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Inherited chilling tolerance in somatic hybrids of transgenic *Hibiscus rosa-sinensis* × transgenic *Lavatera thuringiaca* selected by double-antibiotic resistance

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Summary. Improvement of *Hibiscus rosa-sinensis* for increased frost tolerance has been attempted through somatic hybridization with the frost tolerant *Lavatera thuringiaca.* Cell suspensions from *Hibiscus* and *Lavatera* were transformed with *A. tumefaciens* harboring plasmids containing selectable genes coding for kanamycin and hygromycin resistance, respectively. We provided evidence that *H. rosa-sinensis* and *L. thuringiaca* were transformed by strong selection of transformed calluses in medium containing antibiotics, by GUS activity determination in protein extracts and by molecular confirmation of chromosomal integration and expression of the selectable genes. Protoplasts isolated from a kanamycin-resistant *Hibiscus* callus and from a hygromycin-resistant *Lavatera* callus were fused and selected in medium containing both antibiotics. We determined unambiguously that the regenerated double-antibiotic resistant clones obtained are indeed somatic hybrids through analysis of acid phosphatase zymograms and nuclear DNA content. Plant regeneration through somatic embryogenesis was accomplished from both isolated protoplasts and transgenic calluses of *L. thuringiaca.* However, regeneration from the double-antibiotic resistant fusant calluses was unsuccessful. Analysis of the somatic hybrids at the callus level showed that chilling and freezing tolerance are governed by independent genetic components. The somatic hybrids displayed significant improvement for chilling tolerance at conditions lethal to *H. rosa-sinensis,* although frost tolerance was not expressed.

key words: Acid phosphatase - Embryogenesis - Freezing tolerance - Nuclear DNA content - Protoplast fusion - *Agrobacterium*-mediated transformation

Introduction

Hibiscus rosa-sinensis L. (Malvaceae) is widely appreciated as ornamental shrub in tropical and subtropical regions, but has limited use as outdoor ornamental plant in temperate regions because of its chilling sensitive character. Exposure to temperatures below 11 °C causes reduced growth rate, severe leaf damage and plant death (Karlsson et al., 1991). Significant improvement of *H. rosa-sinensis* for increased chilling tolerance by conventional breeding methods seems unlikely because the genetic components may be absent in this species.

Plant breeding programs, based on repeated cycles of intergeneric crosses and selection, have focused mainly on crop species such as wheat and potato, in attempts to further improve their tolerance. These studies have indicated that the chilling and freezing tolerance traits are typically multigenic and are governed by different pools of genes (Levitt, 1980; Guy, 1990). Furthermore, the complex genetics is reflected in contradictory reports on whether chilling and frost tolerance are dominant characters. Freezing tolerance and cold acclimation are reported as partially recessive traits in *Solanum* spp. (Stone et al., 1993; Palta and Simon, 1993), while in wheat freezing tolerance is controlled by both dominant and recessive genes (Sutka, 1981). On the other hand, little work has been done to improve tropical and subtropical plants for these traits.

To our knowledge, there are no previous reports on the *Agrobacterium*-mediated transformation of both *H. rosa-sinensis* and *L. thuringiaca.* We therefore developed a strategy to select true somatic hybrids of *H. rosa-sinensis* x *L. thuringiaca* based on the double-antibiotic resistance approach. Our analysis for inherited chilling and freezing tolerance in the somatic hybrids demonstrates the feasibility to improve tropical plants such as *H. rosa-sinensis* for increased chilling tolerance.

Materials and methods

Agrobacterium-mediated transformation. Cell suspension cultures were established from calluses of *Hibiscus rosa-sinensis* cv. Okinawa 1 and *Lavatera thuringiaca*, in a modified MS medium as described previously (Vazquez-Tello et al., 1995). One-ml aliquots of 3-day old cell suspension from *H. rosa-sinensis* were washed and resuspended in 1 ml of *A. tumefaciens* LBA4404 grown overnight ($O.D._{600}$ =0.5), harboring pBI121 containing the chimeric CaMV35S-GUS reporter gene and the pnos-NPTII gene that confers kanamycin resistance (Jefferson et al., 1987). *L. thuringiaca* cells were similarly inoculated with *A. tumefaciens* LBA4404 harboring pMM454 (a gift from Drs. K. Shimamoto and J. Imamura, Plantech Research Institute) containing the pnos-*hpt* chimeric construct conferring hygromycin resistance. After incubation for 2 hours at room temperature, the cell suspensions were gently washed and plated on the modified MS medium with 0.6 % agar, and further cocultivated for 2 days at 28 °C. *Hibiscus*

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and *Lavatera* cells were transferred to fresh agar plates containing carbenicillin and cefotaxime at 500 and 200 mg/l, respectively to kill *A. tumefaciens*. Kanamycin and hygromycin were added at 60 mg/l for *Hibiscus* and *Lavatera*, respectively. Incubation was done at 25 °C under a 14-hour light regime. Subculture steps were done at 10-day intervals for 2 months.

GUS activity determination. β-glucuronidase (GUS) activity was determined in protein extracts prepared from *H. rosa-sinensis* calluses resistant to kanamycin, as described by Jefferson et al. (1987). Untransformed callus was used as control.

Northern hybridization analysis. Total RNA was isolated from two hygromycin-resistant calluses of *L. thuringiaca*, and from leaves of untransformed *Lavatera* plants, by the method of Chomczynski and Sacchi (1987). Total RNA was isolated from the untransformed callus and the most vigorously growing callus of kanamycin-resistant *H. rosa-sinensis,* from which protein extract had been shown to contain GUS activity. Ten μg of total RNA were separated in a denaturing agarose gel and transferred to Nytran membranes (Schleicher & Schuell, Inc.). Full-size NPT II and *hpt* genes were restricted from pBI121 and pMM454, respectively, isolated from agarose gels and ^{32}P-radiolabeled by random-primed reaction (BcaBest radiolabeling Kit, Takara, Japan). Northern blots were hybridized following standard techniques (Ausubel et al., 1987).

Protoplast isolation. Protoplasts were isolated as described previously (Vazquez-Tello et al., 1995), from the most vigorously growing calluses of kanamycin-resistant *H. rosa-sinensis* and hygromycin-resistant *L. thuringiaca*, which had been confirmed to be transformed by GUS activity analysis and by chromosomal integration and expression of the selectable genes by northern analysis.

Protoplast fusion. Hibiscus and *Lavatera* protoplasts were mixed at ratio 1:1 to a final density of 1 x 10^6 protoplasts per ml. Protoplast fusion experiments were done according to the method of PEG and DMSO at high pH (Menczel and Wolfe 1984). The fused protoplasts were recovered by centrifugation (50Xg for 5 min) and cultured according to the agarose-embedded method as described (Vazquez-Tello et al., 1995).

Protoplast culture and selection of somatic hybrid calluses. After 40 days of culture, double-antibiotic selection was applied by subculturing the agarose droplets with developing microcolonies on 0.6% agar plates of the modified MS medium containing BAP or kinetin at concentrations ranging from 0.05 to 0.15 mg/l, excluding the auxin 2,4-D. Kanamycin and hygromycin were added at 50 mg/l each. Subculturing of individual calluses with double-antibiotic resistance was done every 10 days. After subculture for two months under these conditions, the calluses displaying vigorous growth were further cultured in double-antibiotic selecting agar plates containing NAA at 0.1 mg/l and either 2ip (6-(γ,γ-dimethylallylamino) purine) or 4CPPU (N-(2-chloro-4-pyridyl)-N'-phenylurea) at concentrations ranging from 0.5 to 5.0 mg/l.

Embryogenesis and plant regeneration from transgenic L. thuringiaca cells. A transgenic *L. thuringiaca* callus resistant to hygromycin shown to express the *hpt* gene by Northern analysis, was transferred to agar medium with the same composition as for selection of transformed calluses, but with the concentrations of 2,4-D and kinetin reduced to 0.01 and 0.05 mg/l, respectively. After two subculture steps in the same medium, developing globular embryos were transferred either in medium devoid of growth regulators, or containing GA_3 at 1 mg/l for embryo maturation and shoot development.

Analysis of acid phosphatase isozymes. Calluses (about 50 mg) from transgenic *H. rosa-sinensis*, *L. thuringiaca* and from the double-antibiotic resistant somatic hybrids were ground in one volume (50 μl) of cold extraction buffer (50 mM HEPES at pH 7.5, 1 mM EDTA, 0.1% CHAPS) in a microcentrifuge tube with a fitting pestle. After centrifugation (15,000 rpm / 10 min), the proteins in 1-2 μl of supernatant were separated according to their charge by IEF-PAGE (gradient pH 5 to 8, Phast System, Pharmacia). Acid phosphatase activity was detected by submerging the IEF gel in a filtered solution containing 50 mM sodium acetate (pH 5.0), 1 mg/ml Fast Garnet GBC (Sigma Co., USA) and 1 mg/ml α-naphtyl phosphate (Sigma, Co., USA), with gentle shaking in the dark at room temperature for 30 min.

Determination of nuclear DNA content. Cell suspensions from the untransformed parent cells and somatic hybrids were established and protoplasts were isolated as described previously (Vazquez-Tello et al., 1995). Intact nuclei were isolated from protoplasts as described by Arumuganathan and Earle (1991) and used directly for determination of DNA content with an Epics C flow cytometer (Coulter Co., USA). Chicken red blood cells were used as internal standard.

Chilling and freezing tolerance tests. Chilling tolerance determination was done by incubating calluses of the double-antibiotic resistant somatic hybrids, in agar plates at 6°C from 10 to 20 days under constant illumination. As control, transgenic *H. rosa-sinensis* and *L. thuringiaca* calluses were used. At the end of the incubation period, the percentage of cell survival as compared to that of identical samples kept at 25 °C, was determined by the TTC reduction method (Towill and Mazur, 1975) and by regrowth tests in which the chilling-stressed calluses were transferred to fresh plates and incubated at 25 °C.

Freezing tolerance determination was done with callus samples in microcentrifuge tubes (approximately 50 mg suspended in 50 μl water), and treated at several temperatures: 25, 5, 0, -1, -2.5 and -5 °C for one hour. At first, the tubes with the samples were equilibrated at the specified temperatures for 5 minutes, and then a minute amount of dry ice was added to initiate ice formation. After the cold treatment for one hour, the samples were defrosted slowly at 6 °C, then the percentage of cell viability was determined according to the TTC reduction method (Towill and Mazur 1975). The transgenic *Lavatera* cells were cultured at 6 °C for 6 weeks to attain maximal cold acclimation.

Results

Agrobacterium-mediated transformation of Hibiscus and Lavatera cells. Preliminary experiments were done to determine the minimal concentration of antibiotics to inhibit growth of untransformed callus of *Hibiscus* and *Lavatera*. Concentrations of either hygromycin and kanamycin were tested from 10 to 100 mg/l for both types of calluses, and we observed an inhibitory effect on more than 70% calluses compared to the controls without antibiotics, at a concentration as low as 15 mg/l, while 100% growth inhibition was observed for calluses from both *Hibiscus* and *Lavatera* at a concentration of 30 mg/l of either kanamycin or hygromycin. Therefore, each antibiotic was added to the culture medium at 60 mg/l for the selection of transformed calluses.

One month after transformation, small green *Lavatera thuringiaca* calluses resistant to hygromycin became visible but

their growth rate was rather slow, requiring several weeks of subculture to produce enough cell biomass for protoplast isolation. In the case of *H. rosa-sinensis*, small microcalluses yellow-cream in color resistant to kanamycin were visible some 35 days after transformation, and its growth rate was faster than that observed for *Lavatera*.

Detection of GUS activity in transgenic Hibiscus callus. β-glucuronidase (GUS) activity was tested in protein extracts from kanamycin-resistant *H. rosa-sinensis* calluses by the method of Jefferson et al. (1987). The GUS activity of the control untransformed callus was 22 pmole 4-MU/min/mg protein, while in the case of a kanamycin resistant callus displaying vigorous growth it was on average 752 pmole 4-MU/min/mg protein.

Confirmation of cell transformation and expression of NPT II and hpt genes in transgenic calluses. We checked by Northern hybridization whether the kanamycin-resistant *H. rosa-sinensis* callus with GUS activity and the hygromycin-resistant *L. thuringiaca* callus expressed constitutively the NPT II and *hpt* genes, respectively (Fig. 2). The NPT II probe detected no transcript in the untransformed *H. rosa-sinensis* control (Fig. 2a, lane 1), while in the case of the kanamycin resistant callus the probe hybridized to at least 3 transcripts with different sizes. The most probable explanation is that they represent transcripts from the same NPT II gene with different transcriptional initiation and alternative polyadenylation and cleavage processing, a phenomenon which was described in other single-copy genes (Hurt et al., 1992).

In the case of transgenic calluses of *L. thuringiaca*, a single band representing *hpt* transcripts was detected in each of two transformed calluses, but no band was detected in the control untransformed callus (Fig. 2b). These results provided a molecular evidence that the calluses were indeed transformed and expressed constitutively the selectable genes as expected. The most vigorous transformant callus from each parent were further selected by frequent subculturing and used for protoplast isolation and fusion.

Selection of somatic hybrid calluses by double-antibiotic resistance. In a single protoplast fusion experiment, the double-antibiotic resistance strategy allowed to obtain 117 calluses resistant to both hygromycin and kanamycin, derived from a total number of 10^6 protoplasts. It is likely that each colony-derived callus originated from independent fusion events involving at least 2 protoplasts. The relatively low number of fusant clones obtained, about ten times lower to that reported by electrofusion of tobacco protoplasts (Komari et al., 1989) may be attributed to a low fusion frequency, rather than to a low plating efficiency: the presence of 0.2 M sorbitol in the fusion solution may have caused reduced fusion frequency as pointed out by Menczel and Wolfe (1984), but the osmoticum was essential to prevent *Hibiscus* protoplasts from bursting. We previously established the optimal conditions for protoplast culture from both species and obtained plating efficiencies up to 30 % with the agarose-embedded system (Vazquez-Tello et al., 1995).

The double-antibiotic resistant calluses were subcultured every 10 days in auxin-free medium containing both antibiotics and either kinetin or BAP at 0.05 mg/l for two months but somatic embryogenesis could not be induced. The calluses were then transferred to plates with several growth regulators and concentrations (see: Materials and Methods) in an attempt to induce adventitious organogenesis. Under the conditions described, the somatic hybrid calluses became dark-green and showed variable phenotype: some developed as soft tissue while other calluses became rather hard and compact. However, after several months of subculture under these conditions, no shoots could be regenerated from them.

Regeneration of plantlets from transgenic Lavatera thuringiaca callus. Hygromycin-resistant *L. thuringiaca* transgenic plantlets were regenerated through somatic embryogenesis in two steps: preglobular proembryos were first induced from undifferentiated callus in medium with low auxin concentration (2,4-D at 0.01 mg/l), and after three weeks, they were transferred to medium containing hygromycin (60 mg/l) and without growth regulators, or in medium containing GA_3 at 1 mg/l to stimulate embryo maturation. Under these conditions plantlets with roots developed (Fig. 1). We have previously reported plant regeneration from *L. thuringiaca* protoplasts through somatic embryogenesis (Vazquez-Tello et al., 1995), and we further show here that the embryogenic potential is conserved in the transgenic cells.

Analysis of acid phosphatase isoenzymes. In order to provide unambiguous proof that the double-antibiotic resistant clones are

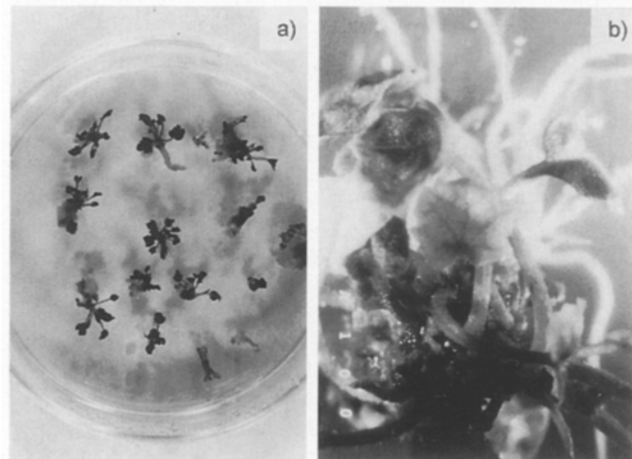

Fig 1. a) Transgenic *L. thuringiaca* plantlets regenerated through somatic embryogenesis in hygromycin-containing medium. b) Developing shoots in rooting medium containing hygromycin.

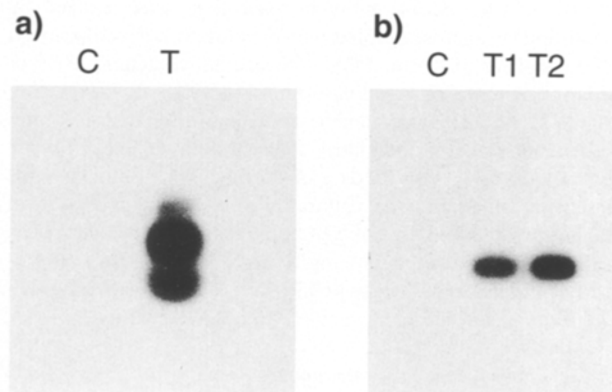

Fig 2. a) Detection of NPT II transcripts by Northern hybridization in *H. rosa-sinensis*. C, control untransformed callus; T, transformed kanamycin-resistant callus. b) Detection of *hpt* transcripts by Northern hybridization in *L. thuringiaca*. C, control untransformed callus; T1, T2, transformed hygromycin-resistant calluses.

real fusants, analysis of the acid phosphatase isoenzymes from callus extracts were performed. The results shown in Fig. 3 demonstrate that *L. thuringiaca* and *H. rosa-sinensis* can be reliably distinguished from each other by their acid phosphatase zymograms by IEF-PAGE. In the case of the parent *L. thuringiaca* at least 14 distinctive polypeptide bands with acid phosphatase activity were detected, the most prominent bands showing more acidic pI, although two faint bands with high pI were also observed (Fig. 3, lanes L). In the case of the parent *H. rosa-sinensis* the pattern of acid phosphatase isoenzymes was totally different from that of *L. thuringiaca*; at least 8 bands with acid phosphatase activity were focused on a smaller area with high pI (Fig. 3, lanes H).

All the double-antibiotic resistant clones analyzed displayed more complex zymograms, consisting of a combination of the isoenzymes observed in both parental cells. These results provide unequivocal evidence that all the double-antibiotic resistant clones tested represent indeed somatic hybrids, and indicate the high efficiency of the selection approach used in this work.

Freezing tolerance of Lavatera is not inherited in the somatic hybrids. We performed freezing tests on several clones with double-antibiotic resistance. Although there were large variations in the percent of cell survival when the hybrid clones were frozen at -1°C, none of them survived after freezing at -2.5 °C for one hour. The transgenic *H. rosa-sinensis* callus did not survive after the same treatment, while *L. thuringiaca* showed 32 % and 71 % cell survival for non-acclimated and cold acclimated callus, respectively (Table 1).

Chilling tolerance is inherited in the somatic hybrids. We performed experiments to test for inherited chilling tolerance by incubating the fusant calluses at 6 °C for 10 and 20 days (Fig. 5). Control experiments with *H. rosa-sinensis* callus showed that initially a rapid decrease of living cells occurred, with 4 days at 6 °C to cause more than 40 % cell death; thereafter the cell death became slower, with 30 % and 13 % cell survival after 10 days and 20 days, respectively (Fig. 4). In another independent experiment, an exposure for 13 days at 6 °C caused 100 % cell death. The viability of the whole callus after chilling stress was also determined in regrowth tests; *H. rosa-sinensis* calluses exposed at 6 °C for 10 days were unable to resume growth when transferred into fresh plates at 25 °C. These results confirm that *H. rosa-sinensis* is a chilling-sensitive species (Karlsson et al., 1991).

In the case of the somatic hybrids, when a chilling stress of 10 days at 6 °C was applied, 50 % of the hybrids analyzed displayed cell survival rates greater than 90 %, the remaining hybrids had survival rates ranging from 20 to 90% (Fig. 5). Under a more severe chilling stress, lethal to *H. rosa-sinensis* (20 days at 6 °C), the distribution of the hybrids among the class frequencies of % cell survival was relatively even (Fig. 5), while *H. rosa-sinensis* and *L. thuringiaca* displayed the lowest (13 %) and the highest (115 %) survival values, respectively (Fig.5 and Table 2). Our results clearly demonstrate that several somatic hybrids displayed chilling tolerance significantly higher than *H. rosa-sinensis*, giving unequivocal proof that they inherited and expressed chilling-tolerance related genes from *L. thuringiaca*. The genes controlling chilling tolerance tend to be dominant in moderate chilling stress, while they are partially dominant in severe chilling stress.

Somatic hybrids represent various fusion products. The DNA content in intact nuclei from the parental cells and somatic hybrids was determined by flow cytometry (Table 2). DNA contents determined for *L. thuringiaca* and *H. rosa-sinensis* cv. Okinawa 1 were 16.7 and 11.8 pg per nucleus (G_0+G_1), respectively. Analysis several somatic hybrids revealed that at least three different

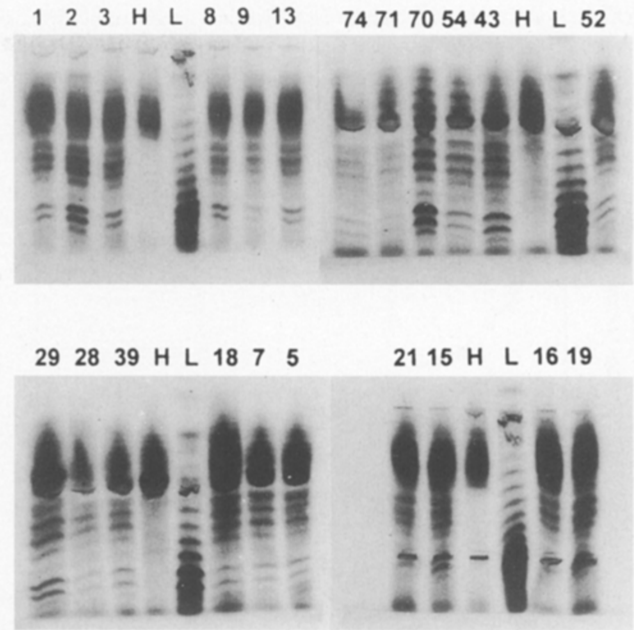

Fig 3. Analysis of acid phosphatase isozyme patterns in extracts from 22 somatic hybrid calluses and from calluses of transgenic *H. rosa-sinensis* (H) and *L. thuringiaca* (L).

Table 1. Percent cell survival[a] determined for Km^r Hm^r fusant calluses, after freezing for one hour at the indicated temperatures. Calluses of *H. rosa-sinensis* (Km^r) and *L. thuringiaca* (Hm^r), which was cold-acclimated (ACC) and non-acclimated (NA), are also included.

Callus	Temperature (°C)				
sample	0	-1	-2.5	-5	-7
Lavatera (NA)	100	nd[b]	32	0	nd
Lavatera (ACC)	100	81	71	60	51
Hibiscus	94	78	0		
H-1	105	34	0		
H-2	108	67	0		
H-3	100	nd	0		
H-4	53	48	0		
H-5	110	77	0	nd	
H-11	104	57	0		
H-15	101	7	0		
H-16	84	nd	0		
H-18	93	85	0		
H-21	81	19	0		

[a] percent cell survival = living cell number of treated sample / living cell number of control sample (25°C)
[b] nd; not done.

fusion products originated by fusion of 2 or 3 protoplasts, resulting in tetraploids and hexaploids, respectively. Moreover, the slight divergence between observed and theoretic nuclear DNA content, suggest that different levels of aneuploidy occurred in some of the hybrids, particularly in the case of somatic hybrids No.10 and 11 (Table 2).

Discussion

Protoplast fusions between *H. rosa-sinensis* and *L. thuringiaca* were performed aiming to obtain somatic hybrid plants able to survive at

chilling or freezing temperatures, which are lethal to the tropical parent *H. rosa-sinensis*. The double-antibiotic selection approach allowed us to obtain more than a hundred hybrid calluses from a single protoplast fusion experiment. Determination of nuclear DNA content by flow cytometry and acid phosphatase zymogram analysis provided unequivocal evidence that the double-antibiotic resistant calluses are somatic hybrids (Fig. 3). These results show that the double-antibiotic resistance approach, using two selectable gene markers inserted into the genome of each parent, is a powerful and efficient selection strategy.

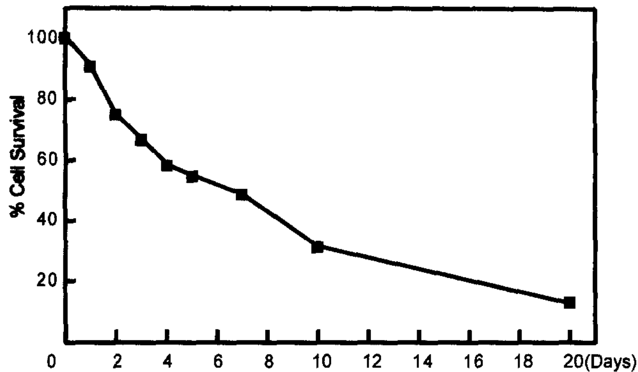

Fig 4. Determination of % cell survival in *H. rosa-sinensis* kanamycin-resistant callus following exposure to chilling stress for up to 20 days at 6°C.

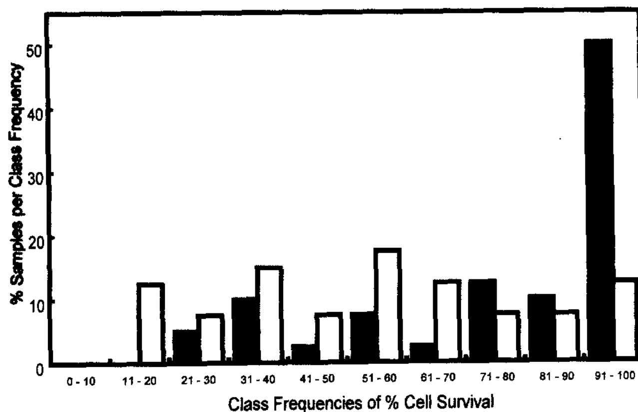

Fig 5. Distribution of 40 calluses of somatic hybrids in class frequencies of % cell survival after exposure to chilling stress for 10 days (black bar) and 20 days (white bar) at 6°C. The transgenic *L. thuringiaca* callus falls in the 91-100% viability class in both treatments. On the other hand, the transgenic *H. rosa-sisnensis* callus falls in the 21-30% viability class when treated for 10 days of chilling stress, but falls in the 11-20% viability class when treated for 20 days.

A major hurdle is that until now plant regeneration from the hybrid clones has not been achieved yet. In the case of the parent *L. thuringiaca* callus we previously reported plant regeneration through somatic embryogenesis (Vazquez-Tello et al., 1995), and we demonstrate here that the embryogenic capability of *L. thuringiaca* transgenic cells is conserved (Fig. 1). Despite our attempts, plant regeneration from the somatic hybrids has been unsuccessful, showing that the embryogenic potential from the parent *L. thuringiaca* is not expressed in the hybrids.

We considered the possibility that chromosomal loss or aneuploidy was related to the loss of regenerative capability. Although our analysis of nuclear DNA content by flow cytometry suggests that chromosome loss could happen in some of the hybrids (Table 2), this hypothesis does not explain the fact that euploid hybrids could not regenerate plants. Furthermore, the analysis of acid phosphatase isozymes pattern gave no evidence of extensive chromosome loss in the hybrids.

Little is known about competence, the ability of a plant cell to respond to specific stimuli during development leading to differentiation and plant regeneration, though it is accepted that both genetic and physiological components are involved (Potrykus and Shillito, 1986). Lack of regenerative capability has been observed in many species; moreover, it has been reported that competent cells can become non-regenerative during culture (Sung and Okimoto, 1981). The parent *H. rosa-sinensis* callus used in this work was produced from leaf tissue about two years ago, and all attempts to induce plant regeneration failed, including experiments with leaf and petiole explants. These observations suggest that the lack of competence in *H. rosa-sinensis* cells has genetic basis. We postulate that lack of expression of morphogenesis in the somatic hybrids may not be related to the absence of some genes, but rather to the presence of a dominant repressing factor inherited from *H. rosa-sinensis* cells, or alternatively by the irreversible inactivation of some regulatory function(s) of the parental *H. rosa-sinensis* callus. The dominance of the impaired function or the repressing factor from *H. rosa-sinensis* over the embryogenic potential of *L. thuringiaca* is evident in the case of the hybrids No.10 and No.56, which issued from two *Lavatera* protoplasts fused to one *Hibiscus* protoplast (Table 2). Our hypothesis support the model of a switch type controlled by a regulating factor, proposed by Sung and Okimoto (1983), in which the hypothetical regulating factor(s) may be the dominant repressing factor or the impaired function. Such regulating factor may act by turning on a set of callus-specific functions while simultaneously turning off another set of embryo-

Table 2. Determination of DNA content in nuclei isolated from transgenic *L. thuringiaca* and *H. rosa-sinensis* cells and their somatic hybrids (G_0+G_1 phase) by flow cytometry.

Samples	DNA content (pg/nucleus)	Type of fusant	Percent cell survival (20 days at 6°C)
Lavatera thuringiaca	16.7 ± 0.1	1 Lavatera	115
Hibiscus rosa-sinensis	11.8 ± 0.2	1 Hibiscus	13
Somatic hybrid no.2	28.4 ± 0.2	1 Lavatera + 1 Hibiscus	41
Somatic hybrid no.5	40.0 ± 0.2	1 Lavatera + 2 Hibiscus	57
Somatic hybrid no.10	42.8 ± 0.3	2 Lavatera + 1 Hibiscus	82
Somatic hybrid no.11	31.5 ± 0.2	1 Lavatera + 2 Hibiscus	20
Somatic hybrid no.56	45.3 ± 0.3	2 Lavatera + 1 Hibiscus	31

specific functions, the switching direction towards differentiation or dedifferentiation depending on the signal or stimuli perceived (Sung and Okimoto, 1983).

Analysis for inherited chilling and freezing tolerance showed that the freezing tolerance trait from *Lavatera* is not expressed in the somatic hybrids, while chilling tolerance behaves in a quantitatively expressed pattern. The distribution of the level of chilling tolerance among the hybrid clones ranged from the chilling sensitive trait of *Hibiscus* to the chilling tolerance of *Lavatera*. These results suggest differential gene expression rates among the hybrids and demonstrates that the chilling tolerance trait is inherited following a complex pattern due to its multigenic nature (Levitt,1980; Guy,1990). Under conditions lethal to the parent *H. rosa-sinensis*, our results provide additional evidence that the genes controlling chilling tolerance are dominant and partially dominant in the hybrids when the chilling stress is moderate and severe, respectively (Fig. 5). Furthermore, these results indicate that the genes controlling chilling tolerance are different from those controlling frost tolerance, the frost tolerant trait being recessive in the hybrids (Table 1). There are contrasting reports on whether freezing tolerance in plants is a dominant or recessive trait (Sutka, 1981; Stone et al., 1993; Palta and Simon, 1993). Nevertheless, our observations on the *Hibiscus* x *Lavatera* hybrids agree with the results of Stone et al. (1993), who found the mode of inheritance of freezing tolerance being partially recessive in F_1 and backcross populations of two potato species. Our work provide unambiguous evidence that improvement for chilling tolerance through somatic hybridization is feasible in tropical species. Moreover, we showed here that there is a relatively high probability to obtain R_0 somatic hybrids with chilling tolerance similar to that of the tolerant parent cells. It is possible to improve chilling tolerance significantly by a single hybridization step, but improvement of tropical plants for freezing tolerance seems to be more difficult and would require overcoming the problems associated with gene recessiveness and gene expression.

In conclusion, we have shown that in protoplast fusion experiments, only one parent cell with regenerative potential provides no guaranty for the successful regeneration of hybrid plants. Protoplast fusion should involve both parent cells having plant regeneration capability. The early selection of the hybrids with best chilling tolerance performance at the callus stage followed by plant regeneration, may considerably decrease both time and effort needed to get a hybrid plant.

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