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Towards genetic transformation of local onion varieties

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Abbreviations: ME: mature zygotic embryos
MSc: callus induction medium
MSr: regeneration medium

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The aim of this work was to explore the possibility of obtaining transgenic plants of onion varieties cultivated in Argentina, starting from calli induced from mature zygotic embryos, using two strains of *Agrobacterium tumefaciens* as transfection vectors. Mature embryos from three varieties of 'Valenciana' onion, Torrentina, Cobriza INTA and Grano de oro were in vitro cultivated for callus induction. After three to four months an average of 57.4% success for the three varieties was reached. Transformation was carried out with Agl1 or LBA 4404 A. *tumefaciens* strains, both carrying a binary vector containing the marker gene *gus a* and the selection gene *nptII*. Selection was done in callus induction medium containing 10 mgL^{-1} geneticin during three subcultures. At the end of the selection period, 342 portions of calli were recovered and transferred to regeneration medium. Of the selected calli evaluated by the expresión of the β-glucuronidase enzyme, 42% presented extensive blue areas or were completely blue. At the end of the first subculture in the regeneration medium, 54 calli were considered potentially organogenic because of the green areas observed. At the end of the wole regeneration period, just one normal plant was obtained, that was negative to PCR analysis using specific primers for *gus a* and *nptII*. All selected calli came from the Torrentina variety and the highest quantity of them were transformed with the strain LBA 4404.

On the frame of a good strategy directed to strengthen the genetic traits introduced by transformation, transgenes should be introduced in plant cultivars which are agronomically superiors and susceptible of being introduced in new plant breeding plans (Lydiate et al. 1995).

Eady et al. (2000), first reported an onion transformation protocol, based on immature zygotic embryos as target tissue. A drawback for this approach is that explant availability is limited to a short period during the year. Recently, Zheng et al. (2001) reported a reliable transformation protocol for onion and shallot (*Allium cepa* L.) using *Agrobacterium tumefaciens* as a transfection vector, and three-week old calli induced from mature zygotic embryos as target tissue. Since seeds are both easy to get and to preserve, for a successful onion transformation would be advantageous to use calli derived from mature zygotic embryos as target explant, because they can be used year-round (Zheng et al. 1998; Zheng et al. 1999).

Hence, the aim of this work was to explore the possibility of obtaining onion transgenic plants from varieties cultivated in Argentina, from calli induced from mature embryos, using two strains of *A. tumefaciens* as transfection vectors.

MATERIALS AND METHODS

Mature zygotic embryos from three onion varieties of Valenciana, *i.e.* Torrentina, Cobriza INTA and Grano de Oro, were *in vitro* cultivated in a callus inductive medium during three culture periods, 40 days each. The MSc was Murashige and Skoog (1962) which also contained 1 mgL^{-1} 2,4- dichlorophenoxiacetic acid, 0,1 mgL^{-1} 6-bencilaminopurine, 30 gL^{-1} sucrose and 8 gL^{-1} agar, pH 5,8. Thousand and fifty ME were cultivated: 662, 178 and 210 of the Torrentina, Cobriza INTA and Grano de Oro varieties, respectively. The cultivation was carried out in Petri dishes each containing 25 mL culture medium and 30 ME were sowed per Petri dish. During the cultivation period, Petri dishes were kept at 25ºC ± 3º in darkness.

The transformation was carried out with either the Agl1 or the LBA 4404 strains of *A. tumefaciens*, both strains carrying a binary vector which contains the gene marker *gus a* (gus intron) and the NPTII gene for selection, both under the control of the 35 S promoter.

The Agl1 strain was cultivated in medium containing 5 gL^{-1} mannitol, 1 gL^{-1} glutamine, 5 gL^{-1} Triptone, 2.5 gL^{-1} yeast extract, 0.25 gL^{-1} KH_2PO_4, 0.1 gL^{-1} NaCl and 0.1 gL^{-1} $MgSO_4$ $7H_2O$ at pH 7. One hundred mgL^{-1} carbenicilin and 50 mgL^{-1} kanamicin were added as selection antibiotics for the bacterium and plasmid, respectively. The LBA 4404 strain was cultivated in minimal medium (Lichtenstein and Draper, 1986) containing 100 mgL^{-1} rifampicin and 300 mgL^{-1} streptomicin as bacterial selection antibiotics and 50 mgL^{-1} kanamicin for plasmid selection.

For transformation, groups of approximately 30 calli were cut into small portions which were inoculated with 4 mL of the bacterial suspension (OD_{600} 0.5 – 0.7), allowing contact for 10 min. Then the bacterial suspension was removed and a sterile absorbent paper was used to eliminate the bacterial suspension excess by gently blotting. The calli sections were then transferred to MSc medium. After four days of co-cultivation, the calli were transferred into the selection medium, *i.e.* MSc containing 10 mgL^{-1} geneticin and 300 mgL^{-1} cefotaxime. The callus portions showing growth were then selected from each subculture and transferred into MSr (Murashige and Skoog medium to which 1 mgL^{-1} of 6 - (γ, γ -dimethylallylamino) purine, 30 g L^{-1} of sucrose and 8 g L^{-1} of agar were added, pH 5) containing geneticin, but no cefotaxime. This plant material was then kept under a 16 hrs of photoperiod (48 μmol s^{-1} m^{-2}). After one month in regeneration medium, the differentiated green areas were selected and cultivated for 60 days more. Sections of calli selected before the cultivation into regeneration medium and also those organs differentiated after the regeneration stage, were also evaluated looking at the possible expression of the *gus a* gene by quantification of the blue stain arising from substrate transformation by the β-glucuronidase activity.

Table 1. Callus induction, transformation, selection and plantlet regeneration from mature embryos of three onion varieties using Agl1 GI and LBA 4404 GI *Agrobacterium tumefaciens* strains as transfection vectors.

Onion Varieties	Cultivated ME	Callus induction (%)	Transformed calli			Selected callus pieces			Calli with green areas			Regenerated plants		
			Agl1	LBA	Total	Agl1	LBA	Total	Agl1	LBA	Total	Agl1	LBA	Total
Torrentina	662	37.0	136	105	241	73	269	342	13	41	54	0	1	1
Cobriza	178	88.7	64	51	115	0	0	0	0	0	0	0	0	0
G. de Oro	210	95.2	85	91	176	0	0	0	0	0	0	0	0	0
Total	1050	57.4	285	247	532	73	269	342	13	41	54	0	1	1

About 0.5 g fresh leaves from putative transgenic *in vitro* plants was collected for PCR analysis. DNA was isolated from these plants via a miniprep protocol. PCR was performed with specific primers for *uidA* and for *hpt*. The PCR cycle was 94ºC for 2 min (1 cycle); 94ºC for 1 min, 56ºC for 1 min, 72ºC for 2 min (35 cycles); 72ºC annealing extension for 10 min. Primers for *uid*A amplify a sequence of 710 bp, while *hpt* primers amplify a sequence of 1.2 kb.

RESULTS AND DISCUSSION

Callus induction, during three to four months, was quite variable among varieties, being particularly low (37%) for Torrentina (Table 1). Calli were mainly of the compact type. Great variability in callus induction and plant regeneration among genotypes was reported previously (Tanikawa et al. 1998; Zheng et al. 1998; Zheng et al. 1999) and also in local varieties (Marinangeli et al. 2005).

At the end of the selection period 342 portions of calli were transferred to the regeneration medium, 73 came from the transformation with Agl1 strain and 269 from the transformation with LBA 4404 *A. tumefaciens* strain, all of them belonging to the Torrentina variety (Table 1). Callus pieces from the other varieties don't shown tissue proliferation in selection medium. This fact indicates that the selection antibiotic's concentration was high for Cobriza and Grano de oro. At the end of the first subculture in MSr, 54 calli were considered potentially organogenic because of the green areas they exhibited, 13 belonged to the transformation with the Agl1 strain and the other 41 calli came from the LBA 4404 strain. At the end of the regeneration period only one normal plant coming from the last transformation was obtained. PCR analysis of this plant was negative, without amplification fragments for *gus a* nor *NPTII*.

The fact of being able to recover calli of only one of the three varieties demonstrates the genotypic variability that this onion varieties exhibited in the tolerance to the antibiotic geneticin and, in this way, to the transformation mediated by A. *tumefaciens*. Zheng et al. (2001) demonstrated that both subspecies (onion and shallot) and cultivar were important factors for a successful transformation: shallot was better than onion and even some varieties better than others.

A different quantity of selected calli was also obtained from the transformation with each one of the two *A. tumefaciens* strains. Probably this may not be the result of a difference in the efficiency of the transgenes transfer to the Torrentina variety, but of a higher aggressiveness of the Agl1 strain which produced death of calli in the first stages of the selection and high proportion of bacterial overgrowth (data not shown). In fact, Zheng et al. (2001) found not differences in onion transformation efficiency working whit the super-virulent EHA 105 and the ordinary LBA 4404 *A. tumefaciens* strains.

Forty two per cent of selected calli evaluated through the glucuronidase expression, presented extensive blue stained areas or were completely blue. On the other hand, the tissues evaluated at the end of the regeneration period did not presented neither partial nor total blue coloration.

The production of a high proportion of calli which were unable to express or just partially express the *gus* gene, could be the result of a low selection pressure which could otherwise produce undesirable transgenic chimerical plants. This limitation could probably be removed by using a higher concentration of the selection agent geneticin from the beginning of selection or even in later culture transfers. Zheng et al. 2001 proved that geneticin was not successful as selective agent in ME derived calli transformation. On the other hand, he proved hygromicin as effective selective antibiotic.

CONCLUDING REMARKS

Although it was not possible to recover transgenic plants yet, this work clearly represents an advance in the endeavour of obtaining a genetic transformation from a

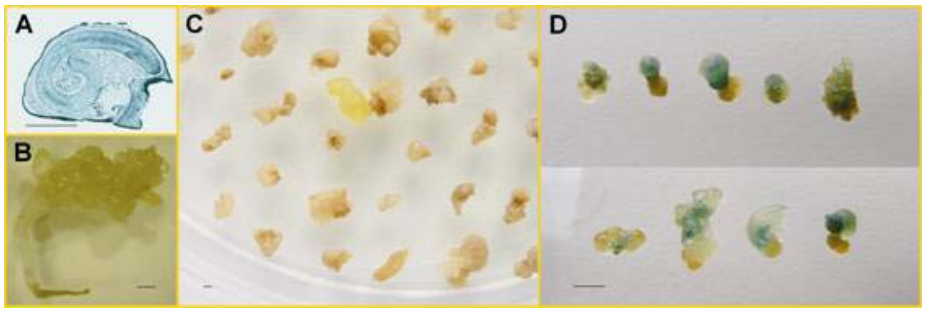

Figure 1. (a) Onion seed cutting showing mature embryos. (Modified from http://www.botany.hawaii.edu/faculty/webb/Bot201/Angiosperm/onion_embryo_developmentM.htm). (b) Callus from ME of Torrentina variety. (c) Calli after three months cultivation in selection medium. (d) Calli pieces selected after four months in selection medium showing β-glucuronidase expression. Bars represent 1 millimetre.

local variety of onion, as it was shown by recovering calli exhibiting a marked glucuronidase expression.

Differences in sensitivity to geneticin among varieties and poor *in vitro* plant regeneration rate shown by this species would be the main limiting steps in the whole process of obtaining transgenic plants of onion local varieties.

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