Theor Appl Genet (2003) 107:462–469
DOI 10.1007/s00122-003-1267-3

P. Ellul · G. Ríos · A. Atarés · L. A. Roig · R. Serrano · V. Moreno

The expression of the *Saccharomyces cerevisiae* HAL1 gene increases salt tolerance in transgenic watermelon [*Citrullus lanatus* (Thunb.) Matsun. & Nakai.]

Received: 5 September 2002 / Accepted: 29 November 2002 / Published online: 29 May 2003

Abstract An optimised *Agrobacterium*-mediated gene transfer protocol was developed in order to obtain watermelon transgenic plants [*Citrullus lanatus* (Thunb.) Matsun. & Nakai.]. Transformation efficiencies ranged from 2.8% to 5.3%, depending on the cultivar. The method was applied to obtain genetically engineered watermelon plants expressing the *Saccharomyces cerevisiae HAL1* gene related to salt tolerance. In order to enhance its constitutive expression in plants, the *HAL1* gene was cloned in a pBiN19 plasmid under control of the 35S promoter with a double enhancer sequence from the cauliflower mosaic virus and the RNA4 leader sequence of the alfalfa mosaic virus. This vector was introduced into *Agrobacterium tumefaciens* strain LBA4404 for further inoculation of watermelon half-cotyledon explants. The introduction of both the neomycin phosphotransferase II and *HAL1* genes was assessed in primary transformants (TG_1) by polymerase chain reaction analysis and Southern hybridisation. The expression of the *HAL1* gene was determined by Northern analysis, and the diploid level of transgenic plants was confirmed by flow cytometry. The presence of the selectable marker gene in the expected Mendelian ratios was demonstrated in TG_2 progenies. The TG_2 kanamycin-resistant plantlets elongated better and produced new roots and leaves in culture media supplemented with NaCl compared with the control. Salt tolerance was confirmed in a semi-hydroponic system (EC = 6 dS m^{-1}) on the basis of the higher growth performance of homozygous TG_3 lines with respect to their respective azygous control lines without the transgene. The halotolerance observed confirmed the inheritance of the trait and supports the potential usefulness of the *HAL1* gene of *S. cerevisiae* as a molecular tool for genetic engineering of salt-stress protection in other crop species.

Keywords Transgenic watermelon · *Agrobacterium*-mediated transformation · *HAL1* gene · Salinity tolerance

Communicated by G. Wenzel

P. Ellul (✉) · A. Atarés · L. A. Roig · R. Serrano · V. Moreno
Instituto de Biología Molecular y Celular de Plantas.
Universidad Politécnica de Valencia-C.S.I.C.
Avenida de los Naranjos s/n. 46022 Valencia, Spain,
e-mail: pellul@upvnet.upv.es
Fax: +34-96-3877859

G. Ríos
Max-Planck Institut fur Zuchtungsforschung,
Carl-von-Linne-Weg 10, D-59829 Koln, Germany

Introduction

Molecular breeding of salinity tolerance has been tackled using different genetic engineering strategies. The osmotic approach was first developed by transferring genes involved in the overproduction of osmolytes and/or osmoprotectants (Tarczynski et al. 1993; Bartels and Nelson 1994; Bonhert et al. 1996; Zhang et al. 2000). In a different approach, several salt-induced transcripts have been detected during salt stress. They encoded proteins that directly or indirectly influence whole plant performance through the metabolic reaction that they initiate (Serrano and Gaxiola 1994). The transfer of the HVA1 LEA gene (late embryogenesis abundant) into rice enhanced salt stress and water-deficit tolerance in progenies of primary transformants (Xu et al. 1996). Zhu (2001) suggested that many of the osmolytes and stress proteins with unknown functions probably detoxify plants by either scavenging reactive oxygen species (ROS) or preventing them from damaging cellular structures. Focusing on this last point, many genes involved in scavenging ROS, such as glutathione S-transferase and peroxidase (Roxas et al. 1997, 2000) or superoxide dismutases (McKersie et al. 1996; Van Camp et al. 1996; Tanaka et al. 1999), enhance salt stress tolerance in crop species.

The strategy tackling the ionic toxicity has also been approached by cloning and overexpressing a vacuolar Na^+/H^+ antiport gene from *Arabidopsis thaliana* (Gaxiola et al. 1999). Increased salinity tolerance was described in Arabidopsis and tomato transgenic plants watered with

NaCl (Apse et al. 99; Zhang and Blumwald 2001). Recently, a H^+-pyrophosphatase gene AVP1 has been overexpressed in *A. thaliana* (Gaxiola et al. 2001), producing salt-tolerant transgenic lines. In a similar way, overexpression of the *Saccharomyces cerevisiae HAL1* gene (Gaxiola et al. 1992) conferred salt tolerance in this yeast by increasing intracellular K^+ and decreasing Na^+ levels by an unknown mechanism (Serrano 1996; Rios et al. 1997). In higher plants, transgenic plants of melon (TG_1) and tomato (TG_2) carrying and overexpressing the *HAL1* gene showed a higher level of salt tolerance in vitro (Bordas et al. 1996; Gisbert et al. 2000). *HAL1* also minimised the reduction in tomato fruit production caused by salt stress under greenhouse conditions (Rus et al. 2001).

With the aim of using watermelon as a receptor material for transferring salt tolerance-related genes, we set out to develop an efficient transformation method that would produce a sufficient number of plants showing predictable and stable transgene expression. The objectives of the investigation reported here were: (1) to optimise conditions for genetic transformation in watermelon in order to obtain an efficient and reproducible *Agrobacterium*-mediated protocol suitable for different cultivars; (2) to introduce and express the salt-related *HAL1* gene from *S. cerevisiae* in watermelon plants; (3) to evaluate the level of salt tolerance of transgenic watermelon and confirm the inheritance of this trait in TG_3 progenies.

Materials and methods

Cloning of the HAL1 gene into an Agrobacterium binary vector

The *Nsi*I fragment containing the *HAL1* coding region was subcloned in the vector pMOG180 as described in Gisbert et al. (2000). This plasmid allows *HAL1* expression driven by the 35S promoter with double enhancer sequence from the cauliflower mosaic virus and contains the RNA4 leader sequence of the alfalfa mosaic virus (Pen et al. 1993). The *Eco*RI-*Hin*dIII fragment was subcloned into a pBIN19 plasmid (Bevan 1984), giving rise to the pPM7 plasmid. The resultant construction was used to transform strain LBA4404 of *Agrobacterium tumefaciens* by electroporation in a BioRad gene pulser (Hercules, Calif.) using the standard protocol for *E. coli* (Dower et al. 1988). The structure of the T-DNA region of the binary plasmids pBI121 used as control plasmid and pPM7 is depicted in Fig. 1.

Plant materials and culture media

Cotyledons of watermelon cultivars Sugar Baby (Asgrow), Crimson Sweet (Intersemillas) and Dulce Maravilla (Sluis & Groot/Sandoz) were taken as the explant source. Mature seeds were decoated, surface-sterilised and grown as described in Bordas et al. (1996). The basal medium (BM) used throughout the experiments consisted of MS inorganic salts (Murashige and Skoog 1962) supplemented with either 3% (w/v) (BM3) or 2% (BM2) sucrose, 100 mg l^{-1} myo-inositol, 1 mg l^{-1} thiamine-HCl. Seeds were placed on GM and incubated at 28 ± 2 °C in the dark. After 2 days, when the radicle emerged and curved into the medium, the test tubes were transferred to a tissue culture chamber maintained at 24 ± 2 °C and a 16/8-h (light/dark) photoperiod (light intensity: about 2,000 lux; cool-white fluorescent lamps); the same photoperiodic conditions were used for all the in vitro cultures. Explants were taken from 3-day-old seedlings by transversally cutting each cotyledon into two segments; only the basal parts were placed into the induction medium and used for shoot regeneration.

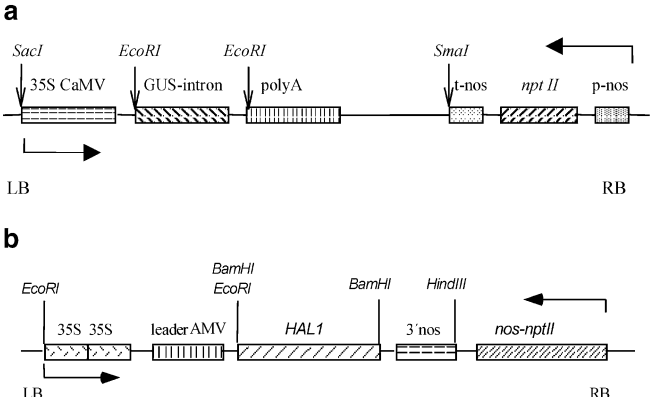

Fig. 1 Structure of the T-DNA region of the modified binary plasmids pBI121 (**a**) and pPM7 (**b**). The fragment between the right (*RB*) and left (*LB*) border defines the beginning and the end of the DNA transferred to the plant, *p-nos* Nopaline synthase gene promoter, *35S CaMV* cauliflower mosaic virus promoter, *AMV leader* sequence of the alfalfa mosaic virus, *nos* nopaline synthase gene, *HAL1* coding region of the *Saccharomyces cerevisiae HAL1* gene, *GUS intron β* glucuronidase coding region, t-*nos* nopaline synthase gene polyadenylation end and transcription terminator site, *nptII* neomycin phosphotransferase II coding region

A three-step protocol was used to regenerate watermelon plants. The caulogenesis induction medium (CIM) consisted of BM3 supplemented with 1.0 mg l^{-1} N^6-benzyladenine (BA). Adventitious shoots were then elongated on SEM (shoot elongation medium) where the BA was substituted by 0.2 mg l^{-1} kinetin. Individual regenerated shoots were rooted on a basal medium containing 1.0 mg l^{-1} indol-butyric acid (IBA). Kanamycin, cefotaxime and acetosyringone were filter-sterilised and added to the autoclaved medium when needed.

Transformation, selective scheme and regeneration

Transformation was performed essentially as described by Bordas et al. (1996), although with some modifications. Bacteria were removed by washing inoculated explants with half-strength liquid MS medium containing 3% (w/v) sucrose and 500 mg l^{-1} cefotaxime. Explants were transferred onto selective caulogenesis induction medium containing 125 mg l^{-1} or 175 mg l^{-1} kanamycin and 300 mg l^{-1} cefotaxime. Identified primary transformants (TG_1) were either maintained in this medium or transferred to a semi-hydroponic system, grown to maturity under greenhouse conditions (40 °C day/26 °C night) and self-pollinated.

DNA preparation and polymerase chain reaction (PCR) analysis

Genomic DNA of 1 g of plant leaves was prepared by the simplified CTAB (cetyl trimethyl ammonium bromide) method of Doyle and Doyle (1990). Yield was 30/60 μg. PCR detection of the neomycin phosphotransferase (*nptII*) and *HAL1* genes was measured with standard methodologies (Taylor 1991). For each sample, 1 μg of DNA was incubated in a final volume of 50 μl with 0.25 μl of 5′ and 3′ primers, 2 m*M* each of dNTP and 2 U thermostable DNA polymerase from *Thermus brockianus* (Dynazyme, Finnzymes, Finland). Reaction cycles (30 cycles) consisted of 30 s at 95 °C for denaturation, 30 s at 60 °C for annealing and 60 s at 70 °C for extension. The *nptII* and *HAL1* primers have been described in Ellul et al. (2002) and Gaxiola et al. (1992), respectively. The results of this analysis are shown in Fig. 2.

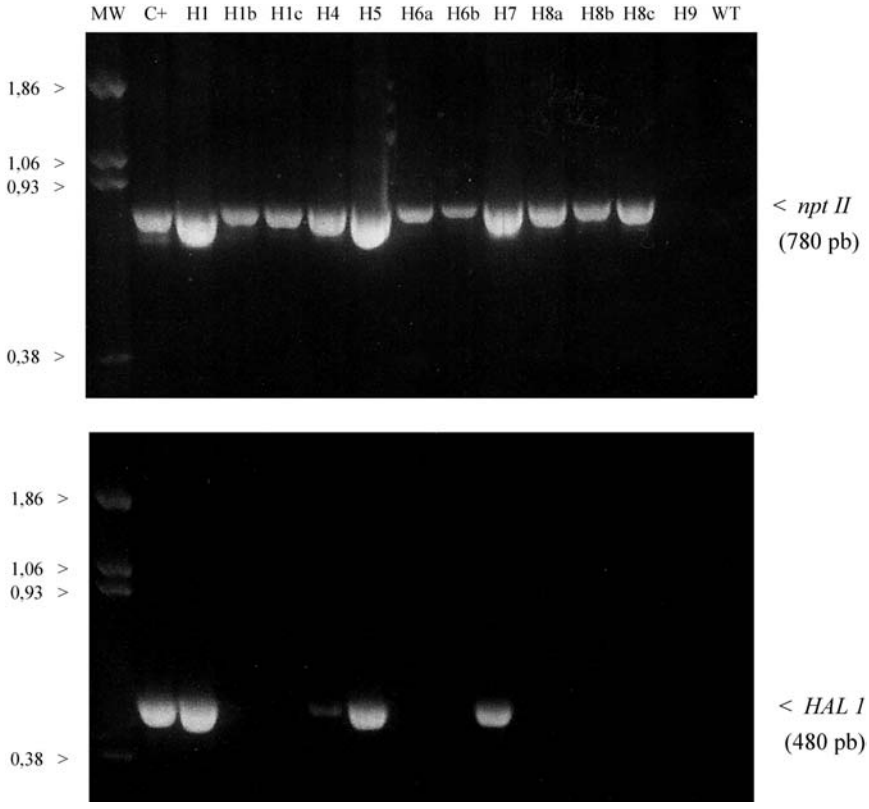

Fig. 2 Electrophoretic analysis of the PCR products of putative transgenic watermelon plants. Aliquots of genomic DNA from the different samples were analysed. *Lanes*: *MW* Molecular size standards (size in kilobases given at the *left*), *C+* positive control, transgenic melon carrying both the *nptII* and *HAL1* genes, *H1–9* putative independent transgenic watermelon plants, *WT* negative control from regenerated watermelon non-transgenic plant

Southern and Northern analysis

Genomic DNA from primary transformed and control plants was extracted using standard procedures (Doyle et al. 1990). A 15-μg aliquot of total DNA was digested with *Hin*dIII and separated by electrophoresis in a 0.7% agarose gel. DNA was transferred to a nylon membrane (Kempter et al. 1991) and hybridised with *HAL1* and *nptII* radiactive probes (Feinberg et al. 1983; Church et al. 1984). *Hin*dIII cuts once within the T-DNA between the *HAL1* and *nptII* genes (Fig. 1b). We used the *Nsi*I digestion fragment containing the coding region of *HAL1* as probe. For the *nptII* probe an internal fragment of the gene was obtained by PCR amplification using the primers described above. Northern analysis was performed essentially as described in Bordas et al. (1996).

Segregation analysis in TG2 progenies

To determine the inheritance of the kanamycin resistance in the progenies of primary transformants, 5-day-old cotyledons of the progenies were transversally cut and cultured in the organogenic medium supplemented with 100 mg l^{-1} kanamycin. Resistance or sensitivity to kanamycin was estimated after 4 weeks by callus growth on the cut edge of the explant.

Evaluation of salt tolerance in transgenic watermelon

Two secondary transformant lines (TG_2) were selected for a preliminary evaluation of salt tolerance. Parental non-transgenic plants of watermelon cv. Dulce Maravilla F_1 were self-pollinated and the progenies (F_2) used as control. Seeds of these three lines (H1, H7 and WT) were sterilised and germinated in vitro. After 7/8 days of germination, 1-cm-long apical primary shoots were excised and transferred to medium supplemented with 1.0 mg l^{-1} IBA and containing 0, 100 and 150 m*M* NaCl. The total number of different genotypes tested varied between 20 and 24 plants for each line and condition. After 4 weeks in the tissue culture room, parameters related to shoot and root growth were measured.

In vivo evaluation of the growth performance under salt stress conditions was carried out using two transgenic TG_3 lines (H1.18 and H7.19) with their two respective isogenic TG_3 control lines (H1.10 and H7.23). In order to obtain homozygous lines, we self-pollinated TG_2 transgenic lines derived from TG_1 that expressed the *HAL1* gene (H1 and H7). The progenies were selected on the basis of expression of kanamycin resistance by cultivating 7-day-old cotyledons on organogenic medium supplemented with 100 mg l^{-1} kanamycin. Shoots corresponding to kanamycin-resistant genotypes were transferred onto MB3 medium supplemented with 1 mg l^{-1} IBA.

Four-week-old shoots, grown in vitro (BM3+IBA), were transferred to the greenhouse and acclimatised. Two weeks later, the plants were transferred to containers with coconut fibre and slow-liberation fertilizer and irrigated for 2 weeks with fresh water (EC = 1 dS m^{-1}) in this semi-hydroponic system. Then, half of the plots remained cultured in the absence of added NaCl and the other half were cultured with 3.5 g l^{-1} NaCl (EC = 6 dS m^{-1}). Irrigation was applied four times each day (8:00, 12:00, 16:00 and 20:00). Vegetative growth appeared healthy and more or less vigorous in all lines, with no apparent diseases. Every 10 days, the number of secondary shoots per plant and the length of each shoot (in centimeters) were measured. The experiment concluded 6 weeks later.

A design experiment with 12 blocks was used for each salinity treatment. Each block comprised two transgenic lines (H1.18 and H7.19) and their two respective isogenic control lines (H1.10 and H7.23), randomly sown. The data mentioned are averages of 12 TG_3*HAL1* plants for each line tested and each salinity condition. The variable "total length of shoot" and "number of secondary shoots per plant" was statistically analysed by the appropriate multiple comparison procedure. Significant differences of the means were determined at the 95% confidence level by using the Fisher's least significantly difference (LSD) procedure.

Table 1 Summary of the transformation experiments of three different watermelon cultivars and two binary plasmids: the pBI121 and pPM7 plasmids

Cultivars	Treatments		Explants					Transformed[c] plants	
	Agrobacterium	Kanamycin (mg l^{-1})	Number	With callus[a]		With shoots[b]		*n*	%
				n	%	*n*	%		
pBI121 plasmid									
Dulce Maravilla	+	125	209	33	15.3	19	9.1	11	5.3
	+	175	165	16	9.7	10	6.0	6	3.6
	–	0	56	56	100	29	51.2	–	–
	–	125	58	0	0	0	0	–	–
Crimson Sweet	+	125	159	59	37.1	24	15.1	5	3.1
	+	175	144	34	23.6	15	10.4	4	2.8
	–	0	60	59	98.3	17	28.3	–	–
	–	125	70	0	0	0	0	–	–
pPM7 plasmid									
Dulce Maravilla	+	125	168	21	12.5	11	6.5	8	4.7
	–	0	36	34	94.4	19	52.7	–	–
	–	125	40	0	0	0	0	–	–
Sugar Baby	+	125	160	14	8.7	1	0.6	1	0.6
	–	0	36	29	80.5	14	38.8	–	–
	–	125	38	0	0	0	0	–	–

[a] Data scored after 4 weeks on the caulogenesis induction medium (CIM)
[b] Data scored after two to three subcultures on the shoot elongation medium (SEM) without antibiotics (cefotaxime and kanamycin)
[c] Characterisation of the putative transgenic plants isolated from independent calli and detected by GUS assays (experiment with pBI 121 plasmid) or PCR analysis with *nptII* and *HAL1* probes (experiment with pM7 plasmid)

Results

Regeneration of watermelon transgenic plants

In control experiments, the highest regeneration rates were obtained with cotyledons from 3-day-old seedlings cultivated without selection pressure (data not shown). Adventitious shoots of cvs. Crimson Sweet, Sugar Baby and Dulce Maravilla were regenerated on CIM at frequencies up to 40%, 65% and 68%, respectively.

In order to determine the possible effect of the transformation protocol on the organogenic response, half-cotyledon explants were treated as in all the transformation experiments but without *Agrobacterium* inoculation and cultivated on CIM without selective pressure. A slight reduction in the organogenic response was observed and regeneration rates were 28%, 39% and 52% for cvs. Crimson Sweet, Sugar Baby and Dulce Maravilla, respectively (Table 1). By culturing the explants not treated with *A. tumefaciens* on CIM supplemented with 125 mg l^{-1} kanamycin, neither cell growth nor organogenic response were observed (Table 1).

Most of the explants inoculated with *A. tumefaciens*, turned to brown and died after 20–24 days on CIM supplemented with 125 mg l^{-1} kanamycin and 300 mg l^{-1} cefotaxime. However, some of these explants produced a green kanamycin-resistant callus with several shoots. The individual green calli were excised and transferred onto SEM without kanamycin (+300 mg l^{-1} cefotaxime). One or two subcultures in the same medium (SEM +300 mg l^{-1} cefotaxime) were necessary for shoot elongation. Individual shoots were then separated, transferred to rooting medium and propagated on this antibiotic-free medium. A total number of 53 and 12 putative transgenic plants were regenerated with the pBI 121 and pPM7 constructs, respectively (Table 1). A rooting test on medium supplemented with 50 mg l^{-1} kanamycin was performed on each genotype, and both histochemical and molecular analysis were assessed on these plants.

In the first experiment (Table 1), 17 and 9 primary transformants resistant to kanamycin and PCR-positive for the *nptII* gene were obtained with Dulce Maravilla and Crimson Sweet, respectively. In the second experiment (Table 1), from 11 *nptII* PCR-positive plants, two primary transformants (H1 and H7) showed a higher ability to form roots and were selected for further evaluation of the number of copies and level of expression of the *HAL1* gene. Southern analysis (Fig. 3) showed that primary transformants H1 and H7 harboured two and one copies of the *HAL1* gene, respectively.

Following molecular analysis and cellular selection, transgenic plants carrying the pPM7 construct and control plants (somaclones and plants obtained from commercial seeds) were acclimatised under greenhouse conditions. The ploidy level was assessed by flow cytometry, and diploidy was confirmed in both somaclones and primary transformants that did not exhibit morphological abnormalities. Moreover, Watermelon plants producing normal female and male flowers and fruits were obtained by hand self-pollination. Fruit yield, fruit quality and number of seeds per plant were the same in somaclones and plants coming from commercial seed (data not shown).

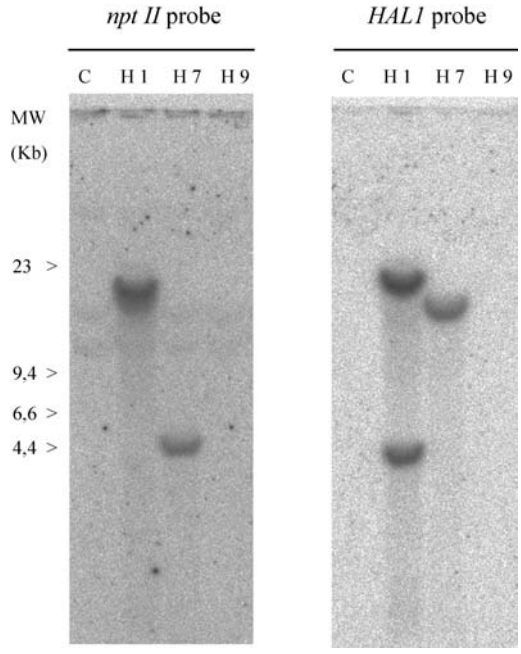

Fig. 3 Southern analysis of *nptII* and *HAL1* integration in putative transgenic watermelon plants. *Lanes*: *C* Negative control, genomic DNA from non-transgenic watermelon (regenerated somaclones), *H1* and *H7* independent *nptII* and *HAL1* PCR-positive watermelon transformants, *H9* negative control, genomic DNA from false transformant

Transformation efficiency

In experiments with the pBI 121 plasmid, selection was performed on culture media supplemented with 125 mg l^{-1} and 175 mg l^{-1} kanamycin. Under these conditions, transformation efficiencies were respectively 5.3% and 3.6% for Dulce Maravilla and 3.1% and 2.6% for Crimson Sweet (Table 1). These results indicated that cv. Dulce Maravilla is slightly more competent for genetic transformation than cv. Crimson Sweet. A slight decrease in the transformation rate was observed in both cultivars when the highest concentration of the antibiotic was used.

In the second experiment with the pPM7 plasmid, the PCR analysis with the *nptII* probe (Fig. 2, Table 1) indicated that cv. Dulce Maravilla was more competent (4.7%) for genetic transformation than cv. Sugar Baby (0.6%). In this experiment, the transformation rate obtained with Dulce Maravilla was similar to that previously obtained with the pBI 121 construct (Table 1), indicating the suitability of the protocol to introduce foreign genes with different constructs in this crop. PCR analysis with both the *nptII* and *HAL1* probes indicated the presence of the selectable gene in all cases and the lack of integration or the loss of the *HAL1* gene in some of them. PCR analysis also indicated (Fig. 2) that different transgenic genotypes may be obtained from the same transgenic callus. For example, plant H1 co-integrated both transgenes, while plants H1b and H1c, both descended from the same callus, apparently lost the *HAL1* gene. This result may be explained by different events of transformation occurring in the cells of the same explant or by a partial integration in the first stage followed by total integration or loss of the left sequence in different parts of the callus derived from the original transgenic cell.

Segregation analysis

Segregation analysis performed with progenies of both transgenic plants on the basis of *nptII* expression (Table 2) was in agreement with the presence of one single copy of the marker gene. This result was unexpected in the case of H1 as the molecular analysis with the *HAL1* probe indicated the presence of two inserts in this plant. In order to clarify this apparent discrepancy, Southern analysis were also performed with the *nptII* probe. The result of this molecular test (Fig. 3) was in agreement with the segregation analysis as only one copy of *nptII* was detected, indicating the probable loss of one of them in the two inserts. On the other hand, Northern analysis (Fig. 4) showed a higher level of expression of the *HAL1* gene in transgenic line H1 than in line H7.

Evaluation of salt tolerance

The TG_2 H1 and H7 lines, obtained by hand self-pollination of the independent transformants H1 and H7, were resistant to kanamycin and expressed the *nptII* and *HAL1* genes (rooting test and Northern analysis, respectively). A previous selection for salt tolerance was performed in vitro by cultivating apical shoots form the transgenic TG_2 and control lines of watermelon in a rooting medium supplemented with NaCl. H1 and H7 TG_2 shoots resistant to kanamycin showed an increased fresh weight and rooting capacity and a twofold greater biomass when compared with the control. Altogether, the results indicate a higher level of salt tolerance of H1 line with

Table 2 Segregation analysis in TG_2 progenies from transgenic watermelon carrying the *pPM7* construct and untransformed plants on rooting medium supplemented with 50 mg l^{-1} kanamycin

TG_2 plant	Observed segregation			Expected Mendelian ratio	X^2 value	P value
	n	kan^R	kan^S			
H1 × H1	43	34	9	3:1	0.380	$0.50 < P < 0.75$
H7 × H7	91	71	20	3:1	0.443	$0.50 < P < 0.75$
H1 × WT	48	26	22	1:1	0.333	$0.50 < P < 0.75$
H7 × WT	46	27	19	1:1	1.391	$0.25 < P < 0.50$
WT (D.M)	46	0	46	All sensitive	–	–

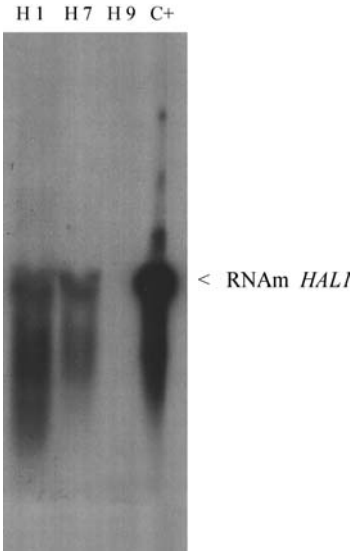

Fig. 4 Northern analysis of *HAL1* expression in transgenic watermelon plants. A 20-μg aliquot of total RNA was applied per lane. *Lanes*: *H1* and *H7* Independent transgenic watermelon, *H9* negative control, RNA from false transformant watermelon, *C+* positive control, RNA from *Saccharomyces cerevisiae* overexpressing the *HAL1* gene. *Arrow* on the *right* indicates the hybridising band of the *HAL1* transgene (0.9 kb) recognised by the *HAL1 S. cerevisiae* radioactive probe

respect to H7, which correlates with the level of expression of the *HAL1* gene obtained by Northern analysis. Under non-stressed conditions, no significant differences were observed between transgenic and control plants.

To determine the inheritance of the halotolerance trait in TG_3 homozygous and azygous progenies, we conducted an in vivo evaluation in both control and saline conditions (50 m*M* NaCl, EC = 6 dS m^{-1}) during 45 days. Several H1 and H7 TG_2 plants were first self-pollinated and homozygous and azygous progenies were then selected on the basis of expression of kanamycin resistance. Two homozygous lines carrying the transgenes (H1.18 and H7.19) and two azygous lines without transgenes (H1.10 and H7.23) were used for the evaluation of salt tolerance.

Under control conditions (without salt stress), we did not observe significant differences between azygous H1.10 and transgenic H1.18 and the same happened for azygous H7.23 with respect to transgenic H7.19 (Table 3). However, after 20 days of treatment with 50 m*M* NaCl, plant growth from azygous lines was severely inhibited, and significant differences were observed between control and transgenic plants at 30 and 40 days of culture. Stem elongation (Table 3) and the production of secondary shoots (Table 3) of salt-treated transgenic plants were significantly greater than those of the non-treated transgenic plants, indicating that watermelon TG_3 plants carrying the *HAL1* gene had an increased ability to tolerate salinity compared with their azygous TG_3 control plants.

Discussion

Agronomically interesting plants pose many more problems than model species for the transfer and expression of salt tolerance-related genes. However, from a breeding point of view, it is necessary to achieve the expression of the candidate gene/s in the crop species where the salt tolerance level would be evaluated.

In order to initiate a breeding programme for improving salt tolerance in watermelon, we have developed a transformation protocol that is useful for different cultivars (Crimson Sweet, Sugar Baby and Dulce Maravilla) of this horticultural species. As previously described by Dong and Jia (1991) and Compton and Gray (1993), the drastic loss of organogenic competence with increasing age of the donor seedlings was a key step in our

Table 3 Evaluation[a] of the growth performance under salt stress conditions in a semi-hydroponic system using two TG_3 transgenic lines (H1.18 and H7.19) with their two respective isogenic control lines (H1.10 and H7.23)

Days of culture	NaCl (0 m*M*)		NaCl (50 m*M*)		NaCl (0 m*M*)		NaCl (50 m*M*)	
	H1.10 (HAL–)	H1.18 (HAL+)	H1.10 (HAL–)	H1.18 (HAL+)	H7.23 (HAL–)	H7.19 (HAL+)	H7.23 (HAL–)	H7.19 (HAL+)
Effect of NaCl on total length of shoots[b] (cm) per plant								
0–10	240^{a}	238^{a}	238^{a}	241^{a}	341^{a}	326^{a}	328^{a}	328^{a}
10–20	$316^{a,b}$	288^{b}	239^{a}	$284^{a,b}$	$478^{c,d}$	$478^{c,d}$	$362^{a,b,c}$	$449^{b,c,d}$
20–30	480^{d}	431^{c}	$355^{b,c}$	$434^{c,d}$	705^{f}	675^{f}	$496^{d,e}$	$613^{e,f}$
30–40	730^{e}	713^{e}	499^{d}	644^{e}	983^{g}	956^{g}	$612^{e,f}$	883^{g}
Effect of NaCl on the number of secondary shoots[b] per plant								
0–10	1.7^{a}	$2.2^{a,b}$	1.8^{a}	1.9^{a}	3.0^{a}	2.7^{a}	3.0^{a}	2.9^{a}
10–20	$3.0^{b,c,d}$	$2.6^{a,b,c}$	$2.3^{a,b,c}$	$3.2^{c,d}$	4.1^{b}	$3.4^{a,b}$	$3.3^{a,b}$	$3.3^{a,b}$
20–30	$4.6^{e,f}$	5.0^{f}	$3.8^{d,e}$	4.7^{f}	5.7^{c}	5.4^{c}	5.1^{c}	5.7^{c}
30–40	6.3^{g}	6.8^{g}	5.1^{f}	6.1^{g}	6.8^{c}	$6.6^{d,e}$	$5.8^{c,d}$	7.1^{e}

[a] These plants were grown in containers with coconut fibre and slow liberation fertiliser and were irrigated for 2 weeks with fresh water (EC = 1 dS m^{-1}). Half of the plots with 8-week-old plants remained cultured in the absence of added NaCl, and the other half were cultured with 50 m*M* NaCl (3.5 g l^{-1}; EC = 6 dS m^{-1}). Every 10 days the number of shoots per plant and the length of each shoot were measured. Data are means of 12–14 replications per condition

[b] Bars with the same letter are not significantly different according to Fisher's LSD test ($P < 0.05$)

transformation protocol, and organogenic calli were only obtained from 2- to 3-day-old seedlings. Watermelon transgenic plants have been regenerated from 5- and 7- to 8-day-old cotyledons using cvs. Sweet Gem and Melitopolski cultivars (Choi et al. 1994 and Srivastratava et al. 1991, respectively). However, neither transformation efficiency percentages nor inheritance and expression values of the transgenes in progenies of the watermelon transgenic plants were reported. In *Citrullus coloccynthis*, Dabauza et al. (1997) described the transformation efficiency as the number of GUS-positive calli per total number of inoculated explants and reported a rate of 14%. In *Citrullus lanatus*, our results indicated that transformation efficiencies ranged from 2.8% to 5.3%, depending on the cultivar. Altogether, these results indicate that the two species of the genus *Citrullus* might be transformed by *A. tumefaciens* LBA4404 strain.

All of the watermelon plants selected on the basis of kanamycin resistance were PCR-positive for the *nptII* gene, indicating that this selectable marker allows a good selection efficiency in *Agrobacterium*-mediated experiments with watermelon cotyledon explants. Moreover, the predominance of the *nptII* gene observed in transgenic plants is not surprising, since selection is based on the marker gene and, furthermore, it is located on the right border of the T-DNA. Our results are in accordance with the directional model of T-DNA transfer from the right to the left border (Wang et al. 1984) and also agree with those obtained in cucumber (Chee and Slightom 1991) and melon (Bordas et al. 1996).

In addition, when designing a transformation protocol for breeding purposes, attention must be paid to the genetic stability of the transgenic plants. Morphological abnormalities and changes in the ploidy level have been frequently reported in both somaclones (melon: Moreno and Roig 1990; watermelon: Compton et al. 1996) and transgenic plants of cucurbit species (cucumber: Trulson et al. 1986; melon: Gonsalves et al. 1992). In our investigation, our use of very young cotyledons (less than 3 days old) might explain the absence of polyploid regenerated plants. Flow cytometric analysis of nuclear DNA contents of 3-day-old cotiledonary tissue of watermelon plantlets revealed the presence of nuclei with three ploidy levels: 2C corresponding to the G1 phase of the diploid cells (79% of the nuclei); 4C values (20% of the nuclei), indicative of the G2/M and the G1 phases of the diploid and tetraploid cells, respectively; the 8C peak (1% of the nuclei), which may be due to the presence of a reduced number of tetraploid cells in G2/M phase of the cycle.

Apart from the age of the explant source, the regeneration and/or transformation protocol could influence the rate of changes in the ploidy level. Recently, we assessed the appearance of numerical changes in both regenerants and transgenic plants obtained by using another regeneration protocol with cv. Dulce Maravilla. Most of the transgenic plants and 15% of the somaclones were tetraploid (Ellul et al., in preparation). These results indicate that the regeneration of tetraploid transgenic plants in watermelon might be procedure-dependent. A similar result was obtained in genetic transformation experiments of tomato: the rate of tetraploid transgenic plants ranged from 20% to 80% depending on both genotype and transformation procedure (Ellul et al. 2002). These results emphasise the necessity to check the ploidy level of transgenic plants before introducing transgenic material into a breeding programme.

With respect to the salt tolerance trait, the overexpression of *AtNHX1*, a vacuolar Na^+/H^+ antiport from *Arabidopsis thaliana*, enabled TG_3 transgenic plants of Arabidopsis to grow in 200 m*M* NaCl (Apse et al. 1999). Transgenic tomato plants with the *AtNHX1* gene (Zhang and Blumwald 2001) or the *HAL1* gene (Rus et al. 2001) were also able to grow, flower and produce fruits in the presence of NaCl. In a similar way, Gaxiola et al. (2001) showed that transgenic plants of Arabidopsis expressing higher levels of the vacuolar proton-pumping pyrophosphatase (AVP1) were more tolerant to salinity than the wild type. In melon, primary transformants (TG_1) carrying the *35S::HAL1* construct showed a higher ability for rooting in NaCl-containing medium with respect to the control, but no clear differences were observed in the aerial parts of the plant (Bordas et al. 1996). Our results with watermelon transgenic plants expressing the *HAL1* gene under control of the 35S promoter with a double enhancer sequence from the cauliflower mosaic virus and the RNA4 leader sequence of the alfalfa mosaic virus indicate that, apart from their beneficial effect on rooting, the expression of *HAL1* gene also improves the development of the aerial part of plant under saline conditions.

In conclusion, we describe here an efficient and reproducible *Agrobacterium*-mediated protocol suitable for transferring agronomic interesting genes in different watermelon cultivars (Dulce Maravilla, Crimson Sweet, Sugar Baby). Moreover, our results show that progenies of watermelon plants expressing the salt-related gene *HAL1* always performed better than non-transformed plants under salt-stress conditions.

Acknowledgements Support for this work was provided by CICYT (Comisión Interministerial de Ciencia y Tecnología, Projects AGL2000-1680-CO2-01) from the Spanish Government. A. Atarés was supported by a fellowship from the Spanish Government. P. Ellul was supported by fellowship from the Ministère de l'Education Nationale de l'Enseignement Supérieur et de la Recherche (French Government).

References

Apse MP, Aharon GS, Snedden WA, Blumwald E (1999) Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiport in *Arabidopsis*. Science 285:1256–1258

Bartels D, Nelson D (1994) Approaches to improve stress tolerance using molecular genetics. Plant Cell Environ 17:271–291

Beck E, Ludwig G, Auerswald EA, Reiss B, Schaller H (1982) Nucleotide sequence and extract localization of the neomycin phosphotransferase gene from transposon Tn5. Gene 19:327–336

Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res 12:8711–8721

Bohnert HJ, Golldack D, Ishitani M, Kamasani UR, Rammesmayer G, Shen B, Sheveleva E, Jensen RG (1996) Salt tolerance

engineering requires multiple gene transfers. In: Collins GB, Shepherd RJ (eds) Engineering plants for commercial products and applications. Ann NY Acad Sci 729:115–125
Bordas M, Montesinos C, Dabauza M, Salvador A, Roig LA, Serrano R, Moreno V (1996) Transfer of the yeast salt tolerance gene hal1 to *Cucumis melo* l. cultivars and in vitro evaluation of salts tolerance. Transgenic Res 5:1–10
Chee PP, Slightom JL (1991) Transfer and expression of cucumber mosaic virus coat protein gene in the genome of *Cucumis sativus*. J Am Soc Hortic Sci 116:1098–1102
Choi PS, Soh WY, Kim YS, Yoo OJ, Liu JR (1994) Genetic transformation of watermelon using *Agrobacterium tumefaciens*. Plant Cell Rep 13:344–348
Church GM, Gilbert W (1984) Genomic sequencing. Proc Natl Acad Sci USA 81:1991–1995
Compton ME, Gray DJ (1993) Shoot organogenesis and plant regeneration from cotyledons of diploid, triploid, and tetraploid watermelon. J Am Soc Hortic Sci 118:151–157
Compton ME, Gray DJ, Elmstrom GW (1996) Identification of tetraploid regenerants from cotyledons of diploid watermelon cultured in vitro. Euphytica 87:165–172
Dabauza M, Bordás M, Salvador A, Roig LA, Moreno V (1997) Plant regeneration and *Agrobacterium*-mediated transformation of cotyledon explants of *Citrullus colocynthis* (L.) Schrad. Plant Cell Rep 16:888–892
Dong JZ, Jia SR (1991) High efficiency plant regeneration from cotyledons of watermelon (*Citrullus vulgaris* Schrad.). Plant Cell Rep 9:559–562
Dower WJ, Miller JF, Ragsdale CW (1988) High efficency transformation of *E.coli* by high voltage electroporation. Nucleic Acid Res 16:6127–6145
Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15
Ellul P, Garcia-Sogo B, Pineda B, Ríos G, Roig LA, Moreno V (2002) The ploidy level of transgenic plants in *Agrobacterium*-mediated transformation of tomato cotyledons (*Lycopersicon esculentum* Mill.) is genotype and procedure dependent. Theor Appl Genet (in press)
Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13
Gaxiola R, de Larrinoa IF, Villalba JM, Serrano R (1992) A novel and conserved salt-induced protein is an important determinant of salt tolerance in yeast. EMBO J 11:3157–3164
Gaxiola RA, Rao R, Sherman A, Grisafi P, Alper SL, Fink GR (1999) The *Arabidopsis thaliana* proton transporters, *AtNhx1* and *Avp1*, can function in cation detoxification in yeast. Proc Natl Acad Sci USA 96:1480–1485
Gaxiola RA, Jisheng L, Undurraga S, Dang LM, Allen GJ, Alper SL, Fink GR (2001) Drought and salt-tolerance plants result from overespression of the AVP1 H^+ pump. Proc Natl Acad Sci USA 98:11,444–11,449
Gisbert C, Rus AM, Bolarin MC, Lopez-Coronado JM, Arrillaga I, Montesinos C, Caro M, Serrano R, Moreno V (2000) The yeast *HAL1* gene improves salt tolerance of transgenic tomato. Plant Physiol 123:393–402
Gonzalves D, Chee P, Provvidenti Seem R, Slightom JL (1992) Comparison of coat protein-mediated and genetically derived resistance in cucumbers to infection by cucumber mosaic virus under fields conditions with natural challenge inoculations by vectors. Biotechnology 10:1562–1570
Kempter B, Luppa P, Neumeier D (1991) A short procedure for Southern blotting on neutral and anionic membranes. Trends Genet 7:109–110
McKersie BD, Bowley SR, Harjanto E, Leprince O (1996) Water-deficit tolerance and field performance of transgenic alfalfa overexpressing suuperoxide dismutase. Plant Physiol 111:1177–1181
Moreno V, Roig LA (1990) Somaclonal Variation in Cucurbits. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry, vol 2. Somaclonal variation in crop improvement 1. Springer, Berlin Heidelberg New York, pp 435–464
Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473–497
Pen J, van Ooyen AJJ, van den Elzen PJM, Quax WJ, Hoekema A (1993) Efficient production of active industrial enzymes in plants. Indian Crops Prod 1:241–250
Ríos G, Ferrando A, Serrano R (1997) Mechanism of salt tolerance conferred by overexpression of the *HAL1* gene in *Saccharomyces cerevisiae*. Yeast 13:515–528
Roxas VP, Smith RK, Allen ER, Allen RD (1997) Overexpression of glutathione S-transferase/glutathione peroxidase enhances the growth of transgenic tobacco seedlings during stress. Natl Biotechnol 15:988–991
Roxas VP, Lodhi SA, Garrett DK, Mahan JR, Allen RD (2000) Stress tolerance in transgenic tobacco seedlings that overexpress glutathione S-transferase/glutathione peroxidase. Plant Cell Physiol 41:1229–1234
Rus AM, Estañ MT, Gisbert C, García-Sogo B, Serrano R, Caro M, Moreno V, Bolarin MC (2001) Expressing the yeast *HAL1* gene in tomato increases fruit yield and enhances K/Na selectivity under salt stress. Plant Cell Environ 24:875–880
Serrano R (1996) Salt tolerance in plants and microorganisms: toxicity targets and defense responses. Int Rev Cytol 165:1–52
Serrano R, Gaxiola R (1994) Microbial models and salt stress tolerance in plants. Crit Rev Plant Sci 13:121–138
Srivastava DK, Andrianov VM, Piruzian ES (1991) Regeneration and genetic transformation studies in watermelon (*Citrullus vulgaris* L. cv. melitopolski). In: Prakash J, Pierik RLM (eds) Horticulture – new technologies and applications. Kluwer, Dordrecht, pp 127–130
Tanaka Y, Hibino T, Hayashi Y, Tanaka A, Kishitani S, Takabe T, Yokota S, Takabe T (1999) Salt tolerance of transgenic rice overexpressing yeast mitochondrial Mn-SOD in chloroplasts. Plant Sci 148:131–138
Tarczynski MC, Jensen RG, Bohnert HJ (1993) Stress protection of transgenic tobacco by production of the osmolyte mannitol. Science 259:508–510
Taylor GR (1991) Polymerase chain raction: basic principles and automatation. In: McPherson MJ, Quirka P, Taylor GR (eds) PCR. A practical approach. IRL Press, Oxford, UK pp 1–14
Trulson AJ, Simpson RB, Shanin EA (1986) Transformation of cucumber (*Cucumis sativus* L.) plants with *Agrobacterium rhizogenes*. Theor Appl Genet 73:11–15
Van Camp W, Capiau K, Van Montagu M, Inés D, Slooten L (1996) Enhancement of oxidative stress tolerance in transgenic tobacco plants overproducing Fe-superoxide dismutase in chloroplasts. Plant Physiol 112:1703–1714
Wang K, Herrera-Estrella L, van Montagu M, Zambryski P (1984) Right 25-pb terminus sequence of the nopaline T-DNA is essential for and determines direction of DNA transfer from *Agrobacterium* to the plant genome. Cell 38:455–462
Xu D, Duan X, Wang B, Hong B, Ho THD, Wu R, Xu DP, Duan XL, Wang BY, Hong BM (1996) Expression of a late embryogenesis abundant protein gene, *HVA1*, from barley confers tolerance to water deficit and salt stress in transgenic rice. Plant Physiol 110:249–257
Zhang HX, Blumwald E (2001) Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. Natl Biotechnol 19:765–768
Zhang JX, Klueva NL, Wu WR, Ho THD, Nguyen HT (2000) Genetic engineering for abiotic stress resistance in crop plants. In vitro Cell Dev Biol 36:108–114
Zhu JK (2001) Plant salt tolerance. Trends Plant Sci 6:66–71