osmoprotectant betaine phosphate (BP) have been examined for their ability to increase the transformation efficiency of Agrobacterium tumefaciens (At), C58CI::pGV3850 harboring the binary vector pKIWI105. This binary plasmid encodes the β-glucuronidase (GUS) gene and was previously shown to be expressed exclusively in plant tissues. Bacteria were grown in one of two previously reported virulence induction media (MS20 and SIM) for 5h and GUS activity was measured fluorimetrically in individual 6 week old leaf discs as a quantitative measure of stable transformation events. Bacteria induced in MS20 supplemented with AS (0.1mM) and BP (1mM) showed a significant increase in GUS activity as compared to media containing AS or BP added singly or control media lacking the supplements. The effects of another osmoprotectant proline (1mM) could replace the beneficial effects of betaine. No significant difference was observed among treatments with respect to the two induction media.

Introduction

Although transgenic apple plants can now be produced [James et al., 1989] the efficiency, even with highly regenerable cultivars, remains low and is usually in the range of 0.1-0.5% on a per explant basis. This has led us to investigate the relationship between Agrobacterium virulence and transformation efficiency rather than examining the tissue culture and regeneration process [Dandekar et al., 1990].

Phenolics, sugars, temperature and pH [Alt-Moerbe et al., 1988; 1989] can affect the virulence of Agrobacterium and presumably its capacity to transform plant cells. Evidence for these observations has usually been obtained by using Agrobacterium with plasmids containing the various vir gene promoter regions fused to the lacZ gene encoding β-galactosidase [Stachel and Nester, 1986]. Different cultural conditions are imposed upon these bacteria and β-galactosidase activity is used to measure the extent to which virulence is induced. We have extended this idea by applying these culture variables to an Agrobacterium strain that harbors disarmed plasmids encoding the β-glucuronidase (GUS) gene [Jefferson et al., 1987] and then measuring stable gene expression by assaying GUS activity in large numbers of apple leaf discs six weeks after infection.

In this paper we have examined only one of four variables mentioned by Alt Moerbe et al., (1989) as being important during the virulence induction phase i.e., the plant phenolic acetosyringone (AS). We also examined the need for including the natural osmoprotectants betaine phosphate (BP) and proline in the virulence induction medium [Le Rudulier et al., 1984]. AS is produced during the wounding of plant cells and has been shown to induce the transcription of the virulence genes of Agrobacterium tumefaciens by interacting with a transmembrane receptor protein [Melchers et al., 1989] encoded by virA [Stachel et al., 1985]. AS has been found useful in experiments aimed at increasing the efficiency of Agrobacterium-mediated plant transformation [Sheikoleslam and Weeks, 1987] while the presence of betaine phosphate has been shown to lead to an increase in the expression of several virulence genes in Agrobacterium [Vernade et al., 1988]. In our experiments, the two compounds have been used either in the presence of a simplified induction medium 'SIM' [Alt-Moerbe et al., 1988] or with the more commonly used MS20 [Murashige and Skoog, 1962] medium. GUS activity was then measured in apple leaf discs transformed with A. tumefaciens
carrying the disarmed binary vector, pKIWI105 [Janssen and Gardner, 1989].

Methods

Bacterial strains and plant vectors. The disarmed Agrobacterium tumefaciens strain CS8C1/pGV3850 harboring the binary vector pKIWI105 [Janssen and Gardner, 1989; Raineri et al., 1990] was used in all experiments. The Agrobacterium tumefaciens strain CS8C1 is a nonmutagenic derivative of CS8 that does not contain the endogenous Ti plasmid pTiCS8 [Van Larebeke et al., 1974]. The plasmid pGV3850 is a disarmed version of the Ti plasmid pTiC58 where the tumor forming genes have been replaced with sequences of the plasmid pBR322 and has been previously used as a cointegrating vector (Zambrzycki et al., 1983). The GUS gene of pKIWI105 has shown negligible activity in Agrobacterium but good activity in plant cells (Raineri et al., 1990).

This vector contains both a chimeric GUS gene under the control of the CaMV35S promoter and the gene encoding the bacterial aminoglycoside phosphotransferase [APH (3')II] under the control of the nos promoter which confers resistance to the antibiotic kanamycin for selection of transformed plant cells. CS8C1/pGV3850 without the GUS gene was used for control infections to measure background fluorescence.

Growth and virulence induction of Agrobacterium. Bacteria were grown at 25°C in liquid yeast-peptone (YEP) medium and allowed to grow to an O.D.420 of 1.5-2.0, centrifuged at 2500rpm and then diluted and resuspended with low pH (5.2) virulence induction medium (MS20 or SIM) to give a final O.D.420 of 0.5. After dilution, bacteria virulence was induced by incubation at 25°C for 5h with agitation at 200rpm. Appropriate antibiotics were present throughout the vegetative growth of the bacteria but were not present during the induction phase.

Induction media. Two induction media were used: i) MS20, this was full strength MS20 [Murshige and Skoog, 1962] medium containing 2% sucrose (w/v) pH 5.2 and II) Simplified induction medium (SIM) [Alt-Moerbe et al., 1988] containing 2% sucrose in 20mM sodium citrate, pH 5.2. AS (0.1mM) with or without BP (1mM) was added to induction media; the pH was adjusted and solutions were filtered sterilized.

Leaf disc transformations, co-cultivation, selection and regeneration. All procedures were carried out as previously described [James et al., 1989] except that discs were grown in 9cm Petri dishes rather than multi-welled dishes. Filter-sterilized AS (0.1mM) and BP (1mM) were included in the co-cultivation medium in one experiment, otherwise they were only included during the 5h induction stage. Transformed cells were selected on a medium containing 25 mg/l kanamycin on which discs were left for 21 days at 25°C in the dark. The regeneration media BNZ511, contained B A at 5mg/l, NAA at 1mg/l, thidiazuron at 1mg/l and cefotaxime at 200mg/l to prevent Agrobacterium growth. After 21 days discs were transferred to the same regeneration medium lacking kanamycin for a further 21 days in the dark at 25°C. At the end of the 42 days discs were destructively assayed to determine GUS activity.

Fluorimetric determination of GUS activity. Determination of GUS activity was carried out as previously described [Jefferson et al., 1987] using 4mM 4-methyl umbelliferol-β-D-glucuronide (MUG) as the substrate. Insoluble polyvinylpyrrolidone and β-mercaptoethanol were added to extraction buffer immediately before homogenization. Assays were performed on individual 7mm diameter leaf discs in a final reaction volume of 0.7ml. Supernatants from crude extracts were used for assay at pH 7.0 and at 37°C. Each treatment contained 45 to 48 leaf discs. Preliminary experiments confirmed that over the assay time used (100 min) zero order kinetics were observed. GUS activity was expressed as pmoles/disc/min.

Table 1. Comparing the two virulence-inducing media SIM and MS20 for their effect on GUS activity in apple leaf discs 42 days after inoculation. The effect of preventing leaf discs from contacting SIM is shown in a comparison of treatment C with D.

Analysis of fluorescence values. Fluorescence values spanned five orders of magnitude within a single experiment. Even after the data were log-transformed they remained very skewed and unsuitable for analysis of variance because of the extreme influence of occasional very large data values. As an alternative and more valid analysis we have classified the fluorescence readings arbitrarily into three classes based on the orders of magnitude of the response. These classifications were 'low' (<10 pmoles/min/disc), 'medium' (10-100 pmoles/min/disc), and 'high' (>100 pmoles/min/disc). For each treatment the number of readings in each category was tabulated and chi-squared tests were used to make comparisons between treatments.

Results

SIM can replace the complex salt mixture of MS in the virulence induction media.

Bacteria were grown on induction media for 5h at 25°C either on MS20 or on SIM with both AS and BP. Compared to MS20, SIM has an improved buffering capacity especially at low pH conditions known to induce vir genes.

To prevent contact between leaf discs and SIM, bacteria were centrifuged after 5h induction in SIM and resuspended in MS20 prior to the infection (treatment D, Table 1). To allow for any detrimental