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Full Length Research Paper

Optimization of particle bombardment conditions by β-glucuronidase (GUS) reporter system in tomato fruit

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Fruits of tomato cultivar R-144 (*Lycopersicon esculentum* Mill. cv. R-144), a variety from Israel, were bombarded on tungsten particles coated with a plasmid containing GusPlus gene that was coded for β-glucuronidase (GUS). Peels of target areas of fruits were removed before bombardment, and as such, after 24 h, the effects of different bombardment parameters were evaluated by comparing the numbers of blue spots which resulted to histological GUS assays. The effects of bombardment pressure, bombardment distance, content of plasmid and bombardment fruit area and fruit maturity stages on GUS expression were investigated. Optimal transient expression of the GusPlus gene was observed after bombardment at 1100 psi, with 0.83 µg plasmid per shoot, and 6 cm between stop screen and fruit. GUS expression decreased with the process of fruit ripening and increased from fruit shoulder (close to the end of the stem) to fruit top (blossom end). The highest number of blue spots was 2456.91/ cm^2 and was observed in the area of fruit separation zone. As such, the optimized conditions of particle bombardment in this experiment would have significance for its further application in genetic transformation.

Key words: Particle bombardment, β-glucuronidase (GUS), tomato, fruit, bombardment parameters.

INTRODUCTION

Since its invention in 1987 (Sanford et al., 1987), particle bombardment has been widely used in plant genetic transformation. Many kinds of plants (for example, coffee, cassava, potato, tomato, etc.) had been bombarded for transient or stable transformation. In those experiment, different tissues and organs were used as bombardment targets; embryogenic callus (Carsno et al., 2008), embryogenic suspensions (Schöpke et al., 1997), hypocotyls (Qutob et al., 2002), stems (Gutiérrez-E et al., 1997), leaves (Strömvik et al., 1999; Gallo-Meagher et al., 1993; Ribas et al., 2005), pods (Strömvik et al., 1999) and fruits (Montgomery et al., 1993; Baum et al., 1997; Endo et al., 2007). Among these plant materials, comparing with its importance in researches and commerce, fruit had not been paid much attention. Particle bombardment of fruit is a useful method for studying the process of fruit ripening, identifying fruit specific pro-moters and analyzing the interaction between pathogens and fruits. Montgomery et al. (1993) identified an ethylene-responsive region as the promoter of E4 by bombarding the pericarp of tomato fruits, whereas Baum et al. (1997) improved on some parameters of bombardment on tomato fruits and identified organ-specific contributions of I-box and G-box to the RBCS2 promoter activity. In the experiments mentioned above, which took fruits as bombardment targets, focuses were on the application of the method and not the optimization of parameters for particle bombardment. An optimal condition for particle bombardment is important and necessary for efficient genetic transformation.

In this study, we used a β-glucuronidase (*GUS*) reporter system and intended to find optimal parameters both in physical and biological parameters for particle bombardment on tomato fruits, which were used as a model for studying fruit ripening and identifying fruit specific promoters. Among the parameters which we had evaluated, 'effect of target area on bombardment' and 'effect of different fruit maturity on bombardment' were never reported. In this study, great attention was paid to keep

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Abbreviations: GUS, β-Glucuronidase; **DPA,** post anthesis days.

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the integrity of the fruits so that they do not need to be cultured in medium after bombardment. At the same time, we used image analysis software to measure the target area on fruit for improving the traditional methods of quantification of GUS blue spots, and as such, all the data obtained from the current work might give some help to the particle bombardment researches on other fruits and also the application of the method itself.

MATERIALS AND METHODS

Plant material

Tomato plants, *Lycopersicon esculentum* Mill. cv. R-144, a variety from Israel with unlimited growth and long harvest period, were grown under standard greenhouse conditions. Fruit maturity stages were determined by post anthesis days (DPA): young fruit stage (13 DPA), mature green stage (25 DPA), turning stage (35 DPA) and red ripening stage (45 DPA). Fruits of uniform sizes were harvested with the fruit calyx at four different maturity stages. Then the fruits were washed and surface sterilized with 75% ethanol for further usage.

Plasmid used for particle bombardment

Plasmid pCAMBIA1305.1 (TAKARA Biotechnology (DALIAN) Co., Ltd.) with the GusPlus gene was used in all particle bombardment. The GusPlus gene was driven by cauliflower mosaic virus (CaMV) 35S promoter. The plasmid was amplified in *Escherichia coli* DH5α cells and purified by 'plasmid purification mini kit-spin column (TAKARA Biotechnology (DALIAN) Co., Ltd.).

Fruit treatment for bombardment

In the preliminary experiments, integrated fruits of four mature stages were used respectively for particle bombardment, and all the areas on fruits were bombarded with different combinations of parameters. Moreover, we obtained no GUS blue spots in any parts of the fruit except the fruit separation zone (Figure 5g), which was the only place where there was no peel. In that case, tomato peel might obstruct the particles from penetrating into the cells of pulp. So before bombardment, the peel of the target area must be removed carefully, keeping the integrality of the cells under the peel. Hard peel is removed by scarifying it with scalpel in the shape of radius (Figure 5a), then removed with forceps from the outside to the center. Sometimes, juice would extravasate from the surface of the uncovered pulp and it must be cleaned before bombardment. Since the target area of the pulp is uncovered with peel, it is easy for moisture and wilt to be lost; so after bombardment, the target area should be covered with parafilm (Parafilm® M) immediately and incubated in the tissue culture room for 24 h at 25°C.

Particle bombardment

Tungsten particles with diameters of 1.0 µm (Ningbo Scientz Biotechnology Co., Ltd, China) were used for bombardment. The preparation of tungsten particles was performed according to Hull et al. (1996), while the final concentration of tungsten particles was 60 $mg \cdot ml^{-1}$ in 50% glycerol. Coating tungsten particles with plasmid was performed at the following steps: 50 µl prepared tungsten particles suspension was added into a 0.15 ml centrifuge tube, and then a certain amount of plasmid (0.5, 1.5 and 5.0 µg, respectively), 50 µl of 2.5 $mol \cdot l^{-1}$ $CaCl_2$ and 20 µl of 0.1 $mol \cdot l^{-1}$ spermine, was also added in the order, while vortexing. The mixture was vortexed for 3 min and left to ice for 5 min. After the mixture was centrifuged at 8000 rpm for 1 min and the supernatant removed, the particles were resuspended in 150 µl of 70% ethanol and vortexed for another 3 min. After that, particles were centrifuged again (8000 rpm, 1 min) and the supernatant was removed, resuspended in 150 µl of 100% ethanol, and the particles were placed on ice for another 5 min and then centrifuged (8000 rpm, 1 min). After the supernatant was removed, tungsten particles were finally resuspended in 60 µl of 100% ethanol, before depositing on the macroprojectiles, which were washed in advance with 100% ethanol. As such, plasmid-coated tungsten particles were vortexed for 30 s and the 10 µl of coated particles was deposited on a macro projectile. Then the macro projectile was placed in a Petri dish which was filled with anhydrous $CaCl_2$ for drying up. The final contents of plasmid per shoot (on each macro projectile) were 0.83, 0.25 and 0.08 µg, respectively.

In this study, a nitrogen-driven particle deliver system Scientz GJ - 1000 (Ningbo Scientz Biotechnology Co., Ltd, China) was used. Mature green stage fruits, whose peels were easily removed, were first used for optimizing the bombardment parameters and the following conditions were evaluated by orthogonal design $L_9(3^4)$: Target area on fruit for bombardment; acceleration pressure, distance between stop screen and fruit and plasmid content per shoot (Table 1). In all the bombardments, the partial vacuum was 71 mm Hg. Then fruits of other three stages were bombarded with the optimal combinations, which were screened from the first experiment, for evaluating the effects of maturity stages on bombardment. As such, each treatment was repeated 5 times.

Histochemical detection of GUS

GUS assay was modified from that of Jefferson (1987). The assay buffer contained three components: (A) Basic phosphate buffer (50 $mmol \cdot L^{-1}$ pH 7.0 sodium phosphate, 1 $mmol \cdot L^{-1}$ $K_3Fe(CN)_6$, 1 $mmol \cdot L^{-1}$ $K_4Fe(CN)_6$, 10 $mmol \cdot L^{-1}$ Na_2EDTA and 0.1% (v/v) Triton X - 100); (B) anhydrous methanol and (C) 20 $mmol \cdot L^{-1}$ X - Gluc: 5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexyl-ammonium salt, solvent was dimethyl formamide. However, the proportion of the three components in the assay buffer was: (A): (B): (C) = 40: 10:1.

After it has been incubated in the tissue culture room for 24 h at 25°C, parafilm was removed from the target area of fruits, and the surface layer of the pulp in the target area was cut into thin slices (2 ~ 3 mm, thickness). Then, it was put in a 5 ml centrifuge tube which contained excessive assay buffer (3 ml), and as such, centrifuge tubes were incubated for 24 h at 37°C. For a better view, green or red pulp should be discolored with 70% ethanol.

Quantification of GUS blue spots

In this study, we evaluated the bombardment effect by the number of GUS blue spots per square unit of pulp. Thin slices of pulp were put on white background and the number of GUS blue spots was counted. Then a digital camera (FUJIFILM FinePinx S5700) was used to obtain images in the same shooting distance (10 cm), zoom (1.0×) and pixels value (7.1 million pixels). Photomicrographs were obtained by a microscope of Olympus DX40. After photographing, all images were stored in a computer and transformed into the format of Tiff (a kind of format for digital images).

For measuring the area of pulp and eliminating the errors caused by shooting distance and lens distortion in photography, standard points (Figures 5a - d) were introduced into photographing at the time of photographing. Standard points were drawn with the software the software of Adobe® Photoshop® CS and printed by Cannon IR6570 printer. As such, the area of each black

Table 1. Effects of factors on bombardment of tomato fruits on mature green stage.

Combination	A Target area on fruit	B Acceleration pressure (psi)	C Distance between stop screen and fruit (cm)	D Plasmid content (μg per shoot)	Number of GUS blue spots per cm^2
$A_1B_1C_1D_1$	Fruit top	500	1.0	0.08	16.89 c C
$A_1B_2C_2D_2$	Fruit top	650	3.0	0.25	33.24 bc BC
$A_1B_3C_3D_3$	Fruit top	1100	6.0	0.83	107.32 a A
$A_2B_1C_2D_3$	Fruit waist	500	3.0	0.83	53.76 b B
$A_2B_2C_3D_1$	Fruit waist	650	6.0	0.08	25.80 bc BC
$A_2B_3C_1D_2$	Fruit waist	1100	1.0	0.25	37.38 bc BC
$A_3B_1C_3D_2$	Fruit shoulder (Fruit separation zone)	500	6.0	0.25	33.69 bc BC(1311.85)
$A_3B_2C_1D_3$	Fruit shoulder (Fruit separation zone)	650	1.0	0.83	45.79 b BC(2456.91)
$A_3B_3C_2D_1$	Fruit shoulder (Fruit separation zone)	1100	3.0	0.08	16.90 c C(523.81)

This table was established by orthogonal design $L_9(3^4)$. Results were tested by Duncan's multiple range test at P = 0.05 (lowercase) and P = 0.01 (majuscule). Parenthesis marks in the last column corresponded to those in the column 'Target area on fruit'.

point was 1 mm^2.

In order to get the number of GUS blue spots per square unit of pulp, the target pulp must be measured. The area of a standard point is known, if we know the number of target pulp areas in the same image. As such, we can calculate the actual target area of the pulp. Areas in the images were measured with the software of ImageMaster™ 2D Platinum (format of Tiff available). All data were analyzed with Microsoft Office Excel and Orthogonally Experiment Assistant (Sharetop Software Studio).

Measurement of fruit firmness

Sclerometer with flat top (FUJIHARA Co., Japan) was used for measuring the fruit firmness. On each maturity stage, 10 fruits were used for measurement, while on each fruit; three areas (fruit top, waist and shoulder) with no peel were measured. The unit of fruit firmness was $kg \cdot cm^{-2}$.

RESULTS AND DISCUSSION

Optimization of bombardment parameters

When using particle bombardment for genetic transformation, three aspects may affect the effect of bombardment: plant material, plasmid and parameters of bombardment apparatus. In this study, 9 combinations were established for optimizing the parameters by orthogonal design $L_9(3^4)$ and the results are shown in Tables 1 and 2. The intuitive analysis of Table 1 shows that among all the combinations, $A_1B_3C_3D_3$ was the best that had significant difference when compared with others (P = 0.01). As the range is shown in Table 2, the order of factor influence power to bombardment effect was: Plasmid content > physical parameters (final pressure and distance between stop screen and fruit) > target area on fruit for bombardment.

With further statistical analysis on experiment data, the utility curves (Figures 1 and 2) were obtained. In Figure 1, shows a clear tendency for an increase in the number of GUS blue spots with an increase in plasmid content. The optimal content of plasmid was 0.83 μg per shoot, which was similar to the content used in Strömvik (1999) study of soybean pods, but was less than the content used in bombarding the embryogenic suspension cultures of cassava in Schöpke et al., (1997) (1 μg plasmid per shoot). When bombarding the pulp of mature green fruits with the optimal plasmid content, the largest number of blue spots in pulp was only 107.32, while the number of blue spots in fruit separation zone was 2456.91 per cm^2 (Table 1), which was more than the largest number (1400 per cm^2) of GUS blue spots obtained in Schöpke et al., (1997). It suggests that the optimal content of plasmid, 0.83 μg per shoot, was sufficient for the bombardment of mature green fruit. More plasmid might increase the number of blue spots, but might also cause more waste. In addition, excessive plasmid would cause the agglomeration of particles (Tuanwu et al., 2005). So the content, 0.83 μg plasmid per shoot, was optimal for bombardment and its visual effect was also good enough for qualitative and quantitative analysis (Figures 5a - 5d).

Acceleration pressure and distance between stop screen and fruit are two important factors that affect bombardment. Acceleration pressure controls the speed of particles and also determines the

Table 2. Variance analysis result of test $L_9(3^4)$.

Average	A Target area on fruit	B Acceleration pressure (psi)	C Distance between stop screen and fruit (cm)	D Plasmid content (µg per shoot)
k_1	52.48	34.78	33.35	19.87
k_2	38.98	34.95	34.63	34.77
k_3	32.13	53.87	55.61	68.96
Range	20.35	19.09	22.26	49.09

This table was obtained from the orthogonal analysis of Table 1.

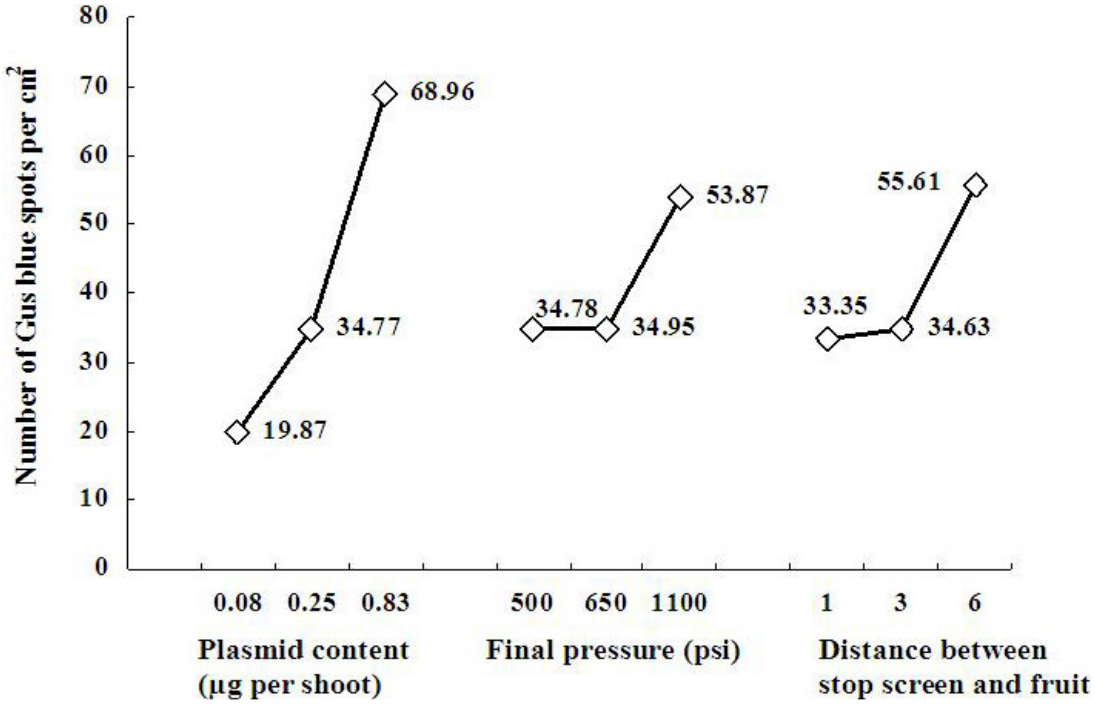

Figure 1. Effects of different factors on bombarding tomato fruits of mature green stage.

penetration depth of particles. The greater the pressure, the deeper the particles will penetrate into the pulp. Since the firmness of mature green fruit was high (Figure 4), high pressure (1100 psi) had better effect than others, and the tendency of GUS blue spots was increased with the increase of acceleration pressure (Figure 1). Moreover, at the same time, high pressure would cause more damages to the pulp and would also lead to the decrease of the dispersibility of tungsten particles (Zhao et al., 2001). To reduce the damages caused by high pressure, the distance between stop screen and fruit must be increased (Batista et al., 2008). Thus, the utility curves of the distance in Figure 1 also support this. The optimal distance between stop screen and fruit, which had the highest number of GUS blue spots after bombardment, was 6 cm and the tendency of high pressure with long distance, were also embodied in Endo et al., (2007). In their research, the plant materials and juice sacs of *Citrus*, which were much easier to be damaged and the combination of pressure and distance was 1350 psi with 9 cm. In order to make the speed not to reduce with the change of distance, bombardment chamber must be partially vacuum and the optimal vacuum degree was 71 mm Hg (Schöpke et al., 1997; Menossi et al., 1997).

Besides plasmid content and physical parameters, plant material was also an important aspect that might affect bombardment. As shown in Figure 2, all the areas on each fruit could express *GUS* gene after bombardment and the utility curve of the GUS blue spots increased from the fruit shoulder (close to the end of the stem) to the fruit top (blossom end) (Figure 2), although the difference between the three areas was not significant. At the same time, the firmness of the three areas on mature green fruits, shown in Figure 4 mature green stages, decreased in the following order: Fruit shoulder, fruit top and fruit waist. As we know, in the same condition, the

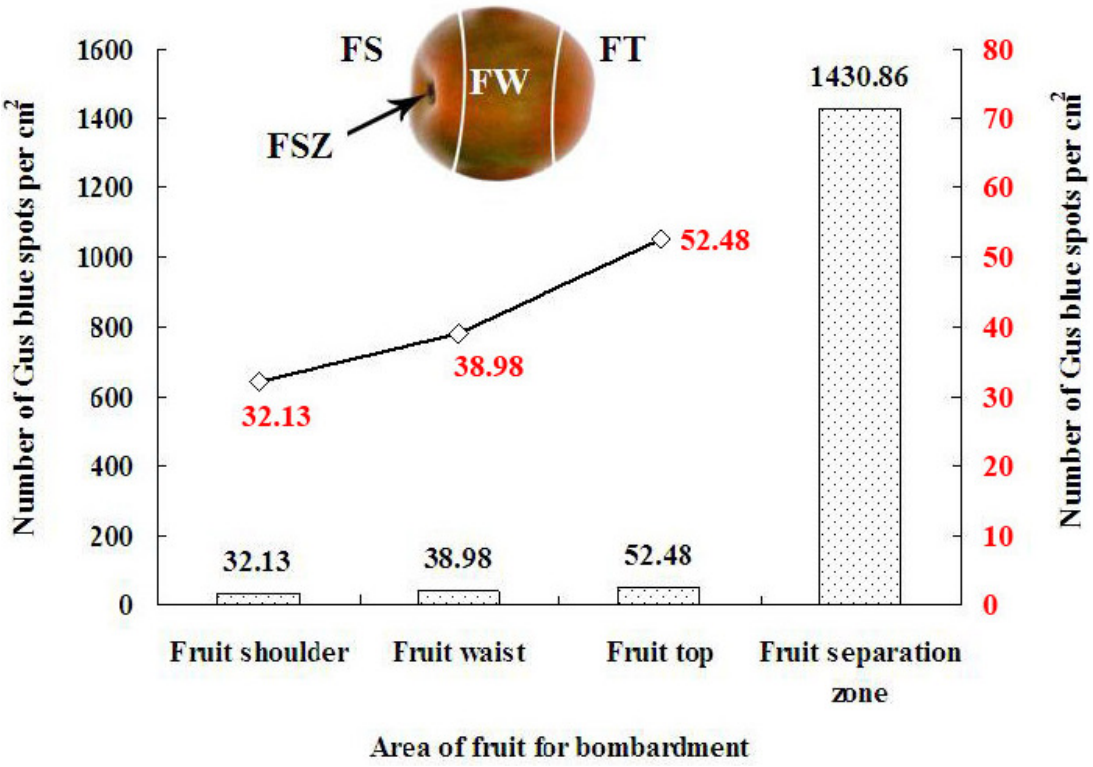

Figure 2. Effects of different target area on bombarding tomato fruits of matured green stage. FSZ, FS, FW and FT represent fruit separation zone, fruit shoulder, fruit waist and fruit top, respectively.

stronger the cell wall, the harder it would be for penetration, and as such, the fruit area with a stronger cell wall would have less GUS blue spots. However, the different trends between GUS blue spots and firmness shown previously, indicated that differences of GUS expressed between the three areas of mature green fruit were not absolutely caused by changes of firmness. The micrographs in Figures 5e and f revealed that, comparing it with cells in fruit shoulder, cells in fruit top and fruit waist were smaller and their geometry was similar to the sphere, while the geometry of cells in the fruit shoulder were much similar to the rectangle. Geometrical shapes of cells in different parts of the fruit would determine the cell number per square unit on target area. In that case, in fruit top and waist, there would be more cells per square unit. At the same time, intercellular spaces would be more in fruit top and fruit waist, because more cells need more cell walls to divide. Ahsana et al. (2002) reported that particles, which were penetrated into the intercellular spaces, could be delivered into zooblast by endocytosis. Although the process had not been reported in plants, endocytosis was pivotal in many biological processes of plants (Ketelaar et al., 2008). In that case, more intercellular spaces and cells in the target area might increase the probability of particles in penetrating and delivering it to the target cells. A similar result, that is, the bombardment efficiency which was not the same between different parts of the same organ, was also found in Gallo-Meagher (1993) research on sugarcane, but the exact reason still need to be investigated in further study.

Comparing this with the difference between the three parts of tomato pulp, the difference between pulp and fruit separation zone, shown in Figure 2, was significant (α = 0.01). In this study, fruit separation zone was bombarded with the fruit shoulder, because of its position that was in the center of the fruit shoulder, but the result was unexpected and the number of GUS blue spots of fruit separation zone was 44.5 times that of the fruit shoulder and 34.7 times that of the average value of pulp (fruit top, fruit waist and fruit shoulder). Fruit separation zone might be the most optimal fruit area for bombardment. The reason, why fruit separation zone had more GUS blue spots than that of the pulp, could be ascribed to the difference both in geometry of cells and treatments. Cells in the fruit separation zone were smaller than that of the pulp (Figures 5e, f and g) and that gave the fruit separation zone more probability to get particles. Besides, treatments were also an important factor that affected the bombardment effect. The operation of removing the peel from the pulp might lead to the injury of cells under the peel, and as such, the injury would cause a decrease in the cell's ability to express foreign genes. On the contrary, fruit separation zone was bombarded immediately after the fruit stalk was removed. Cells in it maintained a better condition and also had a greater ability of expressing foreign genes than that of the pulp. All of these finally led

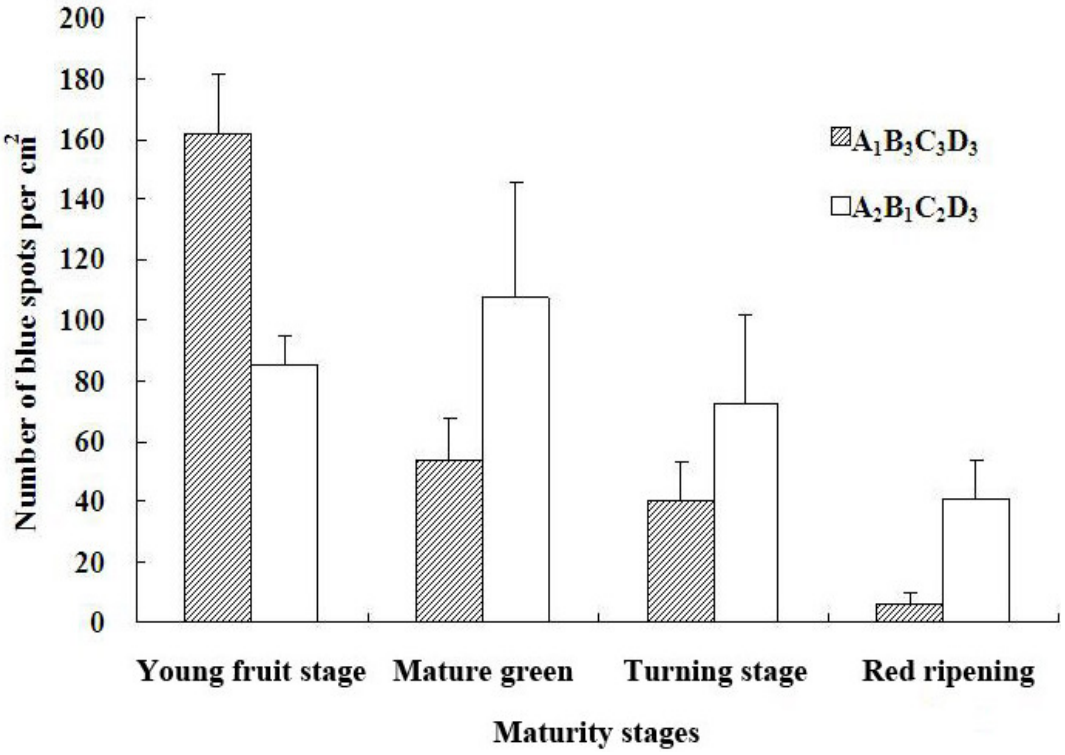

Figure 3. Effects of maturity stages on bombardment of tomato fruits.

to significant difference of GUS expression between pulp and fruit separation zone.

In the experiment, effect of maturity stages on bombardment was evaluated with the optimal combinations which were selected from orthogonal design $L_9(3^4)$. Fruits of four maturity stages (young fruit stage, mature green stage, turning stage and red ripening stage) were used for bombardment. The result showed that the maturity stage of different parts on each tomato fruit were not the same. In the process of tomato ripening, fruit shoulder matures later than fruit top and fruit waist. In other words, fruit shoulder was less 'sensitive' to ripening. For that reason, when evaluating the effect of mature stages on bombardment, fruit shoulder was not used. As shown in Figure 3, there was a clear trend that the expression of *GUS* gene decreased with the process of maturity when taken together. Beside the young fruit stage, the effects of $A_1B_3C_3D_3$ were better than those of $A_2B_1C_2D_3$.

As it is known, fruit firmness decreases with fruit ripening (Figure 4) and the pulp became easier to be penetrated by tungsten particles. As such, the plasmid, which adhered to tungsten particles, would also have more probability to be delivered into the cells and express the very protein it encodes, but as shown in Figure 3, the number of GUS blue spots did not increase with fruit ripening. On the contrary, *GUS* gene expression, decreased with fruit development and ripening. It suggests that, there was another reason, besides the fruit firmness, which might cause the aforementioned phenomenon. Fruit ripening is a process of senescence which associates with the decrease of mechanical properties of cell wall, increase of membrane permeability and reduction of physiological activity of cells. Furthermore, it was reported that there was a strong increase in nuclear ploidy in tomato fruit cells during the development, but a decrease in some processes which was related to the replication of DNA during fruit ripening (Teyssier et al., 2008). At the same time, the senescence of fruit could also be reflected in the diffusion of GUS blue spots on different mature stages, that younger fruits had smaller spots (Figures 5a to d). Although there were more particles and plasmids that penetrated the pulp cells, as a result of the decrease in the ability of gene expression, the number of GUS blue spots on turning stage and red ripening stage did not increase. The special case in Figure 3, which stipulates that the numbers of GUS blue spots of $A_1B_3C_3D_3$ was more than that of $A_2B_1C_2D_3$ in young fruit stage, might have caused the high firmness of pulp which would cumber the particle that penetrated the cells.

Quantification of blue spots

As it is reported, there are two main methods used for estimating the expression of reporter genes after bombardment, physiological evaluation and visual evaluation. With the method of physiological evaluation, products (proteins or pigments) that reporter genes expressed

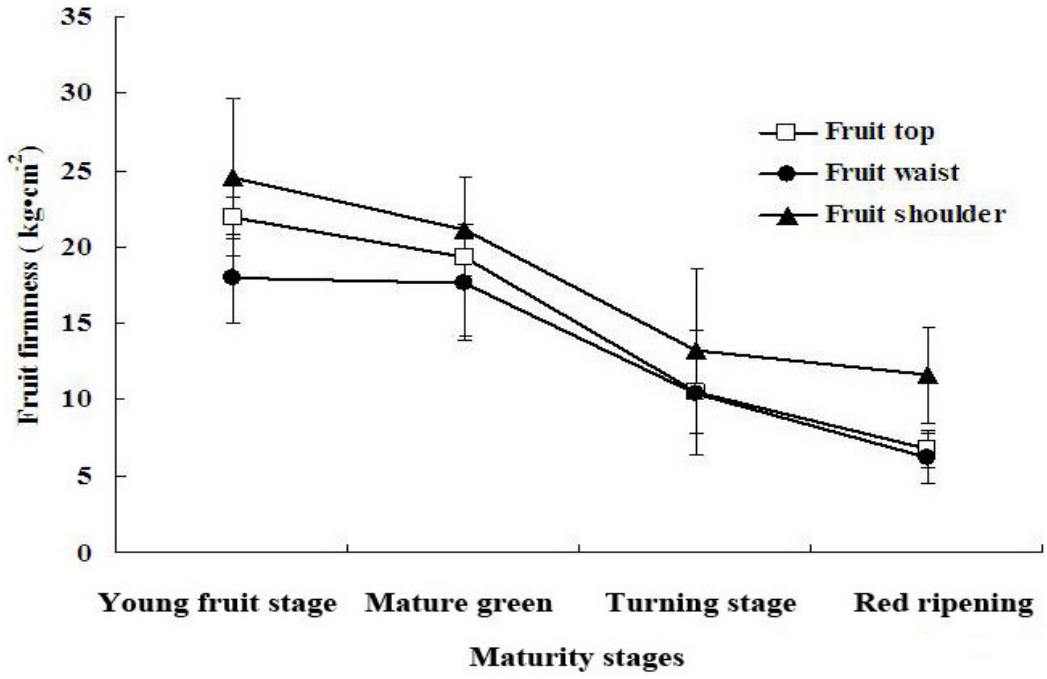

Figure 4. Fruit firmness of different maturity stages on tomato fruits (no peel).

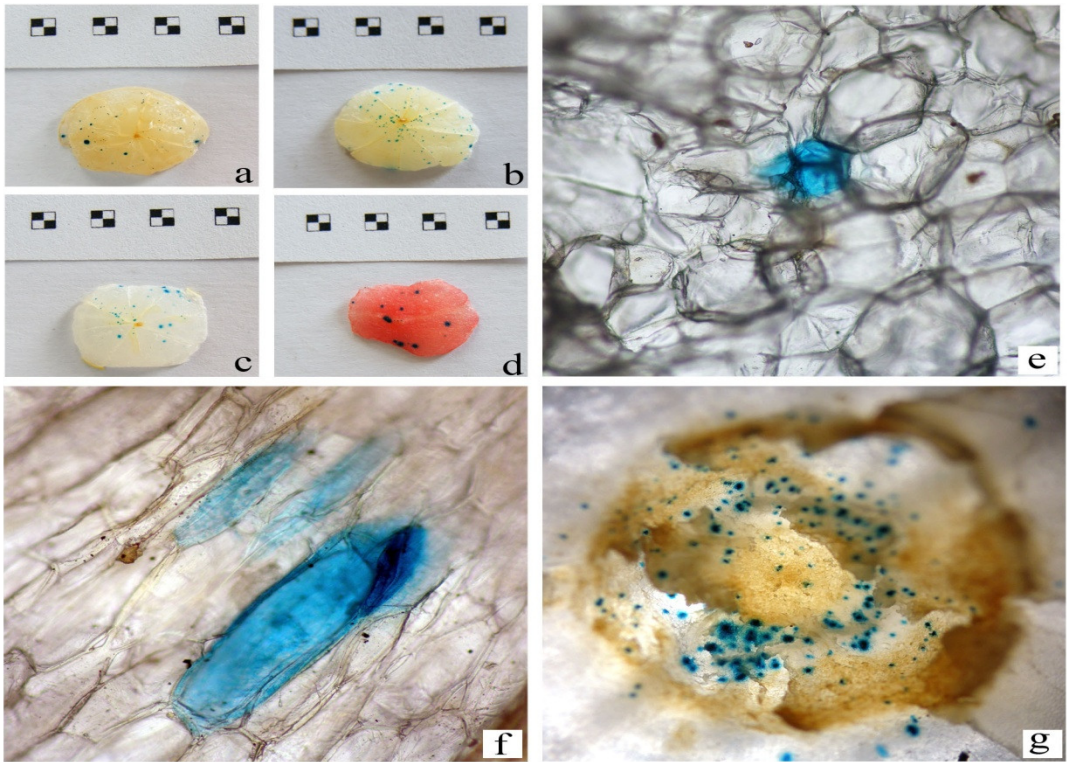

Figure 5. Effect of bombardment on tomato fruits. a-d show the effects of bombardment on fruit top of four maturity stages: (a) Young fruit stage; (b) mature green fruit stage; (c) turning stage and (d) red ripening stage. e (100×) is the fruit waist of mature green fruit after bombardment. Figure 5f (100×) is the fruit shoulder of mature green fruit after bombardment. g (20×) is the fruit separation zone of mature green fruit after bombardment. The blue spots in the images are the production of *GUS* gene expression after coloration of the GUS histochemical assay, while the black squares in Figures 5a to d are the standard points.

should be extracted and measured with corresponding instruments. From the concentration of products, gene expression can be evaluated. Physiological evaluation is suitable and widely used in analyzing the function of promoters (Montgomery et al., 1993; Strömvik et al., 1999; Inaba et al., 2007). Although physiological evaluation is accurate, it also has some disadvantages. First, for extracting proteins or pigments that reporter genes expressed, tissue must be destroyed and cannot be used for protein localization. Secondly, conducting of physiological evaluation is relatively complicated. Visual evaluation is a method which depends on the spots of pigments or fluorescence produced by the expression of reporter genes. Gene expression is evaluated by the number of spots per square unit, while gene localization is determined on the places where the spots are. This method is accurate enough and can indicate the differences of reporter gene expression when the materials are the same on bombardment. Due to the fact that it is simple and intuitive, visual evaluation has been widely used in the optimization of particle bombardment parameters (Schöpke et al., 1997; Carsono et al., 2008), but for the convenience of measurement and calculation, in traditional visual evaluation, same area of bombardment materials was required (Prakash et al., 1992). For the materials which were difficult to be cut as the same area, the number of spots was calculated in the same field of view (Schöpke et al., 1997). The aforementioned limitations were the disadvantages of using visual evaluation and they also limited the use of this method in irregular plant materials. In this experiment, software ImageMaster™ 2D Platinum (GE Healthcare) was used to measure the area of irregular pulp, and with the help of this software, the exact area of the pulp was obtained. Using image analysis software and standard points, it is much easier to measure the area of irregular pulp and also improved the method of visual evaluation. Furthermore, using standard points eliminated the errors caused by shooting distance and lens distortion in photography.

Advantages and limitations

Treatments of tomato fruits for particle bombardment, which had been reported, would cause destruction to the fruit integrity and serious injuries of fruits. For example, in Montgomery and Baum (1997), seeds and locular tissue of tomato fruits were removed and the pericarp of each fruit was cut into relatively flat thin pieces. Plant materials, after these treatments, must be cultured in the medium and the operation environments must be aseptic. Besides, mediums of different kinds and the serious injuries might cause the changes in fruits that we had not known. All of these affections would influence the bombardment effects in some way. In our experiment, the only treatments of fruits were removing the peel at the target area of bombardment and covering it with parafilm. When fruits expressed foreign gene, they should not be cultured in the medium and as such, could be operated in normal environments. So, some researches about gene expression during the process of fruit development and ripening can be done with the integrity of fruits.

Although we had tried our best in maintaining the integrity of fruits, peel still had to be removed because of its hindering effect on tungsten particles. As it is known, any injury to fruits would induce ethylene, and this might affect the use of ethylene induced promoters. In the experiment, we found that fruit separation zone was the suitable place in tomato fruits for particle bombardment, although it was different from pulp. This result might be helpful to future researches on fruit conducting tissue.

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