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A simple protocol for transient gene expression in ripe fleshy fruit mediated by *Agrobacterium*

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Abstract

Fleshy fruits represent a very important economic resource and, therefore, they are an ideal target for biotechnological ameliorations. However, because of their physiological and anatomical characteristics, ripe fleshy fruits represent an extremely difficult material for transient gene expression assays aimed at the study of gene promoters in a short time. To this purpose, a fast and efficient *Agrobacterium*-mediated transient gene expression system was developed for ripe fleshy fruits. A β-glucuronidase reporter gene interrupted by an intron was used in order to prevent the possible expression of GUS activity by the *Agrobacterium* cells. The contemporary use of another reporter gene was used to check the transformation efficiency. This method is based on the injection of an *Agrobacterium* suspension into the fruits, and allows both qualitative and quantitative assays in a wide range of fruits to be carried out.

Key words: Agrobacterium tumefaciens, ripe fleshy fruits, transient gene expression.

Introduction

Fruits are very important for the life of plants since they protect the seeds and play a role in their dispersal. However, due to their nutritional and organoleptic characteristics, fruits may also be important for the animals that feed on them. This economic importance has led many researchers to study the process of fruit development and ripening, though most work has dealt with edible fleshy fruits, while much less is known about the development of non-fleshy, less economically important fruits.

Molecular biology has provided substantial information about genes expressed during ripening, however, most knowledge has been obtained from a limited number of fruit species, with tomato being the most intensely studied crop. Therefore, it could be useful to know, for instance, whether a given fruit-specific gene promoter from tomato might be employed to drive the expression of genes of interest in other fleshy fruits. In other cases, once expression studies have demonstrated the fruit-specificity of a certain gene, it is important to understand the regulatory properties of its regulatory regions, before using it to drive expression of genes of interest in fruits. In other words, knowledge of either a promoter strength or the length of its best regulatory region would be extremely useful for the correct planning of a biotechnological intervention aimed at the improvement of a fruit quality.

Promoters can be studied either in permanently transformed plants or in transient expression systems. In particular, the latter allows a fast analysis since no regeneration of transformed cells into a transgenic plant is required. Such an advantage is especially valuable in those cases where species recalcitrant for regeneration have to be studied, but also in the case of fruits which are normally produced long after the transformation experiments have been carried out.

Protoplasts have often been used for transient expression analyses. Once set up, this method is reasonably fast and does not require any particularly expensive apparatus. However, a number of problems can arise when trying to obtain protoplasts from ripe fleshy fruits as they normally consist of very large parenchymatic cells with huge vacuoles and the centrifugation steps usually required for protoplast preparation (Bilang *et al.*, 1994) may easily disrupt them.

Besides allowing the permanent transformation of many important, and formerly recalcitrant, agronomic

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species, the biolistic method has become very common in transient expression studies. However, contrary to protoplasts, this method requires a special apparatus and presents a number of physical and biological parameters which need to be considered (Christou, 1994), thus making it not particularly simple to use. In particular, this can be true in the case of ripe fleshy fruits due to their anatomical and physiological peculiarities.

Because of the difficulties in doing homologous transient gene expression using ripe fleshy fruits, fruit promoters have mostly been studied in permanently transformed plants (Deikman *et al.*, 1992; Blume and Grierson, 1997), thus limiting sensibly the number of fruit specific promoters analysed so far. Moreover, the difficulty of transforming and regenerating the woody plants which yield many popular fleshy fruits (e.g. apple, peach, pear, orange, etc.), has made it necessary to assay promoters in heterologous systems (Atkinson *et al.*, 1998).

In this paper it is demonstrated that transient expression can easily be performed in ripe fleshy fruits. The method described here is inexpensive and does not requires any particular apparatus since it makes use of *Agrobacterium* as the transforming agent. Furthermore, it has proved to be valuable in a number of different fruits where it could be used for both qualitative and quantitative assays.

Materials and methods

Plant material

Commercially ripe fruits [apples (*Malus × domestica*), pears (*Pyrus communis*), tomatoes (*Lycopersicon esculentum*), peaches (*Prunus persica*), strawberries (*Fragaria × ananassa*), and oranges (*Citrus sinensis*)] were purchased at a market in Padova (Italy). They were rinsed thoroughly in water added with Tween 20 (0.05%) before injecting them with an *Agrobacterium* suspension. After the incubation time and prior to assaying the reporter activity, the injected tissues were sampled and halved. One half was used immediately for the histochemical assay while the other half was frozen in liquid nitrogen and stored at –80 °C for subsequent use in the quantitative assay.

Preparation of Agrobacterium *for the fruit infection*

Plasmids used for transformation experiments contained the GUS reporter gene interrupted by a plant intron (described by Vancanneyt *et al.*, 1990). This gene can be driven by the CaMV 35S promoter in plasmid p35SGUSINT or it can be used without promoter, as in plasmid pPR97 (Szabados *et al.*, 1995), to carry out negative controls. The intron containing *LUCint* gene (Hanson *et al.*, 1999) was cloned, under the control of a double 35S promoter, in the pISV2678 vector supplied by Dr M Schultze, Department of Biology, University of York, UK.

Growth and induction of *Agrobacterium* was carried out according to Kapila *et al.* (Kapila *et al.*, 1997). A culture of *Agrobacterium* GV3101 (pMB90) was grown at 28 °C in YEB medium (5 g l^{-1} beef extract, 1 g l^{-1} yeast extract, 5 g l^{-1} peptone, 5 g l^{-1} sucrose, and 2 mmol l^{-1} $MgSO_4$), buffered with 10 mmol l^{-1} MES (2-(*N*-morpholino)ethanesulphonic acid) to pH 5.6 and rifampicin (100 mg l^{-1}), gentamycin (25 mg l^{-1}), kanamycin (100 mg l^{-1}), and acetosyringone (20 μmol l^{-1}) were added. When the culture reached an OD_{600} of about 0.8 it was centrifuged and the pelleted bacteria were resuspended (up to a final OD_{600} of 2.4) and incubated 1 h at 22 °C in MMA medium (MS salts, 10 mmol l^{-1} MES pH 5.6, 20 g l^{-1} sucrose, 200 μmol l^{-1} acetosyringone). In the case of peach and strawberry, after incubation the suspensions of *Agrobacterium* transformed with the two different reporter genes were mixed in a 1 (luciferase) to 3 (GUS) ratio due to the much higher sensitivity of the luciferase assay. Then, the resulting mixture was used in the injection experiments.

Infiltration of fruits

The *Agrobacterium* suspension was evenly injected throughout the entire fruits by means of a sterile 1 ml hypodermic syringe. The thinness of its needle, besides minimizing the wound damages, allowed very fine control of the injections in fruits with epicarps easy to pierce, such as those used in this work. In the case of tomatoes, the injection was made trying to avoid the locules where most of the bacteria could concentrate, thus making infection of the fruit less effective. As a consequence, both locules and seeds were discarded when sampling tissues for the quantitative assays.

The outside of the injected fruits was dried to get rid of excess bacteria and the fruits were placed on moistened filter paper at 22 °C for 2 d with a 16 h light photoperiod. The 2 d incubation time was chosen after a number of trials since it was found to be sufficient for detecting significant GUS activity while avoiding the formation of moulds on the fully ripe strawberries which were the most sensitive to this damage.

Histochemical assay of GUS activity

For the histochemical assay (Jefferson *et al.*, 1987) the injected tissues were sampled and immersed into 1 mmol l^{-1} X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide), 100 mmol l^{-1} phosphate buffer pH 7.2, 0.1% Triton X-100, 0.5 mmol l^{-1} $K_3Fe(CN)_6$, 0.5 mmol l^{-1} $K_4Fe(CN)_6$, 10 mmol l^{-1} EDTA, and 20% methanol. After a vacuum treatment of 5 min to facilitate the penetration of the dying solution, the immersed tissues were kept for 12 h in the dark at 37 °C. Destaining was made with 70% ethanol.

Assay of GUS and luciferase activity

Frozen tissues were ground in a mortar and protein extracted in 1.7 ml g^{-1} fresh weight of modified CCRL buffer (100 mmol l^{-1} K-phosphate pH 7.8, 1 mmol l^{-1} EDTA, 10% glycerol) added before use with 7 mmol l^{-1} β-mercaptoethanol, 0.1% Triton X-100 (Luehrsen *et al.*, 1992) and 2% polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged twice at 32 000 *g* for 15 min and the clear supernatant was used for either protein (Bradford, 1976) or reporter activity quantification.

The GUS assay was carried out by incubating 50–200 μl of protein extract with the substrate 4-methylumbelliferyl-β-D-glucuronide (MUG) at 37 °C. The released 4-methylumbelliferone (4-MU) was quantified with a Hoefer TKO 100 mini-fluorometer according to the manufacturer's instructions. The GUS activity was expressed as nmol 4-MU released min^{-1} μg^{-1} protein (Jefferson *et al.*, 1987).

The luciferase assay was carried out on an aliquot of the same protein extract used for the GUS assay. The used luminometer (TD-20/20 Luminometer, Turner Design) automatically injected 100 μl of substrate (Luciferase Assay Reagent, Promega) onto 30 μl of fruit protein extract. After a 3 s incubation, the emitted light was measured over a period of 10 s. The luciferase activity was expressed as pg luciferase μg^{-1} protein. The standard consisted of 100 pg of purified luciferase (Sigma) dissolved in 30 μl of a protein extract from fruits transformed with a vector lacking the luciferase gene.

Results and discussion

Ripe fleshy fruits usually have very large cells whose walls undergo marked changes in their structure. In other words, cell walls are partly dismantled by the activity of different enzymes, and the result of this process is the softening of the fruits.

The above-mentioned physiological and anatomical peculiarities can cause difficulties when fruits have to be used as transient expression systems to analyse promoters. For instance, the sugars present in the huge vacuoles, which add to those released by the activity of cell wall hydrolases during the softening process, might cause problems to both particle delivery transformation and protoplast preparation. In fact, it might be difficult precisely to calibrate the molarity of the solutions needed either to pre-plasmolyse fruit slices to be used in particle delivery experiments (Sanford *et al.*, 1993) or as incubation medium for protoplast preparation. Moreover, it is known from the literature that sugars (Jang and Sheen, 1997) and osmotic stress (Mikolajczyk *et al.*, 2000) can modulate gene expression, so the high molarity solutions used might affect the results of transient gene expression experiments.

To overcome the aforesaid problems, the method for *Agrobacterium*-mediated transient gene expression developed for intact leaves (Kapila *et al.*, 1997) was tried. However, in spite of its simplicity (the method is based on the vacuum infiltration of intact leaves with a suspension of *Agrobacterium*), it was ineffective with fleshy fruits. The dense structure of fleshy fruits did not allow any significant penetration of *Agrobacterium* through the epidermis into deeper mesocarp cells. Also the use of vacuum infiltration with slices of fruits led to very poor results since the surface-sterilization with sodium hypochlorite and the vacuum treatments brought decay of the slices (data not shown).

Therefore, the problem of keeping the fruit tissues in more physiological and viable conditions had to be solved. This could be achieved by keeping the fruits intact throughout the whole period of *Agrobacterium* penetration and subsequent reporter gene expression, so the fruits would be cut just before performing the assay of reporter activity. All the problems related to the handling of the fragile fruit tissues would therefore be minimized, and the entire procedure would be hastened.

The difficulty of *Agrobacterium* penetration into the mesocarp of whole intact fruits could be overcome by injecting them with a sterile syringe, and taking advantage of the loose cell-to-cell contacts present in these fruits as a consequence of the softening process.

In order to make sure that the observed GUS activity was not due to its expression inside the *Agrobacterium* cells, the reporter used was the GUS-intron gene developed earlier (Vancannayet *et al.*, 1990). This reporter is interrupted by a plant intron which prevents expression of GUS activity in the prokaryote *A. tumefaciens*. At the same time, it allows its expression in plant cells due to their ability to splice out the intron and to produce a functional GUS mRNA. Control experiments were carried out with a plasmid (pPR97) containing a GUS-INT reporter gene without any promoter (Szabados *et al.*, 1995). When fruits were transformed with such a construct, GUS activity was not detected (not shown).

GUS activity assays were performed 2, 3 and 5 d after carrying out the injection in order to assess the time period necessary for measuring reporter expression. Two days at 22 °C was usually the maximum time after which the fully ripe strawberries started to form moulds, even when not injected, so this time period was chosen for the present study.

A number of commercially ripe fleshy fruits (i.e. apple, pear, tomato, peach, strawberry, and orange) was transiently transformed with a construct formed by a 35S promoter fused to the GUS-intron reporter gene. After 2 d incubation, the tissues injected with the *Agrobacterium* suspension were sampled and used for reporter assays.

Though some variability could be observed in the apparent amount of expressed GUS activity, as deduced by the intensity of the blue colour, all the analysed fruits showed a clear expression of the reporter gene (Fig. 1). The pattern of blue staining was different in different fruits so that it appeared evenly distributed in apple, pear and tomato, while it was uneven and patchy in peach and, especially, in strawberry.

The penetration of the *Agrobacterium* suspension was facilitated by the loss of cell-to-cell contacts that occurs during fruit ripening, so the differences in blue dye distribution might reflect differences in pattern of fruit softening and anatomy. Such differences certainly exist among fruits from different species, but also among fruits produced by different cultivars of the same species. A clear example of the latter is visible in Brett and Waldron where SEM fracture surfaces of both a very crisp and a very mealy apple are presented (Brett and Waldron, 1996). Also the peculiar pattern of GUS expression in the orange fruit (Fig. 1F), where the blue

colour was mostly limited to the albedo region, confirms the above idea. The pattern was, in fact, due both to the way the injection was made (the fruit segments formed by enormous and extremely juicy cells were deliberately excluded) and the anatomy of such tissue which caused a preferential penetration of the bacterial suspension throughout it because of its spongy consistency.

This should not be particularly relevant in studies aimed at understanding whether a fruit-specific promoter from a given species is also active in fruits of another

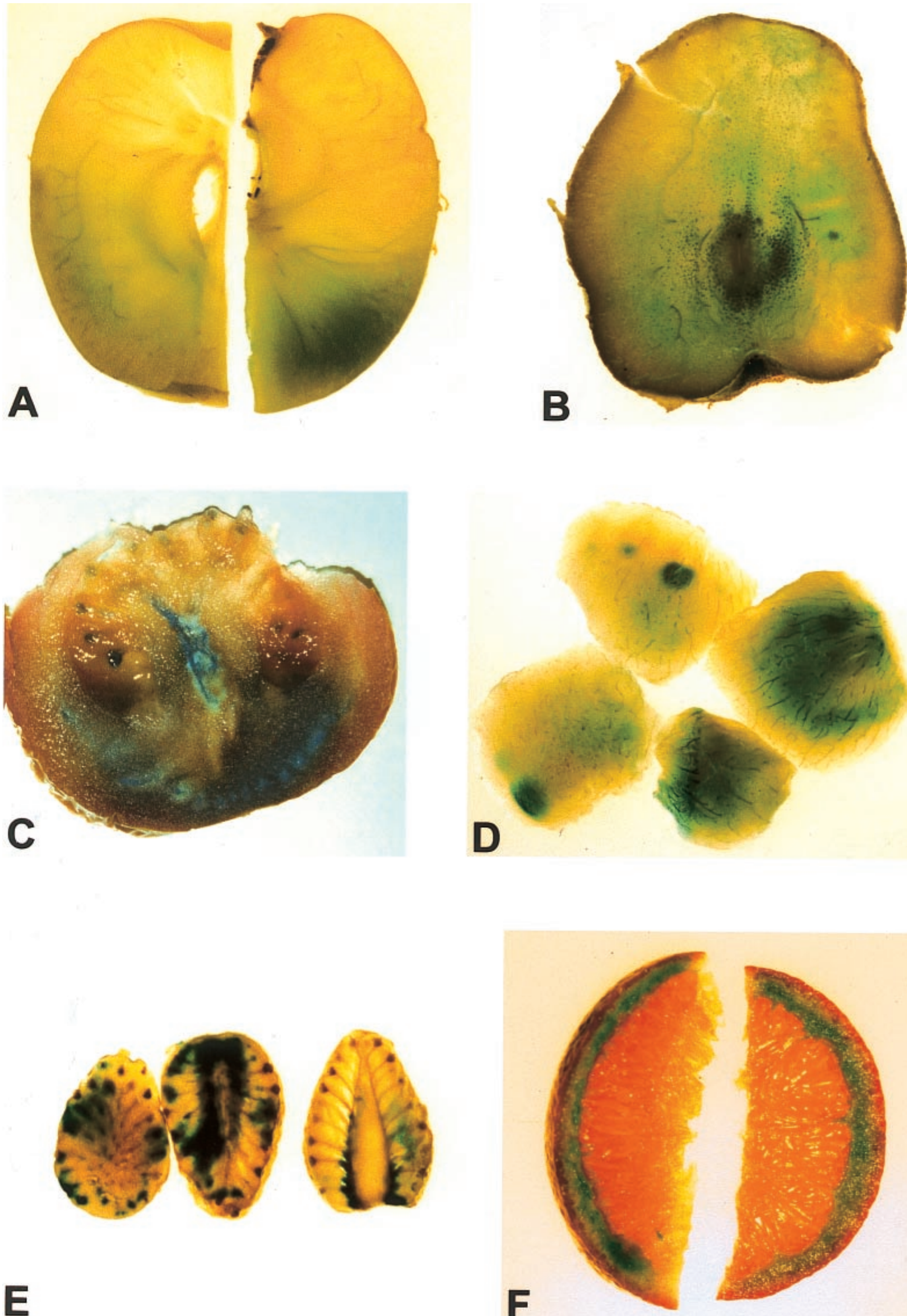

Fig. 1. Examples of GUS staining in sections of fruits transiently transformed with an *Agrobacterium* suspension. The positive regions appear blue in the pictures (A, apple; B, pear; C, tomato; D, peach; E, strawberry; F, orange).

species or aimed at the evaluation of a promoter strength. In fact, in these cases it would be more important to observe the presence/absence of the reporter gene product rather than the actual pattern of its distribution that might be influenced by the anatomical characteristics of the examined fruit.

Quantitative analyses of GUS activity (Fig. 2) were also performed in the case of strawberry and peach that represent the two crops of interest for this laboratory. To this purpose, it has to be emphasized that for quantitative analyses the extraction of proteins from fruits represents a crucial step, so the biochemical characteristics of the examined fruits such as, for instance, the presence of either phenols or high amounts of sugars, might influence both yield and quality of extracted proteins. In other words, it is believed that for any fruit examined an optimization of protein extraction should be done before performing quantitative reporter assays.

When studying a given promoter in a transient expression system, the lack of reporter expression might be due to either inability of that promoter to drive expression of the reporter gene or to failure of the transformation process. In order to check the transformation efficiency, a second reporter gene can be used. Results of such an experiment are shown in Fig. 3 for strawberry and peach. Both GUS and luciferase genes were used in the same transformation experiment and the related activities were measured in the same protein extract. It is interesting to note the different results obtained with the two different fruits. In strawberry the reporter activity related to protein amount was higher for GUS than for luciferase, while the opposite occurred in the case of peach (Fig. 3). These results confirm that each fruit is a system apart and requires its own protocol optimization but, more importantly, they show that a normalization of data can be achieved by using two different reporters in the same transfection experiment, thus allowing reliable information to be obtained on the promoter studied.

Genes can be expressed in living organisms either constitutively or in a tissue- and/or state-of-development-specific manner. The knowledge of the latter genes is of particular interest, because they can be used for biotechnological purposes specifically to modify a given physiological process. For instance, the finding that a tapetum-specific promoter from tobacco could work also in *Brassica* allowed Mariani and co-workers to produce male sterile plants of both tobacco and *Brassica* (Mariani *et al.*, 1990).

The knowledge and characterization of genes that are specifically expressed in fruits during the ripening proper is particularly important. In fact, the ripening of fruits is a process of relevant physiological and economical interest. However, while tomato represents a model plant since its fruits are consumed worldwide and transformation and regeneration protocols are common, the situation is quite different for those fleshy fruits that are produced either by woody plants (i.e. apple, pear, peach, and others) or by plants whose regeneration and growth until the stage of fruit production is long (e.g. strawberry). In such cases, the analysis of fruit specific promoters is more easily done in heterologous systems. So, the study of ripening specific ACC-oxidase and polygalacturonase genes from apple has recently been performed in tomato plants (Atkinson *et al.*, 1998).

In this paper it has been shown that promoter analysis in homologous systems can also be made with fleshy fruits without the need of any expensive apparatus and/or time-consuming methods.

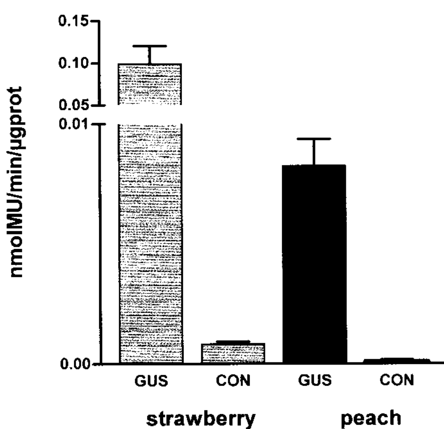

Fig. 2. β-glucuronidase activity measured in protein extracts from either transformed or untransformed fruits of peach and strawberry. The GUS activity measured in transformed fruits is highly significant compared to the negligible activity measured in the untransformed fruits. The activity is expressed as nmol of MU released in a minute per μg of protein. Each value represents the mean of six independent measurements. Bars represent standard errors.

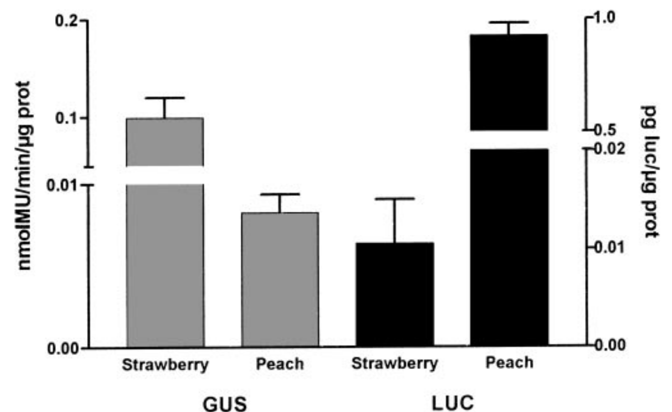

Fig. 3. β-glucuronidase (expressed as nmol of MU released min^{-1} μg^{-1} of protein, left) and luciferase (expressed as pmol of luciferase per μg of protein, right) activities measured in the same protein extract from either strawberry or peach. Each value represents the mean of four independent measurements. Bars represent standard errors.

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