Histological study of shoot organogenesis in melon (Cucumis melo L.) after genetic transformation

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Abstract

Leaf and cotyledon explants from two melon genotypes ‘Vedrantais’ and ‘Paul’ were cultured on different media and their regeneration was followed during 60 days. High frequency of plant regeneration was observed with ‘Vedrantais’ cotyledons cultured on MS medium added with BAP and 2-iP. Using this protocol, Agrobacterium mediated transformation was developed and a significant reduction of the plant regeneration rate was observed. Explants histological analysis revealed the presence of transformed meristematic areas but a rapid and premature cell vacuolization was observed. During plant regeneration, meristematic cells were affected by important cytological damages: cell dislocation accompanied by cell death and final collapsus. This disruption of meristematic areas may be related to an effect of A. tumefaciens transformation on melon cells and would explain the difficulties encountered to regenerate plants.

INTRODUCTION

The two most important pre-requisites for genetic transformation through Agrobacterium tumefaciens are the availability of an optimized plant regeneration system and a suitable method for transformation. Regeneration by organogenesis or somatic embryogenesis has been described on a wide range of Cucumis melo L. cultivars (Guis et al. 2000; Akasaka-Kennedy et al. 2004) and investigations have been focused on the effect of plant genotype, growth regulation and explant source. The regeneration response is genotype dependent (Galperin et al. 2003) and the presence of meristematic protuberances that fail to develop into elongated normal shoots has frequently been observed (Liborio Stipp et al. 2001). Melon is still considered as being difficult to regenerate (Pech et al. 2007).

Melon transformations with marker genes as well as viral resistance or fruit quality genes have been reported (Fang and Grumet 1990; Yalcin-Mendi et al. 2004; Gaba et al. 2004) but generally with poor efficiency. The lack of an efficient regeneration system remains a major factor preventing the development of gene transfer on melon.

The objective of the present study was to develop an efficient regeneration system in order to establish an efficient Agrobacterium transformation method. In this...
context, we examined the regeneration of melon explants (infected or not) and the origin of shoot buds was studied at different steps by histological techniques.

MATERIAL AND METHODS

Plant material

Seeds of the *C. melo* subsp. *melo* ‘Védraintais’ (charentais type line from Vilmorin Company) and ‘Paul’ (hermaphrodite line of oriental type) were used in this study.

Plant regeneration and transformation

Young leaves from 13 days-old seedling or cotyledons from 5 days-old seedling germinated aseptically on half concentration of MS (Murashige and Skoog 1962) were used as explants. Leaves and seeds were sterilized 20 min in 2.5% calcium hypochlorite containing 0.1% tween 20, rinsed three times in sterile water, cut in four pieces and cultivated on two regenerating media described respectively by Guis et al. (2000) and Yalcin-Mendi et al. (2004): MReg (MS medium with 0.2 mg.L⁻¹ BAP and 0.2 mg.L⁻¹ 2-iP) or MR medium (MS medium with 1.12 mg.L⁻¹ BAP, 0.88 mg.L⁻¹ IAA and 0.26 mg.L⁻¹ ABA). Explants were sub-cultured every two weeks and shoots were excised and placed on two elongation media ME (MS medium with 0.2 mg.L⁻¹ BAP and 0.1 mg.L⁻¹ GA₃) or MM (MS medium with 0.67 mg.L⁻¹ BAP) during 2-3 weeks. They were then placed on the rooting medium MSR (MS medium without plant growth regulators). All media were supplemented with 30 g.L⁻¹ sucrose, 7 g.L⁻¹ Bacto agar (Difco Laboratories) and the pH was adjusted to 5.7 before autoclaving (120°C, 20 min). In vitro cultures were incubated at 25-28°C under 16-h photoperiod (60 µmol.m⁻².s⁻¹ fluorescent white light). *A. tumefaciens* strain C58 containing the binary plasmid pBI101 that harbour the β-D-glucuronidase GUS uidA reporter gene and the nptII selective gene for kanamycin resistance under the control of the CaMV 35S promoter was used for transformation. Explants were incubated during 20 min in an overnight *Agrobacterium* suspension adjusted to O.D₆₀₀ = 0.3 in MS medium with 200 mg.L⁻¹ KH₂PO₄, 0.9 mg.L⁻¹ thiamin and 39 mg.L⁻¹ acetosyringone. After rinsing and drying on sterile filter paper, explants were placed on co-culture medium (MReg or MR medium with 39 mg.L⁻¹ acetosyringone) in dark during two days and then transferred to MReg or MR medium with timentin (225 mg.L⁻¹) and kanamycin (100 mg.L⁻¹); shoots were transferred on ME or MM medium supplemented with the same antibiotics and then on MSR medium.

Histochemical GUS assay and histological study

The *Agrobacterium* infection frequency was determined on ten explants 2, 7, 14, 21, and 30 days after co-culture by performing a histochemical assay for β-D-glucuronidase GUS *uidA* activity as described by Jefferson et al. (1987). The same explants were then immersed in cold fixative constituted by formalin-acetic acid-alcohol (5:5:90, V/V/V) for routine observations. After 48 h fixation at 4°C the specimens were rinsed in distilled water and stored in 70% alcohol until required. They were then dehydrated in alcohol series and embedded in methacrylate resin (Kit Technovit 7100, Wehrheim, Germany). Sections (3 µm thickness) were serially cut using a retraction microtome, collected on microscope slides and allowed to dry. Slides were stained with Toluidine Blue and observed with a light microscope.
(Optiphot-2, Nikon). Plants obtained on rooting medium were also tested by GUS assay.

RESULTS AND DISCUSSION

Effect of genotype, explant source and culture medium on regeneration

Leaf and cotyledon explants from the two genotypes ‘Vedrantais’ and ‘Paul’ were cultured on the different culture media and their regeneration was followed during 60 days. No plant regeneration was obtained with the different explant sources of the two genotypes placed successively on MR and MM medium (Tab.1) except with ‘Vedrantais’ cotyledon explants revealing a regeneration rate (0.07) similar to that reported on ‘Kirkagac 637’ by Yalcin-Mendi et al. (2004). On the other hand, the regeneration rates were significantly optimized when explants were cultured successively on MReg and ME media. In these conditions, cotyledon explants of ‘Vedrantais’ showed a particularly high competence for plant regeneration: 70% of explants gave at least one shoot and a rate of 1.48 plants regenerated by explant was obtained.

Table 1. Effect of culture medium, genotype and explant source on melon regeneration

<table>
<thead>
<tr>
<th>Culture media</th>
<th>Genotype</th>
<th>Explants source</th>
<th>Number of explants in culture</th>
<th>Number of explants with shoots</th>
<th>Number of regenerated plants</th>
<th>Regeneration ratea</th>
</tr>
</thead>
<tbody>
<tr>
<td>MReg/ME/MSR</td>
<td>Vedrantais</td>
<td>Leave</td>
<td>70</td>
<td>8</td>
<td>8</td>
<td>0.11</td>
</tr>
<tr>
<td>(Guis et al 2000)</td>
<td>Paul</td>
<td>Cotyledon</td>
<td>150</td>
<td>106</td>
<td>223</td>
<td>1.48</td>
</tr>
<tr>
<td>MR/MM/MSR</td>
<td>Vedrantais</td>
<td>Leave</td>
<td>70</td>
<td>3</td>
<td>3</td>
<td>0.04</td>
</tr>
<tr>
<td>(Yalcin-Mendi et al 2004)</td>
<td>Paul</td>
<td>Cotyledon</td>
<td>71</td>
<td>14</td>
<td>10</td>
<td>0.14</td>
</tr>
</tbody>
</table>

z regeneration rate: number of regenerated plants / number of explants in culture

In a second experiment (Tab. 2) an exceptional regeneration rate (3.44) was also obtained from ‘Vedrantais’ cotyledon explants, clearly superior to those previously reported ranging from 0.85 (Guis et al. 2000) to 2 (Galperin et al. 2003).

Effect of genetic transformation on regeneration

‘Vedrantais’ cotyledon explants were cultured on MReg and ME media after Agrobacterium co-culture or not (regeneration control). Bacterial infection didn’t influence the number of explants developing shoots but it induced a significant reduction of the regeneration rate: about 0.30 plants were obtained per explant after Agrobacterium infection instead of 3.44 for uninfected control explants (Tab. 2).
Presence of kanamycin in the culture medium seemed not modify the regeneration rate obtained.

Histochemical assays realised on explants 14 and 30 days after co-culture showed that 60 to 90% of explants expressed a high level of GUS activity. However 60 days after the beginning of the culture only six plants obtained on selective medium expressed GUS activity; they need to be tested by PCR. These results indicate that our method didn't eliminate escapes particularly common in melon transformation experiments and suggest that Agrobacterium infection inhibits drastically plant melon regeneration.

Table 2. Effect of Agrobacterium infection on regeneration of ‘Vedrantais’ cotyledon explants

<table>
<thead>
<tr>
<th></th>
<th>Agrobacterium</th>
<th>Kanamycin in medium</th>
<th>Number of explants</th>
<th>GUS-positive explants (%)</th>
<th>Number of regenerated plants</th>
<th>Regeneration rate</th>
<th>Number of GUS-positive plants泽</th>
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</thead>
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<tr>
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<td>J+14</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>absent</td>
<td>none</td>
<td>150</td>
<td>-</td>
<td>-</td>
<td>517</td>
<td>3.44</td>
<td>nt</td>
</tr>
<tr>
<td>present</td>
<td>none</td>
<td>200</td>
<td>60</td>
<td>70</td>
<td>54</td>
<td>0.27</td>
<td>0/25</td>
</tr>
<tr>
<td>present</td>
<td>yes</td>
<td>200</td>
<td>80</td>
<td>90</td>
<td>62</td>
<td>0.31</td>
<td>9/32</td>
</tr>
</tbody>
</table>

*number of GUS positive plants obtained/number of regenerated plants tested (in progress)

Histological analysis of the regeneration process

Cytological observations of explants at different regeneration stages showed that epidermal cells are the source of organogenesis and many small independent areas are involved. After 7 days in culture these cells formed meristematic structures and protuberances which developed shoots and plants in 21 to 30 days. Similar results were obtained by Yalcin-Mendi et al. (2003) on watermelon.

Cytological studies of explants after Agrobacterium infection showed that genetic transformation occurred on epidermal and sub-epidermal cells two days after co-culture and reached the meristematic structures expressing a high level of GUS activity. However these structures showed premature cell vacuolization (after 15 days in culture) and lose their cohesion and organization over three weeks. The disorganization of the meristematic areas may be related to an effect of Agrobacterium transformation on the melon cells and this would explain the difficulties to regenerate plants after genetic transformation.

CONCLUSION

We developed an efficient protocol for melon regeneration showing the importance of the genotype and the explant source and very performing results were obtained from ‘Vedrantais’ line. However melon genetic transformation mediated by A. tumefaciens remained difficult and this may be explain by a disorganization of the meristematic structures after bacterial infection, revealing an incapacity for melon
cells to be competitive for both regeneration and transformation. These results suggest that the choice of the *Agrobacterium* strain may be an important factor (Galperin et al. 2003) to take into consideration for future experiments.

**Literature cited**


