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A New Selection System for Pepper Regeneration by Mannose

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Abstract

We report the development of a new selection system for the transformation of pepper plants by mannose. In order to achieve this, we first tested several factors related to regeneration conditions. Among the 30 inbred lines examined, line P915 was able to generate shoots at the highest rate from both cotyledons and hypocotyls in MS media. A dosage curve for optimizing the selection conditions was established by mixing mannose (range 0-50 g/L) and sucrose (range 0-30 g/L). The least selection pressure on shoot formation was created by a mixture of sucrose and mannose at 20 g/L and 15 g/L, respectively, and the threshold for ultimate tissue death was 50 g/L of mannose irrespective of the sucrose concentration. However, we found that mannose itself was not the sole inhibitor of pepper shoot development. High concentrations of sucrose (30 g/L) contributed additively to the inhibition of shoot formation at higher mannose concentrations. Genotype preference is a major factor that enhances regeneration ability in mannose media, as was observed in MS media. P915 and P410 line had high regeneration rates under mannose selection conditions in the presence of *Agrobacterium* infection. Different virulence levels of *Agrobacterium* strains did change the regeneration rates, probably due to interaction with the specificities of the inbred lines. Taken together, P915 offers the best pepper inbred line for transformation and we recommend a selection condition of 20 g/L of sucrose and 15 g/L or more of mannose up to 50 g/L in media.

Introduction

The ability to distinguish between plant cells with an integrated transgene and the bulk of non-transformed cells is a decisive step in the production of transgenic plants. One way of achieving this goal involves the use of antibiotic and herbicide resistant genes as selective markers within the vector, so that cells that do include the selective genes survive under the effects of selection agents. In recent years, the use of antibiotics and herbicide resistant genes for selecting transformed plants has generated widespread public concern because of inadequate evidence of the transformed gene's impact on human health and the environment. Alternative strategies for selection without marker genes have been developed to avoid the above problems. One of these involves the use of mannose as a carbon source in media. Mannose, a hexose sugar, strongly inhibits root growth, respiration (Stenlid, 1954; Morgan and Street, 1957) and germination (Pego et al., 1999; Matheson and Myers, 1998) because plants can not metabolize mannose to other sugars. Phospho-mannose isomerase (PMI, EC 5.3.1.8) catalyzes the reversible interconversion of mannose 6-phosphate and fructose-6 phosphate. PMI is common in nature and found across kingdoms and in humans. However, PMI is not present in plants except in soybeans (Lee and Matheson, 1984). Recently, PMI has been used as a selectable marker for the transformation of plant species due to Pi sequestration by phosphorylating mannose into mannose-P (Privalle et al., 2000; Brouquisse et al., 2001). The *PMI* gene was isolated from *E. coli* (Miles

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and Guest, 1984), and it has been successfully transformed into sugar beet (Joersbo et al., 1998), maize (Wang et al., 2000; Wright et al., 2001), wheat (Wright et al., 2001) and cassava (Zhang and Puoni-Kaerlas, 2000); moreover, the mannose selection has an increased transformation frequency compared to kanamycin selection (references herein).

In order to apply this new selection system to pepper plants, which are recognized as one of the most difficult crops to transform with the present means of selection pressure, we examined shoot regeneration in media containing different concentrations of sucrose and mannose. Here we report, for the first time, the best conditions for the regeneration of pepper shoots in vitro under mannose selection pressure.

Materials and Methods

Shoot formation rate comparison among pepper inbred lines

Seeds from 30 pepper inbred lines (Nong Woo Bio Co. proprietary) were surface-disinfested in 50% bleach for 10 min and rinsed three times with sterilized water. Sterilized seeds were placed in MS medium (Murashige and Skoog, 1962) and allowed to germinate in light at 25°C. Cotyledons and hypocotyls from 7-9 day-old plants were excised and used as explants, which were transferred to a regeneration medium consisting of modified MS medium supplemented with zeatin 2 mg/L, NAA 0.05 mg/L. The shoot formation rate was measured by counting the shoots transferred to elongation medium from the total explants.

Establishment of mannose selection pressure

The explants were transferred to regeneration medium consisting of modified MS medium supplemented with zeatin 2 mg/L, and NAA 0.05 mg/L. Sucrose and mannose concentrations were mixed in the range 0-30 g/L sucrose and 0-50 g/L mannose in order to find an appropriate selection condition. After 4 weeks of culturing, the surviving multishoots were dissected to individual shoots and transferred to the elongation medium to which cefotaxime 300 mg/L had been added. The regeneration rate was measured by counting the numbers of plantlets present in the elongation medium with respect to the total explant numbers.

Transformation of pepper

The explants were pre-cultured in MS with zeatin 2 mg/L and NAA 0.05 mg/L in a culture room for one day. *Agrobacterium* strains containing a vector (p3635 or p3634) harboring the *E. coli PMI* gene (provided by Syngenta) were cultured until the O.D. value reached 0.6 0.8. The bacterial suspension was then diluted with YEP to O.D. 0.1 and used to inoculate the explants for 10 min. The bacterial suspension and explants were co-cultured in the dark for 2 days and the explants were washed with cefotaxime 500 mg/L three times. Explants were incubated on selection medium for 4 weeks and transferred to elongation medium. Resistant shoots were then transferred to hormone and sucrose free root inducing media.

Results and Discussion

Shoot formation rate among pepper inbred lines

Cotyledons and hypocotyls from 30 commercially important inbred lines were incubated in MS medium and allowed to generate shoots (Table 1). Shoot formation rates varied among the inbred lines and differences between the highest and the lowest were broad, from 95% to 10% for cotyledons and from 70% to 0% for hypocotyls. Therefore, it seems to be important that one chooses carefully the line to be used for regeneration and/or transformation. Generally, the shoot formation rate from the hypocotyls, as explants, was much lower than that of the cotyledons. In particular, line P915 was capable of generating shoots at the highest rate from both cotyledons and hypocotyls.

Shoot formation rate under mannose and sucrose

In order to evaluate the selection capability of man-

Table 1. Shoot formation rates of pepper inbred lines.

Line	C	H	Line	C	H
P915	95	70	P2404	60	30
P409	85	45	S800	58	16
S1622	85	4	P1557	55	30
P410	81	34	S928	54	0
P1744	80	45	P1947	50	20
P101	80	30	P0564	40	20
S841	77	59	P60	40	28
P2377	75	30	S849	38	18
P49	75	20	S48	32	14
P784	70	40	S885	27	19
Ph240	70	45	S267	20	10
P318	65	25	S788	12	0
P20123	65	22	P50	10	0
P319	60	30	P53	10	0
P2403	60	30	P57	10	0

nose on pepper explants, a dosage curve was established by mixing various concentrations of mannose and sucrose. Shoot formation from cotyledons and hypocotyls from P410 were negatively affected by adding mannose into the medium in a dosage-dependent manner. Figure 1 shows the shoot formation frequency from cotyledons in shooting medium (a) and elongation medium (b) in the presence of sucrose. Higher concentrations of mannose increased the toxic effect on shoot formation and elongation (Figure 2A and B) by browning the tissues. Without sucrose in the medium, the shoot formation rate from cotyledons decreased dramatically with 5 g/L of mannose, to one third of that with 0 g/L mannose. Shoot formation from cotyledons was not inhibited completely until the mannose concentration reached 50 g/L in combination with 20 or 30 g/L of sucrose. This value is the highest quoted for the selection of other plants with mannose (Joersbo et al., 1998; Zhang and Puonti-Kaerlas, 2000; Wang et al., 2000; Negrotto et al., 2000; Write et al., 2001), indicating that pepper tissues are resilient to the mannose effect.

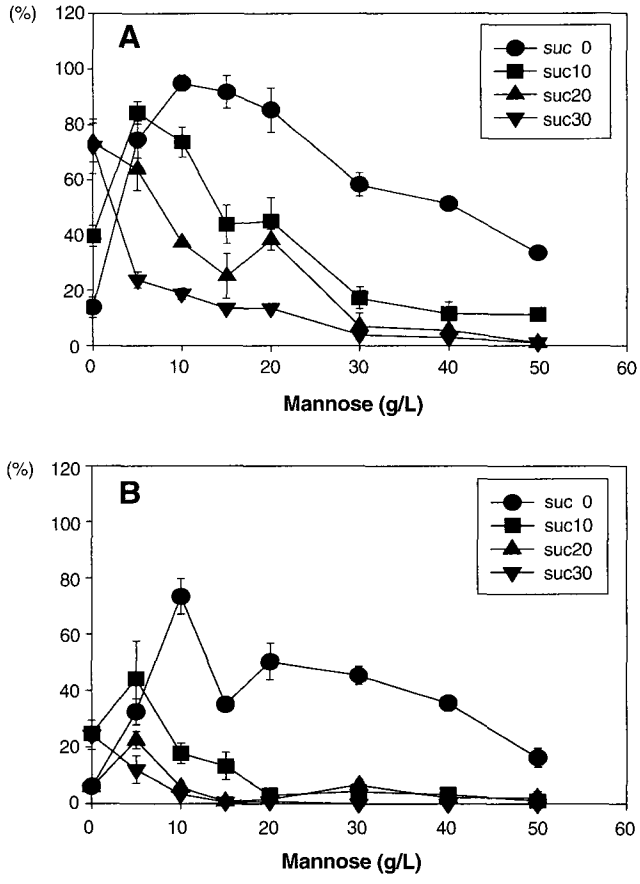

Figure 1. Shoot formation efficiency from cotyledons of P410 in shooting media (A) and survivability of regenerated shoots in elongation media (B).

Interestingly, higher concentrations of sucrose were found to have an additive role in terms of lowering the shoot formation rate in both shooting and elongation media. These data are contrary to previous results, which found that toxic effects of mannose increase with decreasing sucrose concentration (Joersbo et al., 1998, 1999; Zhang and Puonti-Kaerlas, 2000) and that sucrose omission drastically reduced regeneration. Generally the shoot formation rates from hypocotyls were much lower than those of cotyledons (Figure 3A and B, Figure 4A and B). Twenty g/L of mannose in combination with 20 or 30 g/L sucrose completely inhibited the growth of shoots, indicating that the hypocotyls are more susceptible to mannose than the cotyledons. The interaction between mannose and sucrose is also important in shoot formation from hypocotyls. Higher concentrations of sucrose contribute an additive effect to the inhibition of shoot formation at higher mannose concentrations. The reason for the additive effect of sucrose could be possibly due to a lack of Pi in cells when sugars from sucrose breakdown sequester Pi levels by being phosphorylated, which may cause lower ATP production (Privalle et al., 2000; Brouquisse et al., 2001). Taken together, mannose itself does not seem to be sole inhibitor of pepper shoot development. Sucrose should also be present in the medium in order to generate a selection pres-

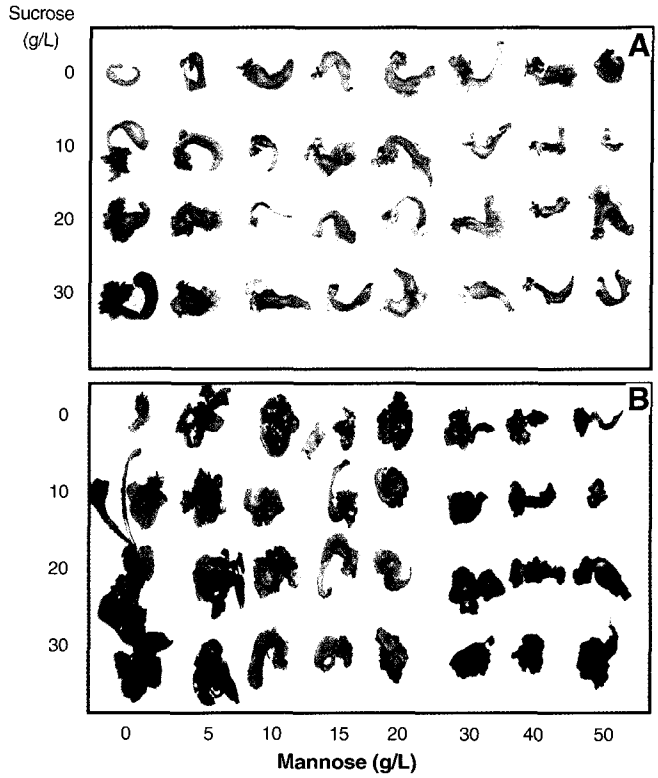

Figure 2. Regenerated shoots from cotyledons of P410 in shooting media (A) and elongation media (B).

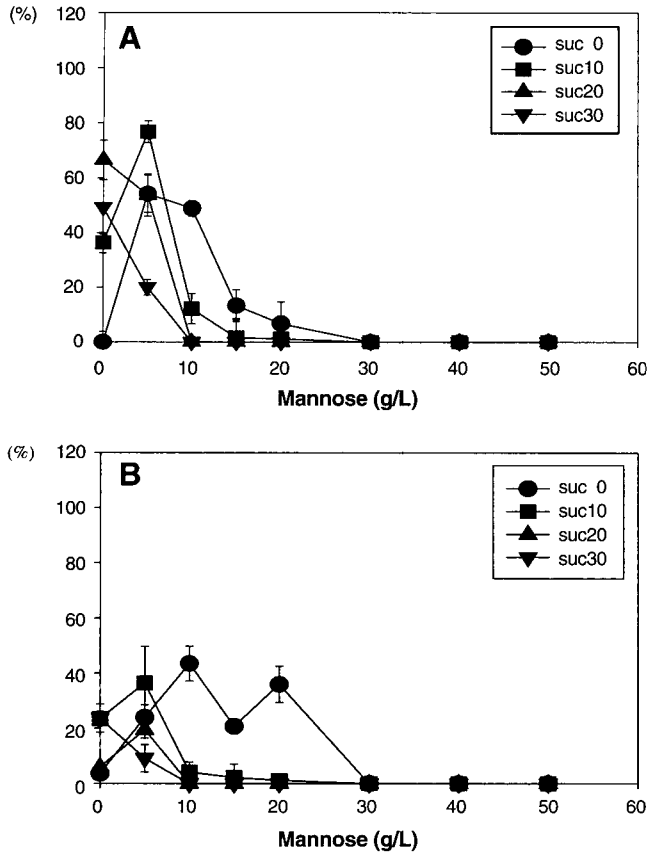

Figure 3. Shoot formation efficiency from hypocotyl of P410 in shooting media (A) and survivability of regenerated shoots in elongation media (B).

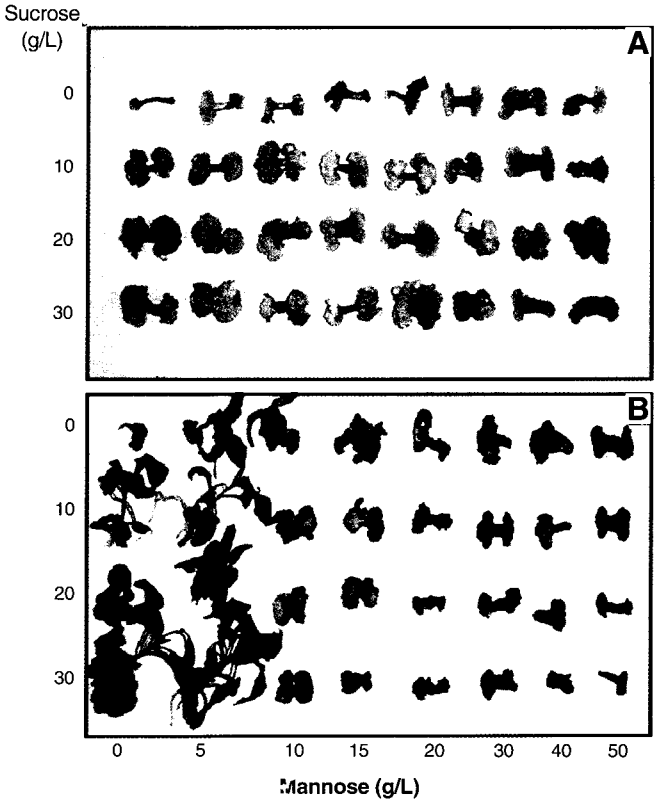

Figure 4. Regenerated shoots from hypocotyls of P410 in shooting media (A) and elongation media (B).

Table 2. Elongation efficiency (%) among inbred lines in 20 g/L sucrose and 15 g/L mannose in elongation media.

Line	Cotyledon (%)	Hypocotyl (%)
P915	0.5	3
P410	1.0	5
P319	0.25	0.5
P101	0.05	0.5
P2404	0	0
P1744	0	0
P2377	0.04	0.08
P60	0	0
P57	0	0
P53	0	0

Table 3. Regeneration rates at different levels of virulence and at different line specificities.

Strain	P410	P915	P2377	P49
A136	0.4	4.4	0.2	0.1
A1567	0.2	1.3	0.2	0.1
GV3101	0.8	2.2	-	-
LBA4404	0.4	2.6	0.4	0.2

sure. Least selection pressure on shoot formation was observed for a mixture of sucrose and mannose at 20 g/L and 15 g/L, respectively.

Regeneration frequencies among inbred lines

Cotyledons and hypocotyls from a total of 10 pepper inbred lines were cultured in a medium containing 15 g/L of mannose with 20 g/L of sucrose to identify inbred lines that are able to generate high frequencies of regeneration (Table 2). The shoots grown in the shooting media under mannose pressure were transferred to elongation media and four weeks later, the surviving shoots were counted. Five inbred lines regenerated from cotyledons were also regenerated from hypocotyls; the other five inbred lines did not form any shoots from either cotyledons or hypocotyls. The elongation frequencies of shoots from hypocotyls were much higher than those of cotyledons (2-10 times). Inbred line 410 showed the highest elongation frequency for both cotyledons and hypocotyls while 2377 showed the lowest. It is interesting that P915 line, which had the highest shoot formation rate among the 30 inbred lines in MS media (Table 1), showed half the frequency of line 410. Again, the genotype and the selection media were

found to be very important factors in the control of regeneration capability.

Agrobacterium-mediated transformation under mannose pressure

Four inbred lines (p410, p915, p101 and p319) were chosen based on the data of Table 2 and the explants from these 4 lines were co-cultured with *Agrobacterium* strain LBA4404 harboring the PMI vector. After 2 days of co-culture, the explants were incubated on a selection medium containing sucrose (20 g/L) and mannose (15 g/L). Table 3 shows the frequencies of regenerated shoots obtained from the mannose selection medium with different strains and lines. Surprisingly, a large difference in the regeneration rate of strains with a line and among lines with a strain was observed. This was attributed to different virulence levels among strains and specificities among lines. Based on our results, we conclude that p915 is the best line for regeneration and transformation. It gave the highest regeneration rate of any strain used for co-culture, it had the highest shoot formation rate among lines (Table 1) and a relatively high regeneration rate (Table 2), as well as aiding shoot survival in the presence of *Agrobacterium* infection. Breeders are using this line as a male donator for producing commercially valuable F1 seeds (personal communication with Dr. SH Choi).

Multishoots were excised and plated on higher con-

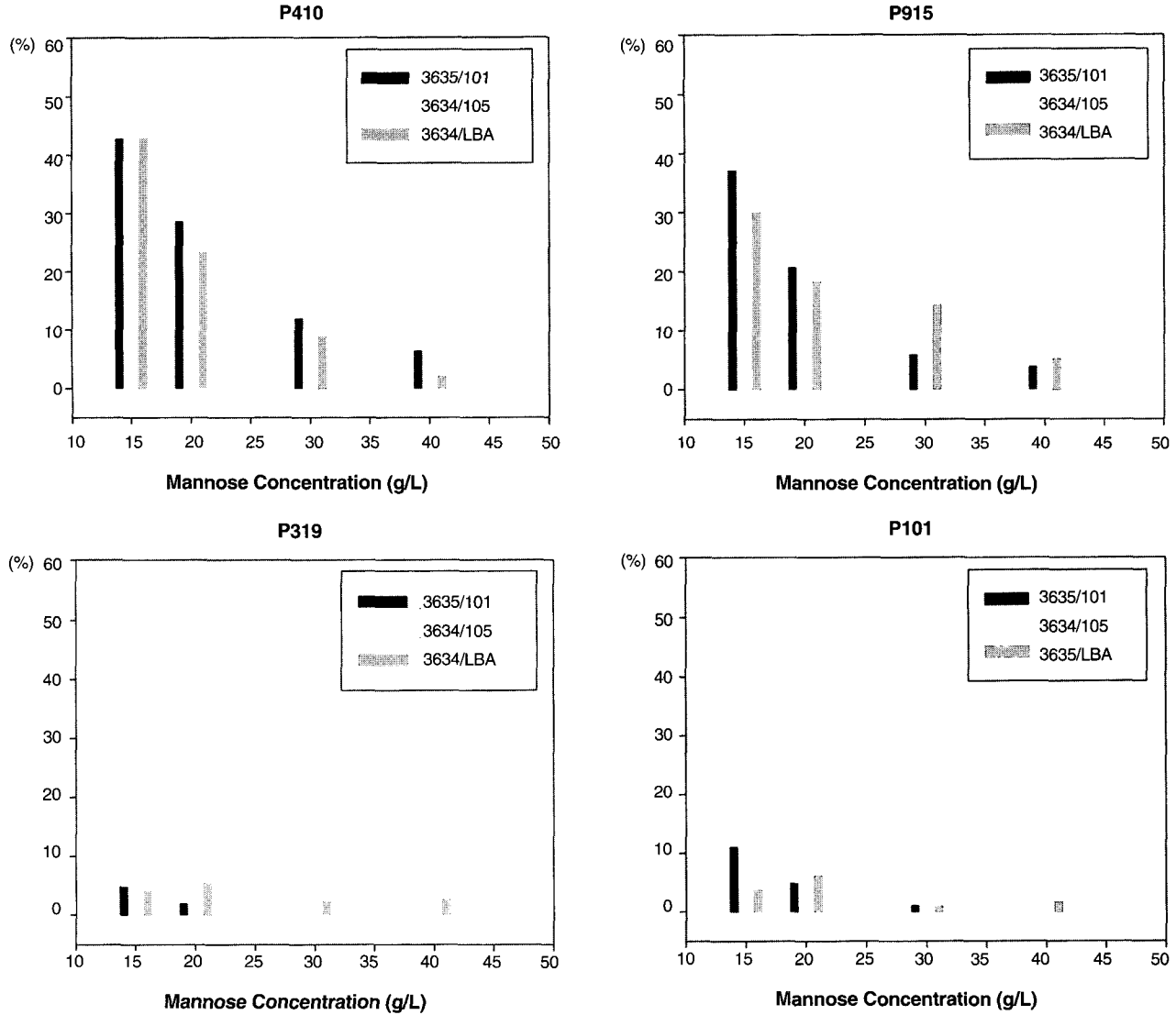

Figure 5. Shoot formation efficiency (%) among different *Agrobacterium* strains, EH 101, EH 105 and LBA 4404. Two PMI vectors, p3635 and p3634, were used for transformation.

centrations of mannose (20, 30 and 40 g/L) in combination with 20 g/L of sucrose to test if selection pressure helps shoot formation efficiency after co-culturing with *Agrobacterium*. The explants from the inbred line p915 produced the highest regeneration rates while explants of lines 319 and 101 regenerated at very low rates (Figure 5). We found that some resilient shoots survived even after mannose 40 g/L treatment and all of those shoots appeared phenotypically normal. However, no significant differences in shoot producing efficiency were found when the co-cultures were performed with different strains.

In elongation media, ultimately the regenerated shoots from both hypocotyls and cotyledons did not survive well at sucrose and mannose concentrations mix of 20 g/L of each or higher. Taken together, this new method should work for the effective screening of transformed peppers. We are currently screening elongating shoots by PCR analysis and we have not obtained the transformed pepper yet.

Acknowledgement

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